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Mechanisms of type 1 diabetic serum-induced hyperactivation of Ca_V1 channels in the pancreatic β cell

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Mechanisms of type 1 diabetic serum-induced hyperactivation of Ca_V1 channels in the pancreatic β cell

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

The pancreatic β cell relies on appropriate Ca^{2+} entry through voltage-gated calcium (Ca_V) channels to accomplish its unique function insulin secretion and to guarantee its viability. Well-regulated β cell Ca_V channels are critical to ensure adequate functional β cell mass, thereby maintaining adequate insulin release and glucose homeostasis in the body. When β cell Ca_V channels mediate insufficient or excessive Ca^{2+} influx due to either inherited or acquired defects, β cell becomes malfunctioning and even dies. Type 1 diabetic (T1D) serum hyperactivates β cell Ca_V1 channels driving Ca^{2+}-dependent β cell apoptosis via previously unappreciated mechanisms. The present PhD work has mechanistically dissected T1D serum-induced hyperactivation of Ca_V1 channels in the β cell by combining patch-clamp techniques, confocal microscopy, as well as molecular and cellular approaches. It reveals the following findings:

Functional $Ca_V1.3$ channels reside in 20 % of mouse islet $Ca_V1.2^{-/-}$ β cells. They characteristically show a large unitary Ba^{2+} conductance with long-lasting openings in plasma membrane patches of islet cells endowed with undetectable voltage-gated Na^+ currents, larger cell capacitance (> 7 pF) and insulin mRNA. These observations pinpoint β cell-specific $Ca_V1.2^{-/-}$ mice as a convenient small animal model for investigation of human β cell $Ca_V1.3$ channel-related disorders such as T1D serum-induced hyperactivation of β cell $Ca_V1.3$ channels.

T1D serum hyperactivates both $Ca_V1.2$ and $Ca_V1.3$ channels by elevating their conductivity and number in the β cell plasma membrane. This finding emphasizes that both $Ca_V1.2$ and $Ca_V1.3$ channels are potential druggable targets for prevention of Ca^{2+} overload-induced β cell death.

Apolipoprotein CIII (ApoCIII) in T1D serum is electrophysiologically validated to be the actual factor enhancing Ca_V channel currents in the β cell. This validation opens up the possibility to deplete or neutralize ApoCIII in T1D serum for medical intervention of Ca_V channel hyperactivation-driven β cell destruction.

ApoCIII activates both PKA and Src kinase in a scavenger receptor class B type I/β1 integrin-dependent fashion to selectively hyperactivate β cell Ca_V1 channels without altering β cell Ca_V1 channel expression. ApoCIII-induced hyperactivation of β cell Ca_V1 channels results from the enriched density and increased activity of functional Ca_V1 channels in the β cell plasma membrane. This newly-identified signaling pathway shows great potential as a set of novel druggable targets for prevention of Ca^{2+}-dependent β cell death in association with diabetes.

The key endocytic protein syndapin I/PACSIN 1 (PCS1) is richly expressed in β cells to govern endocytic activity. PCS1-mediated endocytosis acts as a homeostatic control system to fine-tune the Ca_V1 channel density in the β cell plasma membrane. These findings add a new layer of complexity to the mechanisms of β cell Ca_V1 channel regulation.

ApoCIII impairs both constitutive and regulated β cell endocytosis with no influence on PCS1 expression. Consequently, ApoCIII abrogates PCS1-dependent endocytic trafficking, thereby accumulating excessive Ca_V1 channels in the β cell plasma membrane. These results delineate a novel mechanism of Ca^{2+}-dependent β cell destruction in diabetes development and reveal a promising and attractive option to counteract the critical diabetogenic process of Ca^{2+}-dependent β cell death.

Overall, the aforementioned findings depict a mechanistic picture of how ApoCIII renders Ca_V1 channels highly enriched and excessively activated in the β cell plasma membrane, thereby resulting in pathologically exaggerated Ca^{2+} influx and Ca^{2+}-dependent β death. These findings lay the foundation for novel treatment strategies for diabetes.

LIST OF SCIENTIFIC PAPERS

I. Guang Yang*, **Yue Shi***, Jia Yu, Yuxin Li, Lina Yu, Andrea Welling, Franz Hofmann, Jörg Striessnig, Lisa Juntti-Berggren, Per-Olof Berggren, Shao-Nian Yang (2015) $Ca_V1.2$ and $Ca_V1.3$ channel hyperactivation in mouse islet β cells exposed to type 1 diabetic serum. Cell Mol Life Sci 72: 1197-1207 (*equal contribution)

II. **Yue Shi***, Guang Yang*, Jia Yu, Lina Yu, Ruth Westenbroek, William A. Catterall, Lisa Juntti-Berggren, Per-Olof Berggren, Shao-Nian Yang (2014) Apolipoprotein CIII hyperactivates β cell Ca_V1 channels through SR-BI/β1 integrin-dependent coactivation of PKA and Src. Cell Mol Life Sci 71: 1289-1303 (*equal contribution)

III. **Yue Shi***, Guang Yang*, Jia Yu, Yuxin Li, Britta Qualmann, Michael M. Kessels, Lina Yu, Per-Olof Berggren, Shao-Nian Yang (2015) Inositol hexakisphosphate primes syndapin I/PACSIN 1 activation in endocytosis. Manuscript (*equal contribution)

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V. Shao-Nian Yang*, **Yue Shi***, Guang Yang, Yuxin Li, Lina Yu, Ok-Ho Shin, Taulant Bacaj, Thomas C. Südhof, Jia Yu, Per-Olof Berggren (2012) Inositol hexakisphosphate suppresses excitatory neurotransmission via synaptotagmin-1 C2B domain in the hippocampal neuron. Proc Natl Acad Sci USA 109: 12183-12188 (*equal contribution)

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LIST OF ABBREVIATIONS

A1C	hemoglobin A1C
ApoCIII	apolipoprotein CIII
APS	antigen-presenting cell
CalpC	calphostin C
CaMKII	calcium/calmodulin-dependent kinase II
Ca_V	voltage-gated calcium
$Ca_V1.2^{-/-}$	$Ca_V1.2$ subunit-homozygous knockout
$Ca_V1.2^{+/-}$	$Ca_V1.2$ subunit-heterozygous knockout
$Ca_V1.3^{-/-}$	$Ca_V1.3$ subunit-homozygous knockout
$[Ca^{2+}]_i$	cytosolic free calcium concentration
CK2	casein kinase 2
DHP	dihydropyridine
DMSO	dimethyl sulfoxide
EEEE	polyglutamate motif
FoxO1	forkhead box protein O1
GAD65	glutamic acid decarboxylase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GK	Goto-Kakizaki
HBSS	Hank's buffered salt solution
HDL	high-density lipoprotein
HLA	human leukocyte antigen
IA-2	insulinoma-associated protein 2
IA-2β	insulinoma-associated protein 2β
K_{ATP}	adenosine triphosphate-sensitive potassium
LADA	latent autoimmune diabetes in adults
LDL	low-density lipoprotein
LSD	least significant difference
MHC	major histocompatibility complex
MODY	maturity onset diabetes of the young
NC siRNA	negative control siRNA

NF-κB	Nuclear factor κ-light chain-enhancer of activated B cells
NOD	non-obese diabetic
NO siRNA	mock transfection in the absence of siRNAs
OLETF	Otsuka Long-Evans Tokushima fatty
PCS1	syndapin I/PACSIN 1
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PPARα	peroxisome proliferator-activated receptor α
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SR-BI	scavenger receptor class B type I
STZ	streptozotocin
TFA	triflouroacetic acid
T1D	type 1 diabetic or type 1 diabetes
VLDL	very-low-density lipoprotein
ω-Aga IVA	ω-agatoxin IVA
ω-CTX GVIA	ω-conotoxin GVIA
ZDF	Zucker diabetic fatty
ZnT8	zinc transporter 8

1 INTRODUCTION

The voltage-gated calcium (Ca_V) channel resides in all types of excitable cells and some non-excitable cells. It controls a diverse range of physiological processes. Its abnormality causes various pathological conditions leading to neurological disorders, cardiovascular diseases and metabolic dysfunctions [1-4]. The electrically excitable β cell employs multiple types of Ca_V channels to govern its function and viability. The β cell Ca_V channel-mediated Ca^{2+} influx takes center stage in insulin secretion and plays a unique role in glucose homeostasis. Inherited or acquired defects in β Cell Ca_V channels impair β cell function and in particular insulin secretion, and even drive β cell destruction resulting in diabetes [1-4]. Type 1 diabetic (T1D) serum hyperactivates β cell Ca_V1 channels causing Ca^{2+}-dependent β cell apoptosis via previously unappreciated mechanisms [1-3, 5, 6]. The present PhD work has mechanistically dissected T1D serum-induced hyperactivation of Ca_V1 channels in the pancreatic β cell by combining patch-clamp techniques, confocal microscopy, as well as molecular and cellular approaches.

1.1 DIABETES

Diabetes, also known as diabetes mellitus in full, refers to a group of metabolic diseases characterized by hyperglycemia [7, 8]. Marked hyperglycemia results in the specific symptoms of diabetes, such as increased urinary frequency (polyuria), thirst (polydipsia), hunger (polyphagia) and unexplained weight loss [7, 8]. Over time, untreated hyperglycemia often leads to diabetes complications including retinopathy, nephropathy, neuropathy, cardiovascular damage, etc [7, 8]. Hyperglycemia and diabetes are important causes of mortality and morbidity worldwide [9].

1.1.1 Classification of diabetes

Diabetes is caused by insulin deficiency, insulin resistance or both, and categorized into four subgroups: type 1, type 2, gestational diabetes and other specific types [7, 8, 10]. T1D accounts for about 5-10 % of diagnosed cases of diabetes [7, 8]. This type of diabetes is also called childhood-onset diabetes, juvenile diabetes, or insulin-dependent diabetes. It occurs due to pancreatic β cell destruction, usually leading to absolute insulin deficiency [7, 8]. Type 2 diabetes comprises about 90-95 % of diagnosed cases of diabetes [7, 8]. It is also named adult-onset diabetes, obesity-related diabetes, and noninsulin-dependent diabetes. Type 2 diabetes results from a combination of both insulin resistance and β cell dysfunction, but their relative contribution varies not only during the course of the disease, but also among individual patients [7, 8]. Distinctions between T1D and type 2 diabetes are not always clear, both etiologically and clinically [11]. Some adults are initially diagnosed with type 2 diabetes, but slowly progress to T1D characterized by autoimmune β cell destruction [11]. This type of diabetes is referred to as “Latent Autoimmune Diabetes in Adults (LADA)” [11]. Likewise, some obese adolescents and young adults (<25 years of age) can also have the manifestation of traits of both type 1 and type 2, which is termed

"type 1.5" or "double diabetes" [11]. "Type 3 diabetes" or "brain diabetes" has been proposed for Alzheimer's disease in terms of insulin resistance in the brain [12].

Gestational diabetes occurs in about 2-5 % of all pregnancies and may improve or disappear after delivery [7, 8]. It resembles type 2 diabetes and involves a combination of relative insulin deficiency and insulin resistance [7, 8]. Other specific types of diabetes consist of the remaining cases of diabetes, which do not fit into the aforementioned three types [7]. This category arises from a collection of a few dozen individual causes including genetic defects of the β cell, genetic impairments of insulin action, diseases of the exocrine pancreas, pancreatic endocrinopathies, some genetic/immune disorders, drug/chemical insults, and so on [7]. "Maturity Onset Diabetes Of the Young (MODY)" is a typical example in this category of diabetes [7].

1.1.2 Diagnosis of diabetes

According to American Diabetes Association, diabetes should be diagnosed by any of the following criteria [7]:

a. Hemoglobin A1C (A1C) is greater than or equal to 6.5 %. Test scores should be achieved in a laboratory using a method that is certified by National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial assay [7].

b. Fasting plasma glucose is greater than or equal to 126 mg/dl (7.0 mmol/l). A subject should have no caloric intake for at least 8 h [7].

c. Two-hour plasma glucose is greater than or equal to 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test. The measurement should be performed using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water in line with the World Health Organization's guidelines [7].

d. A random plasma glucose is greater than or equal to 200 mg/dl (11.1 mmol/l) when a patient suffers from classic symptoms of hyperglycemia or hyperglycemic crisis [7].

Criteria a-c should be confirmed by repeat testing if unequivocal hyperglycemia is absent [7].

1.1.3 T1D

T1D is classified broadly into immune mediated (type 1 A) and idiopathic (type 1 B) forms [7, 13]. The minority of T1D is idiopathic. This form of T1D shows clear signs of insulinopenia with an unknown etiology and requires insulin replacement therapy. It is strongly inherited, lacks evidence for β cell autoimmunity, and is not human leukocyte antigen (HLA) associated. The majority of T1D is immune mediated as evidenced by the presence of an inflammatory infiltrate in the islets, a strong linkage with certain alleles of the major histocompatibility complex (MHC) and autoantibodies that react with islet cell autoantigens [7, 13]. The present PhD thesis deals with type 1A diabetic serum-induced hyperactivation of β cell voltage-gated calicium (Ca_V) channels. The term "type 1A diabetes" is hereafter replaced by "T1D".

1.1.3.1 Pathogenesis of T1D

The human MHC is called HLA complex, which is located on chromosome 6 and contains over 200 genes encoding HLA class I and class II molecules [7, 13, 14]. Both HLA class I and class II genes are linked to T1D. The class II genes DQA, DQB and DRB by far show the strongest linkage to the disease [7, 13, 14]. A high percentage of sera from newly diagnosed T1D patients contain autoantibodies against islet cells, insulin, the 65-kD isoform of glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2) and 2β (IA-2β) and zinc transporter 8 (ZnT8) [7, 13, 14]. These autoantibodies appear quite a few months or years before the onset of the clinical disease. They have turned out to be excellent diagnostic and predictive markers for T1D. However, it is not conclusively verified, but just generally thought that they play only a minor role, if any, in the actual pathogenesis of the disease [7, 13, 14]. The cell-mediated immune response is believed to be responsible for β cell destruction [13, 14]. The immune-mediated β cell destruction relies on CD8+ cytotoxic and CD4+ helper lymphocytes. Antigen-specific CD8+ T cells recognize islet cell antigens in a complex with HLA class I molecules on the surface of β cells. This leads to up-regulation of a number of co-stimulatory molecules such as FAS/FASL and activation of one or more of several different effector pathways, e.g., FAS/FASL and perforin/granzyme to drive β cell apoptosis [13, 14]. In contrast, antigen-specific CD4+ T cells indirectly kill β cells by recognizing autoantigens in association with HLA class II molecules on the surface of antigen-presenting cells (APCs) such as macrophages and dendritic cells. This recognition up-regulates co-stimulatory molecules, e.g., CD28/CD80 and stimulates the release of a variety of cytokines, such as interferon γ, tumor necrosis factor α and nitric oxide, from both CD4+ T cells and antigen-presenting cells resulting in apoptosis of nearby β cells [13, 14].

1.1.3.2 Triggers for autoimmune β cell destruction in T1D

Triggers for the autoimmune-mediated destruction of β cells are not known. Immunologic, genetic and environmental factors have been implicated [13, 14]. T cells develop immunocompetence in the thymus [14]. Only a small proportion of newly generated T cells, which receive a weak, low affinity signal from an HLA-autoantigen complex, are capable of passing thymic selection as antigen-specific T cells and migrating to peripheral organs throughout the body including the pancreas. Normally, these T cells lie dormant and remain under strict regulatory control [14]. In the scenarios where either they come in contact with cognate autoantigens presented by β cells or APCs in the pancreas or the regulatory controls fail, these dormant, antigen-specific T cells become activated to initiate the autoimmune cascade of β cell killing. Thus, immune dysregulation may act as one of the triggers for autoimmunity in T1D [13, 14]. With regard to genetic factors, a combination of several different genes is involved [13, 14]. More than 40 genomic loci have been linked with influencing T1D risk [13]. Variations in HLA genes are an important genetic risk factor, but alone they do not account for the disease and other genes are involved [13, 14]. In addition, environmental factors such as delivery by cesarean section, high birth weight, vitamin D deficiency, viral infections, obesity and environmental

toxicants, may undergo complex interactions to drive β cell autoimmunity and cause clinical T1D [13, 15].

1.2 APOCIII

Apolipoprotein CIII (ApoCIII) belongs to the family of amphiphilic apolipoproteins in the circulation [16-18]. These apolipoproteins bind to lipid vesicles through their amphiphilic α-helices to create different plasma lipoprotein particles, including chylomicrons, very-low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) [16-18]. Hence, the apolipoproteins are often described as lipid transport vehicles through the aqueous circulatory system in the body [16-18]. Besides lipid transport, the apolipoproteins also serve as receptor ligands, lipid transfer carriers and enzyme cofactors to regulate cellular uptake and metabolism of lipoproteins [16-18].

1.2.1 Molecular and biochemical aspects of ApoCIII

ApoCIII is an 8.75 kDa protein composed of 79 amino acid residues [16-18]. In humans, this relatively small protein is encoded by the ApoCIII gene clustered with the ApoAI, ApoAIV and ApoAV genes on chromosome 11q23 [19]. ApoCIII gene expression is subjected to complex regulation. Insulin signaling inhibits both hepatic and intestinal ApoCIII expression via phosphorylation-dependent nuclear exclusion of the transcription factor forkhead box protein O1 (FoxO1). This transcription factor stimulates hepatic ApoCIII expression by binding to its consensus site in the ApoCIII promoter [20]. Streptozotocin-induced diabetic mice without and with insulin treatment display increased and decreased hepatic ApoCIII mRNA levels, respectively [21]. Both insulin-deficient non-obese diabetic (NOD) mice and insulin-resistant db/db mice exhibit increased ApoCIII levels due to deregulated FoxO1 expression in their livers and intestines [20]. Activation of peroxisome proliferator-activated receptor α (PPARα) with fibrates drastically decreases hepatic ApoCIII expression [22, 23]. Interestingly, the regulatory element of a nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is located 150 nucleotides upstream from the transcriptional start site of the ApoCIII gene [24]. Activated NF-κB binds to this regulatory element to up-regulate ApoCIII expression [24].

ApoCIII is synthesized predominantly in the liver and to a minor extent in the intestine. Liver and intestinal cells release this apolipoprotein into the blood [16, 17]. Recent work has also revealed that ApoCIII can be produced in mouse islet cells [25, 26]. ApoCIII can be posttranslationally glycosylated at threonine 74 and exists as three isoforms: $ApoCIII_0$, $ApoCIII_1$ and $ApoCIII_2$, which are covalently linked with 0, 1 and 2 sialic acid residues, respectively, and account for 10, 55 and 35 %, respectively, of total ApoCIII in the circulation [27]. ApoCIII contains six amphiphilic α-helixes, each consisting of about 10 residues. The three-dimensional nuclear magnetic resonance structure of ApoCIII has been resolved when it complexes with sodium dodecyl sulfate micelles, mimicking its natural lipid-bound state. The six amphiphilic α-helixes assemble into a necklace-like chain wrapping around the sodium dodecyl sulfate micelle surface [16].

1.2.2 ApoCIII inhibition on triglyceride and cholesterol metabolism

Classically, ApoCIII serves as an effective inhibitor of triglyceride metabolism through the following mechanisms. ApoCIII is situated on the surface of chylomicrons, LDLs and HDLs [16, 17]. It prevents triglyceride-rich lipoproteins from approaching the negatively charged cell surface by displacing apolipoprotein E or changing apolipoprotein E conformation [16, 28]. Consequently, the cell surface-attached lipoprotein lipase and plasma membrane-localized apolipoprotein E receptors can no longer reach triglycerides and apolipoprotein E in lipoproteins to carry on triglycerides hydrolysis and the endocytic clearance of triglyceride-rich lipoproteins, respectively. ApoCIII displaces lipoprotein lipase from lipid droplets and competes with the lipoprotein lipase activator apolipoprotein CII for the lipid/water interface, thereby suppressing lipoprotein lipase activity [29]. ApoCIII also impedes the selective uptake of cholesteryl esters from LDLs and HDLs by binding to the scavenger receptor class B type I (SR-BI), and hampers the endocytosis of cholesterol-rich LDLs by prevention of apolipoprotein B binding to LDL receptors [30-32].

1.2.3 ApoCIII-induced dyslipidemia and inflammation

ApoCIII inhibition on lipoprotein lipase results in impaired triglyceride metabolism and clearance [19, 33]. Mice carrying a disrupted ApoCIII gene show 70 % reduction in triglyceride levels [19]. Transgenic mice overexpressing human ApoCIII manifest hypertriglyceridemia [33]. Elevated plasma ApoCIII concentration is a feature of dyslipidemia in obesity and strongly related to cardiovascular risk [34]. A group of Ashkenazi Jews with reduced plasma ApoCIII concentration maintains cardiovascular health and reaches exceptional longevity [35]. This is not only because of ApoCIII-induced dyslipidemia, but also ApoCIII-mediated inflammation [19, 33, 36]. For example, ApoCIII promotes the development of arteriosclerosis by facilitating adhesion of monocytes to vascular endothelial cells through activation of the inflammatory cascade PKCα/NF-κB/β1-integrin [37-39]. Actually, ApoCIII is a multifaceted player in cell signaling [30, 37, 39, 40]. It can bind to distinct cell surface receptors including SR-BI and uncharacterized binding sites relaying corresponding signals to their downstream effectors, e.g., β1 integrin, pertussis toxin-sensitive G proteins, NF-κB and protein kinases [30, 37, 39, 40]. This suggests that ApoCIII participates not only in the inflammatory process, but also other molecular and cellular events.

1.2.4 ApoCIII and diabetes

Earlier studies indicate that plasma ApoCIII levels are associated with diabetes. There is an increase in this apolipoprotein in both T1D and type 2 diabetes [6, 41]. The Ashkenazi Jews with decreased plasma ApoCIII levels display greater insulin sensitivity with age [35]. A haplotype block carrying genetic variants within the regulatory region of the ApoCIII gene promoter is closely associated with T1D. This genetic association likely results from increased expression of ApoCIII driven by the genetic ApoCIII gene haplotype [42]. *In vivo* suppression of ApoCIII delays onset of diabetes with doubling the time in the BioBreeding rat, a rat model for human T1D. [43]. Interestingly, in ob/ob mouse islets, local insulin

resistance promotes ApoCIII production and β cell failure [25, 26]. Although a T-lymphocyte-mediated autoimmune attack plays a crucial role in the development of T1D, diabetogenic serum factors undoubtedly aggravate the disease development on top of the autoimmune attack [1-3, 5, 6, 44]. Elevated ApoCIII has been demonstrated to act as such a serum factor to drive β cell destruction in T1D [1-3, 5, 6, 45].

1.3 Ca_V CHANNELS

Ca_V channels appear in both prokaryotes and eukaryotes, being more diversified in higher forms of life. Mammals accommodate multiple types of Ca_V channels with different gating kinetics. Distinct types of Ca_V channels are selectively distributed among different cell types and in different special regions of the plasma membrane to control specific cellular activities [1-3, 46-48].

1.3.1 Function of Ca_V channels

Ca_V channels are tightly controlled Ca^{2+}-conducting pores in the plasma membrane. Membrane depolarization drives a rapid conformational switch of Ca_V channels from an impermeable state to a highly permeable pore. The highly permeable pore allows extracellular Ca^{2+} to rapidly enter the cytoplasm, where Ca^{2+} functions as a second messenger to couple electrical signaling to Ca^{2+}-dependent protein-protein interactions and enzymatic responses [1-3, 46-48]. Therefore, the Ca_V channel-mediated Ca^{2+} influx governs a broad range of cellular processes including exocytosis, endocytosis, muscle contraction, synaptic transmission, metabolism and cell fate. It participates in fertilization and regulates proliferation, differentiation, and development through the alteration of protein phosphorylation, gene expression, and the cell cycle [1-3, 46-49]. Pathophysiologically, hyperactivated Ca_V channels mediate exaggerated Ca^{2+} influx resulting in Ca^{2+}-dependent cell destruction through initiation of apoptosis and necrosis. Disturbed function of Ca_V channels, caused by mutation, altered expression and autoimmune insults, gives rise to a series of clinical signs and symptoms, named as calcium channelopathies or calcium channel diseases [1-3, 50-53].

1.3.2 Physiological and pharmacological properties of Ca_V channels

Classical voltage clamp analysis divides Ca_V channels into low- and high-Ca_V channels in terms of their activation thresholds [54]. The establishment of advanced patch-clamp techniques and the discovery of selective Ca_V channel blockers enable in-depth characterization of Ca_V channels [1-3, 46, 47, 55, 56]. Low-Ca_V channels display a tiny unitary conductance and a transient kinetics of inactivation. Therefore, low-Ca_V channels are also called T-type Ca^{2+} channels (T for tiny and transient) [1-3, 46, 47, 55, 56]. High-Ca_V channels show distinct electrophysiological and pharmacological features which subdivide these channels into L-type, N-type, P/Q-type and R-type Ca^{2+} channels [1-3, 46, 47, 55, 56]. L-type Ca^{2+} channels have a larger unitary conductance, mediate long-lasting currents (L for larger and long-lasting) and exhibit the typical dihydropyridine (DHP) sensitivity [1-3, 46, 47, 55, 56]. N-type Ca^{2+} channels display smaller unitary conductance,

lower activation threshold, and faster inactivation rate than L-type Ca^{2+} channels, but larger unitary conductance, higher activation threshold, and slower inactivation rate than T-type Ca^{2+} channels. Such Ca^{2+} channels are neither T nor L. Additionally, they were only found in neurons. Therefore, these channels were classified as a distinct type named N-type (N for neither, nor and neuron). N-type Ca^{2+} channels are blocked by the peptide blocker ω-conotoxin GVIA (ω-CTX GVIA), but are insensitive to DHPs [1-3, 46, 47, 55, 56]. P-type Ca^{2+} channels were originally revealed in cerebellar Purkinje cells (P for Purkinje). Sequentially, Q-type Ca^{2+} channels were identified in cerebellar granule cells (Q chosen according to alphabetical order). Both are blocked by ω-agatoxin IVA (ω-Aga IVA). The former is somewhat more sensitive to ω-Aga IVA and inactivates slightly slower than the latter. However, it is difficult to distinguish them. Hence they are combined as P/Q-type Ca^{2+} channels [1-3, 46, 47, 55, 56]. R-type Ca^{2+} channels were discovered in cerebellar granule cells that showed a residual Ca^{2+} current resistant to the multiple Ca_V channel blockers nimodipine, ω-CTX GVIA, and ω-Aga IVA (R for residual and resistant). R-type Ca^{2+} channels are now no longer resistant to "everything." They are blocked by the purified peptide toxin SNX-482 [1-3, 46, 47, 55, 56].

1.3.3 Molecular architecture of Ca_V channels

Biochemical examination and molecular biological investigation have substantially characterized the architecture of Ca_V channels [1-3, 46, 47]. The Ca_V channel is a multi-subunit protein complex. Typically, it consists of α1, α2δ, β and γ subunits [1-3, 46, 47]. A hydrophobic α1 subunit noncovalently associates with a disulfide-linked α2δ dimmer, an intracellular phosphorylated β subunit and a transmembrane γ subunit [1-3, 46, 47]. In this complex, the α1 subunit contains four homologous transmembrane domains forming a Ca^{2+} conducting pore. Hence, the α1 subunit is known as the pore-forming or principal subunit. The homologous transmembrane domain of the α1 subunit consists of six transmembrane segments (S1 to S6) and a membrane-associated pore loop (P-loop) between the S5 and S6 segments, three intracellular linkers, and N- and C-termini. The four homologous domains are structured into a Ca^{2+}-conducting pore equipped with activation and inactivation gates and controlled by voltage sensors. Four pairs of the S5 and S6 segments along with the P-loops line the Ca^{2+}-conducting pore. The Ca^{2+} selectivity of the pore relies on four glutamic acid residues situated in the four P-loops of high-Ca_V channels or two glutamic and two aspartic acid residues localized in the corresponding positions of low-Ca_V channels. The S4 segments function as the principal voltage sensors in Ca_V channels. The depolarization-evoked movement of the voltage sensors results in the conformational change and/or physical reposition of activation and inactivation gates to open the channel and to reduce channel permeability, respectively [1-3, 46, 47].

So far, ten α1 subunits have been identified. They are α1A, α1B, α1C, α1D, α1E, α1F, α1G, α1H, α1I and α1S subunits and mediate L- (α1C, α1D, α1F and α1S subunits), P/Q- (α1A subunit), N- (α1B subunit), R- (α1E subunit), and T-type Ca^{2+} currents (α1G, α1H and α1I subunits), respectively [1-3, 46, 47]. Other subunits, β, γ and α2δ subunits, do not directly participate in the formation of the Ca^{2+} conducting pore, but regulate the function

and trafficking of α1 subunits. Therefore, these subunits are referred to as auxiliary or regulatory subunits. To date, four α2δ subunits (α2δ1-4), four β subunits (β1-4) and eight γ subunits (γ1-8) have been identified [1-3, 46, 47].

1.3.4 Phylogenetic classification of Ca_V channels

Comparative amino acid sequence analysis satisfactorily builds the phylogenetic tree of $Ca_V\alpha1$ subunits, which shows a close relationship between $Ca_V\alpha1$ subunit structure and function [56]. This phylogenetic tree creates the most accurate and widely-accepted nomenclature of Ca_V channels. The nomenclature categorizes Ca_V channels into three families, Ca_V1, Ca_V2 and Ca_V3. The first one has four members: $Ca_V1.1$ (α1S), $Ca_V1.2$ (α1C), $Ca_V1.3$ (α1D) and $Ca_V1.4$ (α1F). The second one consists of $Ca_V2.1$ (α1A), $Ca_V2.2$ (α1B) and $Ca_V2.3$ (α1E). The third one comprises $Ca_V3.1$ (α1G), $Ca_V3.2$ (α1H) and $Ca_V3.3$ (α1I) [56]. Some of them are selectively expressed in specific cell types, e.g., $Ca_V1.1$ in skeletal muscle and $Ca_V1.4$ in retina and T-lymphocytes, while others are widely distributed in various cell types throughout the body. They execute unique functions in different cell types, such as muscle contraction, neurotransmitter release, and hormone secretion [1-3, 46, 47, 55, 56].

1.3.5 β Cell Ca_V channels

Electrically excitable β cells as a major cellular component of islets of Langerhans control glucose homeostasis by secreting insulin, which is the only hormone capable of lowering blood glucose in the body [1-4, 57]. The most elementary β cell activity glucose-stimulated insulin secretion indispensably relies on β cell Ca_V channels. General speaking, $Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$, $Ca_V2.2$, $Ca_V2.3$, $Ca_V3.1$ and $Ca_V3.2$ channels are present in β cells where they conduct all known types of Ca_V channel currents including L-type, N-type, P/Q-type, R-type and T-type [1-4, 57-59].

1.3.5.1 Heterogeneous expression of Ca_V channels in the β cell

The major type of Ca_V channels in the β cell is the Ca_V1 channel, which conducts L-type Ca^{2+} currents [1-4, 57-59]. The mouse islet β cell is equipped with the Ca_V1 channel as evidenced by the presence of L-type Ca^{2+} currents recorded at the whole-cell and single channel level. Whole-cell or unitary L-type Ca^{2+} currents have been well characterized in rat islet β cells. Like other species, human islet β cells also employ the Ca_V1 channel to conduct the principal Ca^{2+} currents as revealed by whole-cell and single channel analysis [1-4, 57-59].

The β cell also possesses other types of Ca_V channels such as the $Ca_V2.1$ channel, which mediates the P/Q-type Ca^{2+} currents [1-4]. $Ca_V2.1$ channels have been verified in the mouse islet β cell by patch-clamp analysis in conjunction with pharmacological dissection [60]. The rat islet β cell is also equipped with the $Ca_V2.1$ channel [61]. This channel has been identified in human islet β cells where 25 % of whole-cell Ca^{2+} currents are blocked by the $Ca_V2.1$ channel blocker ω-Aga IVA [62]. The identity and function of the $Ca_V2.2$ channel, a conduit of N-type Ca^{2+} currents, are most controversial in β cells [1, 2]. The null effect of

the $Ca_V2.2$ channel blocker ω-CTX GVIA on the mouse β cell Ca^{2+} currents denies the presence of the $Ca_V2.2$ channel [63]. As suggested from cytosolic free calcium concentration ($[Ca^{2+}]_i$) measurements in the presence of ω-CTX GVIA, the $Ca_V2.2$ channel is present in rat islet β cells [64]. It is uncertain whether $Ca_V2.2$ channels are expressed in human islet β cells [1, 2]. The genetic ablation of the $Ca_V2.3$ subunit gene in combination with pharmacological manipulations demonstrates that the $Ca_V2.3$ channel is expressed in the mouse islet β cell and mediates R-type Ca^{2+} currents [4, 65].

Although the low voltage-activated Ca_V3 channels, conduits of T-type Ca^{2+} currents, are expressed in a wide range of cell types, even in nonexcitable cells, T-type Ca^{2+} currents are absent in the normal mouse islet β cell [1, 2]. However, Ca_V3 channels appear as a phenotype of the diabetes-prone mouse islet β cells from NOD mice [66]. This suggests that the Ca_V3 channel genes are silenced in the normal mouse islet β cell. Such silenced genes can be activated in the diabetes-prone milieu in the NOD mouse islet β cell [1, 2, 66]. Rat islet β cells are equipped with Ca_V3 channels [67]. However, whole-cell T-type Ca^{2+} currents mediated by Ca_V3 channels in the rat islet β cells cannot be easily discriminated from high Ca_V channel currents [1, 2]. Single channel analysis convincingly shows the presence of the Ca_V3 channel in rat islet β cells [68, 69]. Both whole-cell and unitary T-type Ca^{2+} currents have been clearly observed in human islet β cells [1, 2, 59].

The Ca_V1 channel is the major β cell Ca_V channel in all species tested. However, obvious interspecies differences in expression of Ca_V channel types have been verified in β cells [1-3]. Primary β cells in different species are equipped with distinct sets of Ca_V channels. For example, Ca_V3 currents are clearly recorded in the human and rat β cell, but not in the mouse β cell [1-3]. It is worthwhile to note that much controversy still remains with respect to interspecies differences in expression of Ca_V channel types in β cells [1-3]. One has not yet reached consensus on species-specific expression of $Ca_V2.3$ channels in the human β cell. Some observations speak in favor of the presence of $Ca_V2.3$ channels, whereas others argue for the absence of these channels [1-3, 59]. $Ca_V1.2$ and $Ca_V1.3$ channels are visualized in β cells including human and rat islet β cells as well as the insulin-secreting cell lines HIT-15T, INS-1, RINm5F and βTC3 [1-3]. However, it is still uncertain whether functional $Ca_V1.3$ channels are present in mouse islet β cells [1-3]. This question was tackled during my PhD studies.

1.3.5.2 Physiological roles of Ca_V channels in the β cell

β cell Ca_V channels mediate Ca^{2+} influx to generate a key electrical signal across the β cell plasma membrane and a versatile second messenger in the β cell [1-4]. The former constitutes the upstroke or depolarization phase of the β cell action potential [1-4]. The latter manifests itself as $[Ca^{2+}]_i$ in the β cell. $[Ca^{2+}]_i$, resulting from Ca_V channel-mediated Ca^{2+} influx and its evoked Ca^{2+} release from intracellular stores, varies both spatially and temporally to form a variety of dynamic signals [1-4]. These signals activate Ca^{2+}-dependent enzymes and Ca^{2+}-binding proteins [1-4]. By means of these signals, β cell Ca_V channels participate in the regulation of all known molecular and cellular events including gene transcription, protein phosphorylation, exocytosis, endocytosis, proliferation,

differentiation, metabolism, survival and growth to guarantee β cell function and viability [1-4]. Genetic or pharmacological ablation of β cell Ca_V channels leads to overt defects in β cell development [2, 4, 65, 70]. The $Ca_V1.3$ and $Ca_V2.3$ channel knockout markedly disturbs postnatal pancreatic β cell generation and evidently impedes islet cell differentiation, respectively [4, 65, 70]. The Ca_V1 channel blockers D-600 and diltiazem significantly retard β cell proliferation [71, 72]. Hyperpolarization of β cells with the selective K_{ATP} channel opener diazoxide noticeably slows down β cell growth [71]. Expression of numerous β cell genes critically depends on Ca^{2+} influx through Ca_V channels [2, 73-76]. For example, Ca_V channel blockers effectively abrogate insulin gene transcription induced by glucose stimulation [2, 73, 74]. Most importantly, β cell Ca_V channels take center stage in glucose-stimulated insulin secretion to maintain glucose homeostasis [1-4]. For further details, see the section Roles of Ca_V channels in insulin secretion below.

1.3.5.2.1 Roles of Ca_V channels in insulin secretion

β Cell Ca_V channels participate in formation of a molecular network to precisely govern glucose-stimulated insulin secretion [1-4]. Blood glucose enters β cells via low-affinity/high-capacity glucose transporters. Subsequently glucose metabolism occurs in these cells leading to activation of a series of signal transduction events [1-4]. An increase in the ATP/ADP ratio, resulting from glucose metabolism, shuts off ATP-sensitive K^+ (K_{ATP}) channels, resulting in plasma membrane depolarization. This in turn leads to opening of Ca_V channels, allowing extracellular Ca^{2+} to enter the β cell. The consequent increase in $[Ca^{2+}]_i$ initiates direct interactions between exocytic proteins situated in the insulin secretory granule membrane and those localized in the plasma membrane. Such protein-protein interactions cause fusion of insulin secretory granules with the plasma membrane and thereby insulin secretion [1-4]. Actually, pancreatic islets release insulin in a biphasic manner in response to a step increase in glucose. This dynamic process displays a rapid initial phase, lasting for about 10 minutes, followed by a nadir, and subsequently a gradually increasing second phase, reaching a plateau after a further 25 to 30 minutes [2, 4, 77, 78]. Available observations support that all types of β cell Ca_V channels participate in the regulation of glucose-stimulated insulin secretion and that different types of β cell Ca_V channels selectively regulate the two distinct phases of dynamic insulin secretion [1-4, 58, 77-80].

The Ca_V1 channel plays a predominant role over other types of β cell Ca_V channels in Ca^{2+} triggered insulin exocytosis. Ca^{2+} influx through the Ca_V1 channel underlies 60-80 % of glucose-induced insulin secretion from mouse, rat and human islets [1-4]. Ca_V1 channels regulate both phases of insulin secretion but take a more prominent part in triggering insulin release during the first phase in the mouse islet [1-4]. The distinct contribution of $Ca_V1.2$ and $Ca_V1.3$ subtypes to insulin exocytosis in the human and rat islet β cell is not known, due to lack of appropriate experimental approaches [1-4].

$Ca_V2.1$ channels are heavily involved in glucose-stimulated insulin secretion from rat and human β cells [1-4]. The $Ca_V2.1$ channel blocker ω-Aga IVA partially inhibits high

Ca_V channel currents in the rat β cell and consequently reduces the DHP-resistant component of glucose-induced insulin secretion by about 30 % [1-4]. The human islet displays about 31 % reduction in the glucose-induced secretory response following incubation with ω-Aga IVA [59]. No definite verdict can be reached on the role of $Ca_V2.2$ channels in insulin secretion [1-4]. It has been shown that glucose-induced insulin secretion in human islets is insensitive to the $Ca_V2.2$ channel blocker ω-CTX GVIA. On the contrary, it has also been revealed that this blocker inhibits insulin secretion from human islets and the second phase glucose-induced insulin secretion from rat islets. However, it was speculated that the inhibitory effect of ω-CTX GVIA on the second phase insulin secretion may be attributed to toxic effects on the secretory process rather than effects on the $Ca_V2.2$ channel-mediated Ca^{2+} entry. Nevertheless, this is unlikely since the ω-CTX GVIA-treated islets exhibit normal first phase insulin secretion and intact high K^+-evoked insulin exocytosis. Further investigations demonstrate that block of β cell $Ca_V2.2$ channels by ω-CTX GVIA significantly dampens the generation of ATP. It is therefore likely that $Ca_V2.2$ channel-mediated Ca^{2+} entry underlies Ca^{2+}-dependent glucose metabolism and thereby facilitation of the generation of ATP, which is important for second phase insulin secretion [1-4]. Application of the $Ca_V2.3$ channel blocker SNX-482 manifests that $Ca_V2.3$ channels regulate insulin secretion from both INS-1 cells and mouse islets [2-4, 65]. Islets incubated with SNX-482 or isolated from $Ca_V2.3^{-/-}$ mice display a severe impairment in second phase glucose-stimulated insulin secretion, but a normal profile of first phase insulin secretion. The above results indicate that unlike the β cell Ca_V1 channels, which are situated at the exocytic site, the $Ca_V2.3$ channel seems to be distant from these sites in the β cell. Ca^{2+} influx through the $Ca_V2.3$ channel selectively regulates second phase insulin secretion, likely due to facilitation of insulin secretory granule trafficking from the reserve pool to the readily releasable pool/immediately releasable pool [2-4, 65].

The Ca_V3 channel-mediated Ca^{2+} entry likely contributes to the regulation of stimulus-secretion coupling in the β cell [1-3]. Block of Ca_V3 channels with lower concentrations of $NiCl_2$ significantly inhibits insulin secretion from INS-1 cells. Both glucose- and K^+-induced insulin secretion in perifused rat islets are attenuated by the nonselective Ca_V channel antagonist flunarizine, which targets both Ca_V1 and Ca_V3 channels [1-4].

It is tempting to speculate that β cell Ca_V1 channels self-organize into triplets and physically associate with syntaxin 1 and SNAP-25 to form local high $[Ca^{2+}]_i$-driven exocytic sites, whereas Ca_V2 channel-mediated Ca^{2+} influx contributes to global $[Ca^{2+}]_i$ to recruit insulin granules to exocytic sites [81, 82]. The differential localization and organization of Ca_V1 and Ca_V2 channels provide a reasonable explanation for selective regulation of first and second phase glucose-stimulated insulin secretion by Ca_V1 and Ca_V2 channels [81, 82].

Ca_V channels not only mediate Ca^{2+} entry to evoke insulin exocytosis, but also serve as mechanical components of the β cell exocytic machinery independent of Ca^{2+} influx [83-85]. The conformational change of the $Ca_V1.2$ subunit, driven by Ca^{2+} binding and depolarization, acts as a trigger to release an appreciable amount of insulin prior to Ca^{2+} influx. This has been substantiated by the following studies. The inorganic Ca_V1 channel

blocker La^{3+} binds to the polyglutamate motif (EEEE) in the selectivity filter of the Ca_V1 subunit and thereby blocking Ca^{2+} influx. Unexpectedly such a La^{3+} binding appreciably potentiates glucose-stimulated insulin secretion from rat pancreatic islets and ATP release in INS-1E cells. The effect is not involved in Ca^{2+} release from intracellular stores and abolished by the organic Ca_V1 channel blocker nifedipine. This indicates that the conformational switch of the $Ca_V1.2$ subunit evoked by La^{3+} binding to the EEEE motif suffices to support glucose-stimulated insulin secretion at the very early phase [84, 85]. Intriguingly, the $Ca_V\beta3$ subunit does not serve as a building block of β cell Ca_V channels. Instead, it interplays with the intracellular Ca^{2+} release machinery in a negative manner to restrain inositol 1,4,5-trisphosphate receptor-mediated Ca^{2+} mobilization from intracellular stores in the β cell and thus hinders insulin secretion [83].

1.3.5.2.2 Regulation of Ca_V channels in the β cell

The conductivity and density of β cell Ca_V channels undergo complex and exquisite regulation to guarantee Ca^{2+}-dependent β cell viability and ensure Ca^{2+}-dependent β cell activity and in particular glucose-stimulated insulin secretion, thereby satisfying various insulin demands of the body under different metabolic conditions [1-4]. Different types of important regulatory mechanisms of β cell Ca_V channels coordinate with each other in the context of physiology [1-4].

1.3.5.2.2.1 Protein phosphorylation

β cell Ca_V channels possess multiple protein phosphorylation sites and are typical substrates of multiple protein kinases and phosphatases. Phosphorylation drives conformational switch of Ca_V channels resulting in alteration in their Ca^{2+} conductivities. Activation of PKA up-regulates the activity of β cell Ca_V1 channels [2, 86-89]. PKC activation brings about complicated regulation on β cell Ca_V channels [90-92]. Pharmacological deprivation of PKC decreases L-, P/Q- and non-N-type Ca^{2+} currents to the same extent in insulin-secreting RINm5F cells. Acute activation of PKC induces a marginal increase in Ca_V channel currents, which are predominantly mediated by Ca_V1 channels, only in a small proportion of RINm5F cells (24 %) [2]. The PKG activator cGMP elevates Ca_V1 channel activity in rat pancreatic β cells [93]. However, the role of PKG-dependent phosphorylation in this case remains to be clarified since cGMP can directly act on Ca_V channels bypassing PKG [2, 94]. Likewise, it is not clear if calcium/calmodulin-dependent kinase II (CaMKII) catalyzes β cell Ca_V channel phosphorylation. The non-specific CaMKII inhibitors KN-62 and KN-93 do abrogate β cell Ca_V channel activity [95, 96]. However, these reagents can produce a direct action on β cell Ca_V channels without CaMKII involvement [2]. Inhibition of protein phosphatases with okadaic acid slightly elevates under basal conditions, but considerably increases Ca_V channel activity on top of activation of PKA in mouse pancreatic β cells [97]. This indicates that mouse β cell Ca_V channels just stay marginally phosphorylated under basal conditions [1-4].

β cell Ca_V channels are subjected to tyrosine phosphorylation [2, 98-101]. The nerve growth factor signaling pathway participates in β cell Ca_V channel regulation [2, 98, 99]. Long-term incubation (5-7 days) with nerve growth factor enriches the density of Ca_V1 channels in the β cell plasma membrane, whereas the acute administration of this nerve growth factor promotes the activity of these channels [2, 98, 99]. Activation of insulin receptors with the insulin mimetic L-783,281 induces an increase in $[Ca^{2+}]_i$ in the mouse pancreatic β cell. The effect is significantly abrogated by Ca_V1 channel blocker but no long occurs in insulin receptor substrate-1-knockout β cells [2, 101]. This opens up the possibility that acute activation of insulin receptors may up-regulate β cell Ca_V channel activity [2]. It is intriguing to verify this possibility with experimental approaches such as patch-clamp analysis.

1.3.5.2.2.2 G proteins and their downstream signals

Various G protein-coupled receptors, such as glucagon, glucagon-like peptide-1, somatostatin, opioid, galanin, adrenergic and muscarinic receptors, reside in the β cell plasma membrane to form complex signal systems with other plasma membrane proteins including Ca_V channels [2, 63, 102-119]. These receptors engage their partner G protein to serve as a brake on β cell Ca_V channel activity through physical association of G proteins with Ca_V channel pore-forming subunits. They also fine-tune β cell Ca_V channel activity by interacting with protein kinases and second messengers. Activation of glucagon, glucagon-like peptide-1 and opioid receptors augments β cell Ca_V channel activity, whereas stimulation of galanin and α_2-adrenergic receptors diminishes it. Concerning muscarinic receptors, their activation can lead to either the enhancement or reduction of β cell Ca_V channel activity dependent on cell types [2].

1.3.5.2.2.3 Syntaxin 1A, SNAP-25 and synatotagmin

Key exocytic proteins such as syntaxin 1A, SNAP-25 and synatotagmin are located in close proximity to β cell $Ca_V1.2$ and $Ca_V1.3$ channels in the β cell plasma membrane where they physically and functionally interact with each other to form an exocytic signalosome in β cells [1-3, 120, 121]. This signalosome bidirectionally conveys messages to regulate both β cell Ca_V1 channel activity and exocytic protein functionality [1-4, 120-122].

Syntaxin 1A have been demonstrated to interact with β cell $Ca_V1.3$ channels [1-3, 121]. Syntaxin 1A and $Ca_V1.3$ subunit not only colocalize in the β cell plasma membrane, but also are physically associated to form the syntaxin 1A/$Ca_V1.3$ subunit complex. Importantly, immunointerruption with anti-syntaxin 1A antibodies, which interferes with the formation of the $Ca_V1.3$ subunit/syntaxin 1A complex, causes a drastic rundown of β cell Ca_V1 channel activity. Furthermore, this treatment also dampens Ca^{2+}-induced insulin exocytosis in permeablized β cells, where Ca_V1 channels can no longer function. This demonstrates that the $Ca_V1.3$ subunit/syntaxin 1A complex mediates bidirectional signals to regulate insulin secretion through maintenance of both Ca_V channel activity and exocytic protein function [1-3, 121].

Syntaxin 1A and synatotagmin also physically associate with β cell $Ca_V1.2$ channels [1-3, 120]. Similar to the $Ca_V1.3$ subunit/syntaxin 1A complex, the $Ca_V1.2$ subunit/exocytic protein complex plays an important role in the regulation of insulin secretion by mediating bidirectional signals. However, signaling through this complex inhibits $Ca_V1.2$ channel activity. The inhibitory action of syntaxin 1A on $Ca_V1.2$ channels is partially counteracted by synaptotagmin. Obviously, the physical association of the $Ca_V1.2$ subunit with syntaxin 1A and synatotagmin delicately regulates $Ca_V1.2$ channel activity. In the β cell, the interruption of the physical association of the $Ca_V1.2$ channel with exocytic proteins completely blocks depolarization-evoked exocytosis, without significant influence on Ca_V channel activity [1-3, 120]. Clearly, the $Ca_V1.2$ subunit/exocytic protein complex also participates in maintenance of the efficacy of exocytic protein interaction.

Both the physical association and the functional interaction of Ca_V1 channels with SNAP-25 have been verified [1-3, 120, 122]. Interestingly, the long N-terminus (SNAP-25(1-197)) and the short C-terminus of SNAP-25 (SNAP-25(198-206)) regulate L-type Ca^{2+} currents differently. The former stimulates whereas the latter inhibits β cell L-type Ca^{2+} currents. It should be noted that inhibition of L-type Ca^{2+} currents by SNAP-25(198-206) is more prominent than stimulation of these currents by SNAP-25(1-197). These findings add an additional level of complexity to the regulation of β cell Ca_V1 channels [122].

1.3.5.2.2.4 Signals generated from glucose metabolism

Typically, glucose metabolism activates β cell Ca_V channels via a K_{ATP} channel-dependent pathway, which has been described in the section Roles of Ca_V channels in insulin secretion. In addition, glucose metabolism also yields a range of signals to regulate β cell Ca_V channels [1-3]. Acute application of high glucose promotes β cell Ca_V channel activity likely through glucose-metabolism-evoked activation of PKC [123]. Chronic exposure to either high or low glucose down-regulates expression of β cell Ca_V channels [2, 124, 125]. Glucose metabolism-derived inositol polyphosphates act as important signaling molecules to fine-tune β cell Ca_V1 channel activity [1, 2, 126]. Elevated inositol hexakisphosphate increases β cell Ca_V1 channel activity via inhibition of protein phosphatases and/or activation of the adenylyl cyclase-PKA cascade [1-4, 126, 127]. Complex changes in inositol polyphosphates by over-expressing multiple inositol polyphosphate phosphatase in a β cell line lead to enhanced Ca_V1 channel activity [1-3, 128].

1.3.5.2.2.5 Transthyretin, fatty acids and cholesterol

Several components in blood plasma are important to β cell Ca_V channels. The serum protein transthyretin up-regulate β cell Ca_V channel activity to augment glucose-stimulated insulin secretion [129]. Free fatty acids serve as ambivalent regulators of β cell Ca_V channels. The most abundant saturated plasma free fatty acid palmitate stimulates and inhibits β cell Ca_V channel currents at lower and higher concentrations, respectively [130]. Cholesterol also executes dual actions on β cell Ca_V channels [131, 132]. Chronic inhibition

of cholesterol biosynthesis gives rise to a significant reduction of β cell cholesterol. The resulting loss of cholesterol in β cells reduces both β cell Ca_V channel activity and insulin exocytosis. Cholesterol repletion efficiently reverses the effect resulting from chronic inhibition of cholesterol biosynthesis [132]. Incubation of mouse β cells with cholesterol-rich medium not only dampens glucose-stimulated mitochondrial ATP production, but also diminishes β cell Ca_V channel currents [131]. These studies emphasize that β cell Ca_V channels need the appropriate amount of serum components such as cholesterol and free fatty acids for its optimal function. They pinpoint that *in vivo* experimental approaches are indispensable to acquire knowledge on β cell Ca_V channels in genuine physiology [3].

1.3.5.3 Pathophysiological roles of Ca_V channels in the β cell

β Cell Ca_V channels become dangerous when they suffer from either inherited or acquired defects. These defective channels undergo exaggerated up- and down-regulation, disappearance and appearance, and inadequate redistribution in the β cell plasma membrane. They mediate insufficient or excessive Ca^{2+} influx resulting in β cell dysfunction and even β cell destruction since β cell viability and functionality critically rely on an appropriate range of $[Ca^{2+}]_i$, considerably contributed by β Cell Ca_V channel-mediated Ca^{2+} influx, at specialized subcellular domains [1-4].

1.3.5.3.1 Abnormal function of β cell Ca_V channels in diabetes

Abnormal function of β cell Ca_V channels occurs in diabetic animal models and diabetic patients (Yang S-N and Berggren P-O, unpublished observations) [1-3]. Both Ca_V1 and Ca_V3 currents markedly increase in β cells isolated from neonatal streptozotocin-induced diabetic (STZ) rats most likely due to an increase in the activity rather than in the number of these channels [133]. In fact, DHP binding sites and expression levels of Ca_V channel subunit genes even decrease in STZ rat β cells [133]. Furthermore, it is difficult to pinpoint the functional consequence of the increased activity of Ca_V channels in STZ rat β cells since glucose-stimulated $[Ca^{2+}]_i$ response and insulin secretion reduce in STZ rat islets [133-135]. Like in the STZ rat, both β cell Ca_V1 and Ca_V3 channels undergo hyperactivation in the Goto-Kakizaki (GK) rat, an animal model of human type 2 diabetes [136, 137]. Indeed, the hyperactivated Ca_V channels mediate an exaggerated Ca^{2+} influx, but trigger diminished exocytosis in the GK rat β cell [136, 137]. It remains elusive whether the hyperactivated Ca_V channels take charge of reduced functional β cell mass, a hallmark feature of the diabetic GK rat. It is tempting to postulate that the hyperactivated Ca_V channels may localize apart from exocytic sites to activate other Ca^{2+} influx-dependent activities, such as Ca^{2+}-dependent β cell death.

On the contrary, β cell Ca_V channels suffer from down-regulation in Zucker diabetic fatty (ZDF) rats [138]. At the prediabetic stage, $Ca_V1.2$ and $Ca_V1.3$ mRNAs in ZDF islets decrease by 28 and 38 %, respectively, and $[Ca^{2+}]_i$ in β cells and insulin-secretory response to glucose stimulation are substantially impaired. Unfortunately, Ca_V channel activity has not been characterized in the prediabetic β cell. The scenario becomes far worse in overtly diabetic ZDF rats. Their islets show 45 and 53 % reductions in $Ca_V1.2$ and $Ca_V1.3$ mRNAs,

respectively, little $[Ca^{2+}]_i$ response to K^+ depolarization or glucose stimulation and undetectable glucose-stimulated insulin release [138]. Similar down-regulation of expression of β cell Ca_V channel subunit genes also occurs in another type 2 diabetic model Otsuka Long-Evans Tokushima fatty (OLETF) rat [139]. $Ca_V1.3$, $Ca_V\beta2$ and β3 subunit mRNAs are considerably reduced in diabetic OLETF rat islets. Glucose-stimulated insulin release in these islets is significantly blunted. However, Ca_V channel currents in the OLETF rat β cell have not been examined [139]. The aforementioned two cases exemplify that the insufficient Ca^{2+} influx resulting from Ca_V channel down-regulation severely impairs β cell function and probably even disintegrates β cells [1-3].

1.3.5.3.2 Phenotypic variation of β cell Ca_V channels in diabetes

The types of Ca_V channels expressed in the β cell are genetically well defined [1-4]. However, the diabetic environment can vary the phenotypes of β cell Ca_V channels [1-4]. Ca_V3 channels do not reside in normal mouse pancreatic β cells, but emerge in pancreatic β cells isolated from 8-10 weeks old NOD mice [66]. The newly-expressed Ca_V3 channels are characterized by a fast inactivation (20-30 ms time constants at -20 mV), a lower threshold for activation (-50 mV), and a maximal activation at -20 mV. These channel-mediated Ca^{2+} influx contributes to a 3-fold increase in basal $[Ca^{2+}]_i$ [66]. This phenotypic variation likely results from cytokine insults surrounding β cells in the NOD mouse, an animal model of human T1D, at 8-10 weeks of age [66, 140-142]. Chronic exposure to a cytokine mixture of interferon-γ and interleukin-1β indeed activates expression of Ca_V3 channels in β cells from Swiss-Webster mice, a compatible control strain for NOD mice [1-3, 140]. In addition, cytokine treatment also evokes apoptotic β cell death [1-3, 140]. Such an apoptotic effect is efficiently diminished by pharmacological ablation of Ca_V3 channels [1-3, 140]. Obviously, in the context of T1D, cytokines selectively switch on expression of β cell Ca_V3 channels to destroy β cells in cooperation with the pathophysiological Ca^{2+} influx mediated by these newly expressed channels [1-3, 66, 140].

1.3.5.3.3 Genetic alteration of β cell Ca_V channels in diabetes

Both T1D and type 2 diabetes are polygenic diseases characterized by a progressive impairment in functional β cell mass [7, 13, 44, 143]. It is reasonable to consider genes critical for insulin secretion and β cell viability, as exemplified by Ca_V channel genes, as possible candidates for diabetes susceptibility [7, 13, 44, 143-146]. Although it is difficult to evaluate correlation between Ca_V channel gene mutations and diabetes, some interesting findings have emerged [1, 2, 145-148].

In a Japanese family, $Ca_V2.1$ subunit gene mutations cause spinocerebellar ataxia type 6 that is highly associated with type 2 diabetes [1, 2, 147]. Thirteen members in this five-generation family have suffered from spinocerebellar ataxia type 6. Three out of the five patients examined were diagnosed with overt type 2 diabetes. This $Ca_V2.1$ subunit mutant carries abnormal CAG repeat expansion encoding a polyglutamine tract [1, 2, 147]. Characterization of biophysical properties and surface expression of this mutant shows

inconsistent results [1, 2, 149, 150]. The work by Toru et al. showed that the polyglutamine-containing $Ca_V2.1$ subunit expressed in HEK 293 cells displayed a negative shift of voltage-dependent inactivation resulting in reduced Ca^{2+} influx [1, 2, 149]. In contrast, Piedras-Renteria et al. reported that the mutant $Ca_V2.1$ subunit did not alter its biophysical properties, but instead it was expressed more abundantly in the plasma membrane in HEK 293 cells, giving rise to higher Ca_V channel current density as compared to the wild-type $Ca_V2.1$ subunit [1, 2, 150]. Unfortunately, it is not known how the mutant $Ca_V2.1$ channel behaves in the β cell to contribute to the development of diabetes concurrent with spinocerebellar ataxia type 6 [1, 2, 147].

Evaluation of human $Ca_V1.3$ subunit gene polymorphisms in 918 Japanese type 2 diabetics and 336 control subjects revealed an ATG repeat expansion of this gene in type 2 diabetics [148]. However, this mutation occurs at low frequency and is not closely associated with the development of common type 2 diabetes. Nevertheless, it may cause a subgroup of this polygenic disease [1, 2, 148]. Association of the single nucleotide polymorphisms (SNPs) rs312480, rs312486 and rs9841978 in the human $Ca_V1.3$ subunit gene with impaired insulin secretion and type 2 diabetes has been examined in 8,987 non-diabetic Finnish and Swedish people and 2,830 Finnish and Swedish patients with type 2 diabetes. It shows that the SNP rs312480 is associated with decreased mRNA expression of the $Ca_V1.3$ subunit gene and reduced glucose-stimulated insulin secretion and that the SNPs rs312486 and rs9841978 are associated with type 2 diabetes [145]. The SNP rs673391 in the human $Ca_V2.3$ subunit has also been revealed to be associated with type 2 diabetes and impaired insulin secretion and in particular reduced second-phase insulin secretion by the Botnia Study Group in collaboration with others [146]. A genomewide linkage analysis has identified a novel neonatal diabetes locus mapped to chromosome 10p12.1-p13 in a large consanguineous family with autosomal recessively inherited neonatal diabetes [1, 2, 151]. This region comprises the $Ca_V\beta2$ subunit gene, which is predominantly expressed in the β cell, [152]. Furthermore, all affected individuals in this family showed low or even undetectable levels of circulating insulin indicating inadequate β cell mass and impaired insulin synthesis and secretion [1, 2, 151]. These findings suggest that the $Ca_V\beta2$ subunit gene should be one of potential susceptibility genes for neonatal diabetes [1, 2, 151]. It may also be true for a subgroup of type 2 diabetes developed from transient neonatal diabetes.

1.3.5.3.4 Destructive action of β cell Ca_V channels on β cells

Ca_V channels act as a central player in control of dynamics and homeostasis of $[Ca^{2+}]_i$ in the β cell [1-3]. In the context of pathology, they execute destructive action on β cells by mediating either deleteriously high or harmfully low Ca^{2+} influxes [1-3]. The former gives rise to Ca^{2+} overload and in turn triggers apoptotic and necrotic β cell death via various Ca^{2+}-sensitive enzymes, e.g., calpain, calcineurin, endonucleases and transglutaminase [1-3, 153-156]. The latter leads to Ca^{2+} deficiency and consequent β cell destruction through extinction of Ca^{2+}-dependent enzymatic reactions critical for β cell viability [1-3]. Additionally, physiological Ca^{2+} influx through β cell Ca_V channels can also serve as a permissive condition for initiation of some apoptosis. For example, interleukin 1β loses its

ability to induce β cell apoptosis upon pharmacological ablation of Ca_V1 channels-mediated Ca^{2+} influx [2, 156].

The Ca^{2+}-dependent, non-lysosomal cysteine protease calpain cleaves and activates caspase-12 and cyclin-dependent kinase 5 to mediate Ca^{2+}-dependent apoptosis [2, 157, 158]. It also stimulates calcineurin by cleaving the endogenous calcineurin inhibitor cain/cabin1 to provoke Ca^{2+}-dependent apoptosis [159]. Calcineurin is also activated by Ca^{2+}/calmodulin. This typical serine- and threonine-specific protein phosphatase dephosphorylates the pro-apoptotic protein Bad to trigger apoptotic events [2, 160]. When Ca_V channels becomes hyperactivated following PKC activation triggered by cytokine insult, calpain and calcineurin undergo sequential activation to execute β cell apoptosis via a cascade consisting of Bad, cytochrome c and caspases [155]. Ca^{2+}-dependent endonucleases cleave chromosomal DNA into oligonucleosomal size fragments, a biochemical hallmark of apoptosis [160]. These DNA cutting enzymes undergo activation to evoke Ca^{2+}-dependent β cell apoptosis when pathologically excessive Ca^{2+} ions flow through Ca_V1 channels into β cells [2, 154]. This is well exemplified by the fact that high glucose exposure induces typical Ca^{2+}-dependent apoptosis which is mimicked by the K_{ATP} channel blocker tolbutamide, abolished by either the K_{ATP} channel opener diazoxide or the Ca_V1 channel blocker D-600, and prevented by the endonuclease inhibitor aurintricarboxylic acid [2, 154]. The Ca^{2+}-activated transglutaminase 2 is ubiquitously expressed in mammalian cells including β cells where it extensively cross-links various cytoskeletal proteins and nucleosomal histones as the final steps of apoptosis [2, 161, 162]. Activation of transglutaminase 2 by lowering intracellular GTP can also initiate Ca^{2+}-dependent β cell apoptosis, which attenuates at low extracellular Ca^{2+} [2, 153, 160]. This suggests that this Ca^{2+}-dependent β cell apoptosis results from Ca_V channel-mediated Ca^{2+} influx [2].

These findings pinpoint β cell Ca_V channels as potential therapeutic targets for the prevention of β cell loss during the development of diabetes. As a matter of fact, it has been verified that systemic application of the Ca_V1 channel blocker verapmil satisfactorily ameliorates and even prevents low-dose streptozotocin-induced progressive diabetes in mice through reduction of β cell apoptosis and promotion of β cell survival and function [163].

1.4 T1D SERUM-INDUCED HYPERACTIVATION OF CA_V CHANNELS IN THE β CELL

Our group has pioneered research into T1D serum-induced hyperactivation of Ca_V channels in the pancreatic β cell and suggested that ApoCIII is likely to act as the actual factor in T1D serum to hyperactivate β cell Ca_V1 channels [5, 6].

1.4.1 T1D serum-induced hyperactivation of β cell Ca_V channels

Our earliest observation demonstrates that T1D serum hyperactivates β cell Ca_V1 channels driving excessive unphysiological Ca^{2+} influx into β cells and consequent Ca^{2+}-dependent β cell apoptosis [5]. In this observation, single channel analysis reveals that β cell Ca_V1

channels open more frequently following treatment with T1D serum. Correspondingly, whole-cell patch-clamp recordings show that a massive increase in Ca_V1 currents occurs in T1D serum-treated β cells. Consequently, such an abnormal Ca^{2+} influx brings about β cell Ca^{2+} overload as verified by ratiometric $[Ca^{2+}]_i$ measurements. The Ca^{2+} overload eventually provokes the appearance of typical apoptotic DNA "ladder" in β cells. The T1D serum-induced β cell apoptosis no longer occurs upon pharmacological ablation of Ca_V1 channels. This verifies that hyperactivated Ca_V channels operate as a conveyor for apoptotic cues from T1D serum to β cells [5]. To extend this observation, we have identified two obvious questions. First, do β cell Ca_V1 channels increase their conductivity, density or both in response to T1D serum exposure? Second, which Ca_V1 channel subtype(s) undergo hyperactivation when insulted with T1D serum?

1.4.2 ApoCIII mimics the action of T1D serum on β cell Ca_V channels

Our extended study reveals that significantly higher levels of ApoCIII arise in T1D sera. This apolipoprotein is capable of mimicking the action of T1D serum on β cell Ca_V channels. Treatment with ApoCIII dramatically elevates whole-cell Ca_V channel currents and $[Ca^{2+}]_i$ in the β cell and evidently induces β cell apoptosis. Immunoneutralization of ApoCIII with anti-ApoCIII antibodies competently abrogates both T1D serum- and ApoCIII-induced increases in $[Ca^{2+}]_i$ and apoptosis [6]. Unfortunately, the scope of the study does not cover full characterization of β cell Ca_V channels exposed to ApoCIII. The present PhD thesis sets out to mechanistically dissect the T1D serum- and ApoCIII-induced Ca_V channel hyperactivation staring with addressing the following two issues. One is which Ca_V channel type(s) are targeted by ApoCIII? The other is do β cell Ca_V channels increase their conductivity, density or both following ApoCIII treatment?

The signaling mechanisms whereby ApoCIII hyperactivates β cell Ca_V channels were not touched in the aforementioned study [6]. Available data do not hint at direct interactions between ApoCIII and Ca_V channels. Obviously, it is important to reveal how ApoCIII signals to β cell Ca_V channels. We proposed a signaling pathway sequentially involving ApoCIII, SR-BI, β1 integrin, PKA/PKC/Src and Ca_V channels in the β cell in terms of the following observations. They demonstrate that SR-BI not only physically associates with ApoCIII in a CHO heterologous expression system but also interacts with β1 integrin in microglial cells [30, 164]. ApoCIII stimulates PKC through indirect interaction with β1 integrin in monocytic cells [37]. Furthermore, β1 integrin activation can also increase Ca_V1 channel activity in neurons, ventricular myocytes and vascular smooth muscle cells through stimulation of PKA, PKC and Src kinase [165-168]. In fact, all these components are expressed in β cells [2, 169-172]. Therefore, we focused on this signaling pathway.

ApoCIII may impair β cell endocytosis to upregulate Ca_V channel density in the β cell plasma membrane. Endocytosis exquisitely internalizes membrane proteins and lipids, and thereby critically controlling the density of structural and functional components in the plasma membrane [173-175]. Mechanistically, endocytosis operates under direct control of a complex molecular network where the interaction of dynamin with syndapin/PACSIN takes center stage [174-178]. Syndapin was originally identified as a synaptic dynamin-

associated protein in the rat brain [179]. Its mouse homolog was independently discovered and named PACSIN symbolizing a protein kinase C (PKC) and casein kinase 2 (CK2) substrate in neurons [180]. Later, this neuron-specific isoform was called syndapin I/PACSIN 1 (PCS1) since other isoforms, syndapin II/PACSIN 2 and syndapin III/PACSIN 3, were detected in other tissues [176, 181-184]. β cell Ca_V channels critically rely on the endocytic machinery for their internalization to exquisitely control their density in the plasma membrane [173-175]. ApoCIII inhibits the endocytosis of cholesterol-rich LDLs [30-32]. Therefore, we have also devoted considerable efforts to investigating the possible involvement of PCS1-dependent endocytosis in ApoCIII-induced hyperactivation of Ca_V channels in the pancreatic β cell.

2 SPECIFIC AIMS

The overall goal of this PhD work is to mechanistically dissect T1D serum-induced hyperactivation of Ca_V1 channels in the pancreatic β cell. It includes the following specific aims:

1) To clarify if functional $Ca_V1.3$ channels are present in mouse islet $Ca_V1.2^{-/-}$ β cells;

2) To determine if T1D serum affects either or both of $Ca_V1.2$ and $Ca_V1.3$ channels in the β cell;

3) To verify if T1D serum alters Ca_V1 channel conductivity, Ca_V1 channel density, or both in the β cell plasma membrane;

4) To electrophysiologically confirm if ApoCIII in T1D serum acts as the actual factor enhancing β cell Ca_V channel currents;

5) To examine if ApoCIII selectively hyperactivates β cell Ca_V1 channels;

6) To elucidate if ApoCIII elevates both conductivity and number of Ca_V1 channels in the β cell plasma membrane;

7) To evaluate if ApoCIII influences β cell Ca_V1 channel expression;

8) To assess if SR-BI and β1 integrin mediate ApoCIII-induced hyperactivation of β cell Ca_V1 channels;

9) To investigate if PKA, PKC and Src kinase participate in ApoCIII-induced hyperactivation of β cell Ca_V1 channels;

10) To characterize if PCS1-mediated endocytosis regulates the number of Ca_V1 channels in the β cell plasma membrane;

11) To explore if ApoCIII impairs PCS1-mediated endocytosis to elevate the density of Ca_V1 channels in the β cell plasma membrane.

3 MATERIALS AND METHODS

3.1 ANIMALS

Adult male and female specific pathogen-free Wistar rats and C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). β Cell-specific $Ca_V1.2$ subunit-knockout ($Ca_V1.2^{-/-}$) mice and their corresponding heterozygous ($Ca_V1.2^{+/-}$) control mice were provided by Dr. Franz Hofmann (Institut für Pharmakologie und Toxikologie, Technische Universität München, Germany) [60]. General $Ca_V1.3$ subunit-knockout ($Ca_V1.3^{-/-}$) mice and wild type control ($Ca_V1.3^{+/+}$) mice were provided by Dr. Jörg Striessnig (Institute of Pharmacy, Pharmacology and Toxicology, University of Innsbruck, Austria) [185]. These knockout mouse lines were created on the C57BL/6 background. The animals were maintained on a regular light-dark cycle (lights on at 07.00 h and off at 19.00 h) in temperature and humidity-controlled rooms. They had free access to food pellets and tap water. All animal experiments were conducted according to the guidelines of the Animal Experiment Ethics Committee at Karolinska Institutet.

3.2 ISOLATION AND CULTIVATION OF ISLETS OF LANGERHANS

Rat islets of Langerhans were isolated using the in situ ductal perfusion technique. Briefly, rats were anesthetized with CO_2 and then killed by decapitation. The rat abdominal cavity was opened to expose the pancreas. About 15 ml of collagenase A solution (1.2 mg/ml; Roche, Basel, Switzerland) was injected into the pancreas through the common bile duct. The distended pancreas was quickly dissected, rinsed with Hank's buffered salt solution (HBSS) (Invitrogen, Carlsbad, CA, USA), placed into the collagenase A solution and digested under mild shaking for about 20 min at 37°C. The digestion was stopped by addition of HBSS. The digested pancreas was passed through 14 gauge needle several times. Dissociated islets were hand-picked up under a microscope.

Mouse islets of Langerhans were isolated using the standard chopped tissue technique. In short, the mice were killed by cervical dislocation. The pancreas was quickly dissected and cut into small pieces. Subsequently, the cut pancreatic tissue was digested with collagenase A (Roche) under vigorous shaking for 10-12 min at 37°C. The digested pancreatic tissue was rinsed twice with a solution containing 125 mM NaCl, 5.9 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, 0.1 % bovine albumin and 3 mM glucose, pH7.4. Dissociated islets were hand-picked up under a microscope.

Obtained islets were subjected to either cultivation in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 100 U/100 µg/ml penicillin/streptomycin (Invitrogen) at 37°C in a humidified 5 % CO_2 incubator or dissociation into single islet cells.

3.3 CELL CULTURE

Islet cell culture was performed according to the following procedures. Rat and mouse islets were dispersed into single islet cells in a Ca^{2+}-free medium containing 125 mM NaCl, 5.9 mM KCl, 2 mM EGTA, 25 mM HEPES and 1 % bovine serum albumin, pH 7.4. The

dispersed cells were incubated in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 100 U/100 µg/ml penicillin/streptomycin (Invitrogen) and maintained at 37°C in a humidified 5 % CO_2 incubator for 2-4 days.

HIT-T15 and RINm5F cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, and 100 U/100 µg/ml penicillin/streptomycin (Invitrogen). INS-1 cells were cultivated in RPMI 1640 medium containing the following additives: 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/100 µg/ml penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 50 µM β-mercaptoethanol (Invitrogen). MIN6-m9 cells were grown in DMEM medium with the following supplements: 10 % fetal bovine serum, 100 U/100 µg/ml penicillin/streptomycin, 11 mM glucose and 0.0005 % β-mercaptoethanol (Invitrogen). The cells were maintained at 37°C in a humidified 5 % CO_2 incubator and grown to approximately 70 % confluency before use.

3.4 PREPARATION OF SERA FROM HEALTHY HUMAN SUBJECTS AND PATIENTS WITH T1D

Blood samples were collected into test tubes from healthy blood donors and T1D patients, respectively. The samples were centrifuged and the resultant sera were aspirated into Eppendorf tubes. The collected sera were heat-inactivated at 56°C for 30 min and stored at -70°C until use. The T1D patients were newly diagnosed and only treated with exogenous insulin when their sera were collected. They had neither ketoacidosis nor severe hyperglycemia. The sodium, potassium and urea values of the collected T1D sera were within the normal range.

3.5 TREATMENTS

Islet cells from $Ca_V1.2^{-/-}$ and $Ca_V1.2^{+/-}$ mice as well as $Ca_V1.3^{-/-}$ and $Ca_V1.3^{+/+}$ mice were incubated overnight in RPMI 1640 medium containing 10 % healthy human serum, 10 % T1D patient serum or 10 % T1D patient serum plus rabbit polyclonal anti-ApoCIII antibodies (100 µg/ml, Academy BioMedical, Houston, TX, USA). Mouse islet $Ca_V1.2^{-/-}$ β cells were acutely exposed to the Ca_V1 channel blocker nimodipine (10 µM, Calbiochem, La Jolla, CA, USA). RINm5F cells were subjected to overnight treatment with ApoCIII, the PKA inhibitors H-89 (Calbiochem) and myristoylated PKI (14-22) (PKI, Sigma-Aldrich, St. Louis, MO, USA), the PKC inhibitor calphostin C (Calbiochem), the Src kinase inhibitor PP2 (Calbiochem) and the Ca_V1 channel blocker nimodipine (Calbiochem) in RPMI 1640 medium at final concentrations of 20 µg/ml, 0.5 µM, 1 µM, 0.1 µM, 0.1 µM and 5 µM, respectively. Mouse β cells were treated with ApoCIII (20 µg/ml) overnight. ApoCIII was dissolved in 0.1 % triflouroacetic acid (TFA) to make a stock solution of 1 mg/ml, PKI was dissolved in water to prepare a stock solution of 0.5 mM, whereas H-89, calphostin C, PP2 and nimodipine were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions of 5 mM, 1 mM, 1 mM and 10 mM, respectively. 0.002 % TFA and/or 0.03 % DMSO were used as corresponding vehicle controls. RINm5F cells were stimulated for 5 min with 10 mM glyceraldehyde plus 2.8 mM glucose or 30 mM KCl to induce regulated endocytosis.

3.6 siRNA SILENCING

21-mer siRNAs targeting the rat β1 integrin (β1 integrin siRNA1, ID127971 and β1 integrin siRNA2, ID127972), rat SR-BI (SR-BI siRNA, ID128929) as well as rat PCS1 (PCS1 siRNA1, ID S132057 and PCS1 siRNA2, ID S132056) were designed and chemically synthesized by Applied Biosystems/Ambion (Austin, TX, USA). Their sequences were confirmed by BLAST searches to ensure their specificity. Silencer® Select Negative Control siRNA (4390843) (NC siRNA), not targeting any gene product, and Silencer® Select GAPDH Positive Control siRNA (4390849), efficiently silencing GAPDH in human, mouse, and rat cells, were purchased from Applied Biosystems/Ambion (Austin, TX, USA). RINm5F cells were transfected with the aforementioned siRNAs using Lipofectamine® RNAiMAX (Invitrogen). In brief, NC siRNA, GAPDH siRNA, β1 integrin siRNA1, β1 integrin siRNA2, SR-BI siRNA, PCS1 siRNA1 or PCS1 siRNA2 was mixed with Lipofectamine® RNAiMAX followed by 20-min incubation at room temperature. Thereafter, cells were added to the siRNA/Lipofectamine® RNAiMAX mixtures followed by gentle agitation and kept at 37°C in a humidified 5 % CO_2 incubator. After 72 h, the transfected cells were grown to about 70 % confluency and knockdown efficiency was evaluated by immunoblot assay.

3.7 SEMIQUANTITATIVE RT-PCR

Total RNA was extracted from RINm5F cells using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA). Oligonucleotide primer pairs for RT-PCR amplification were synthesized by Sigma-Aldrich. The SR-BI primer pair included the forward primer 5'-CAAGAAGCCAAGCTGTAGGG-3' and the reverse primer 5'-CCCAACAGGCTCTACTCAGC-3'. The GAPDH primer pair consisted of the forward primer 5'-TAGACAAGATGGTGAAGG-3' and the reverse primer 5'-TCCTTGGAGGCCATGTAG-3'. 500 ng of total RNA was reverse transcribed using SuperScript® II Reverse Transcriptase (Invitrogen) and Oligo(dT)12-18 Primer (Invitrogen). Polymerase chain reaction was performed using the Platinum® Taq DNA Polymerase (Invitrogen). The reaction underwent 90 seconds at 94°C for completely denaturing templates and activating the Taq DNA Polymerase, followed by 29 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, and ending with a final extension at 72°C for 5 min. The amplified PCR products were analyzed by 2 % agarose gel electrophoresis and ethidium bromide staining.

3.8 SINGLE CELL RT-PCR

The entire islet cell was collected with a glass pipette after a patch-clamp recording and stored in a PCR tube with 10 µl 1x Taq DNA polymerase reaction buffer containing $MgCl_2$ (Promega, Madison, WI, USA) at -70°C for later use. Expression of insulin mRNA in collected single cells was determined using the QIAGEN OneStep RT-PCR Kit (Valencia, CA, USA) according to the manufacturer's instructions. The RNasin Plus RNase Inhibitor (Promega) was used to avoid damaging mRNA. The insulin primer pair for RT-PCR analysis was synthesized by Sigma-Aldrich. It comprised the forward primer 5'-

CAGCAAGCAGGTCATTGTTT-3' and the reverse primer 5'-CAGTAGTTCTCCAGCTGGTAGA-3'. These primers were added at a final concentration of 0.6 μM to the reaction mix. The reaction was amplified for 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min. The amplified PCR products were resolved by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining.

3.9 SDS-PAGE AND IMMUNOBLOT ANALYSIS

Mouse islets and exocrine tissue were isolated using the standard chopped tissue technique. The pancreas, brain, heart, kidney, liver, lung, muscle and spleen were quickly dissected out from cervically dislocated mice. The harvested tissues as well as insulin-secreting HIT-T15, INS-1, MIN6-m9 and RINm5F cells were homogenized on ice in 250 μl of a homogenization buffer (pH 7.4) consisting of 20 mM HEPES, 1 mM $MgCl_2$, 2 mM EDTA, 250 mM sucrose, 1 mM PMSF and a protease inhibitor cocktail (Roche). The resultant homogenates were pelleted at 800 x *g* for 10 min to remove nuclei, unbroken cells and debris. The protein concentration of the pelleted homogenates was measured with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). These homogenate samples were then denatured by heating at 96ºC for 3 min in SDS sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis. In brief, 45-180 μg proteins were separated in discontinuous gels consisting of a 4 % acrylamide stacking gel (pH 6.8) and a 8 % acrylamide separating gel (pH 8.8). The separated proteins were then electrophoretically transferred to hydrophobic polyvinylidene difluoride membrane (Hybond-P; Amersham, Buckinghamshire, UK). The blots were blocked by incubation for 1 h with 5 % non-fat milk powder in a washing buffer consisting of 50 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl and 0.05 % Tween 20 (pH 7.5). The blocked blots were incubated overnight at 4ºC with affinity-purified rabbit polyclonal antibodies against β1 integrin (1:500; Millipore, Billerica, MA, USA), $Ca_V1.2$ (1:200), $Ca_V1.3$ (1:200), SR-BI (1:2500; Novus, Cambridge, UK) and PCS1 (1:1000), respectively, and for 1 h at room temperature with mouse monoclonal antibody to GAPDH (1:4000; Applied Biosystems/Ambion). Subsequently, the blots were rinsed with the washing buffer and incubated with the secondary antibodies (either horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-mouse IgG; 1:50,000; Bio-Rad) at room temperature for 45 min. The immunoreactive bands were visualized with the ECL plus Western blotting detection system (GE Healthcare, Uppsala, Sweden). Anti-$Ca_V1.2$ and anti-$Ca_V1.3$ antibodies were provided by Dr. William A. Catterall (Department of Pharmacology, University of Washington, WA, USA). Anti-PCS1 antibodies were provided by Dr. Britta Qualmann (Institute for Biochemistry I, Friedrich Schiller University Jena, Jena, Germany).

3.10 IMMUNOCYTOCHEMISTRY

Isolated rat and mouse islets as well as cultured rat and mouse islet cells were subjected to double immunolabeling. Isolated islets were fixed with 4 % paraformaldehyde for 90 min. Cultured rat and mouse islet cells on glass coverslips were fixed in 2 % paraformaldehyde for

30 min. Both the fixed islets and cultured cells were incubated with 5 % normal goat serum (Sigma-Aldrich) for 1 h to block nonspecific staining. Subsequently, the specimens were double-labeled with rabbit polyclonal antibodies to PCS1 (1:200) and guinea pig polyclonal antibodies to insulin (1:200; Dako, Glostrup, Danmark) at 4°C overnight. They were then incubated with goat anti-rabbit or anti-guinea pig IgG coupled to Alexa 488 or Alexa 633 (1:200; Molecular Probes, Eugene, OR, USA) for 20 min at room temperature. Negative controls included omission of the primary antibodies, incubation with nonimmune IgG from corresponding species and preabsorption of the anti-insulin (1:200) with bovine insulin (1000 μg/ml). The specimens were mounted in ProLong Gold Antifade (Molecular Probes), coverslipped and sealed with nail polish.

3.11 CONFOCAL MICROSCOPY AND DECONVOLUTION ANALYSIS

Double immunofluorescence-labeled islets and islet cells were visualized with a Leica TCS-SP5II-AOBS confocal laser-scanner equipped with a 405 nm Diode laser, an Argon laser (458, 476, 488, 496, 514 nm lines), a 561 nm DPSS lasers, a 594 nm HeNe laser and a 633 nm HeNe laser and connected to a Leica DM6000 CFS microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Alexa 488 linked to goat anti-rabbit and Alexa 633 conjugated to goat anti-guinea pig IgG were excited by 488 and 633 nm laser lines, respectively, and the resultant emissions were collected at 499-537 and 648-734 nm, respectively. Optical sections were acquired using Leica HCX IRAPO L 25x/0.95 water and HCX PL APO 100x/1.44 oil objectives. The confocal images were processed and deconvoluted with Huygens Essential (Scientific Volume Imaging, Hilversum, Netherlands).

Live-cell confocal imaging of FM1-43 was conducted on RINm5F cells. The cells were incubated for 30 min with RPMI 1640 medium containing no glucose and then for 10 min with extracellular solution consisting of 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 HEPES and 0.1 % bovine serum albumin. Subsequent addition of FM1-43 (Molecular Probes) at a concentration of 5 μM proceeded. Some cells were maintained in the extracellular solution for further 60 min for constitutive endocytosis assay and other cells were stimulated for 5 min with 10 mM glyceraldehyde plus 2.8 mM glucose for regulated endocytosis measurements. FM1-43 accumulation in the cells was visualized with a Leica TCS-SP2 confocal laser-scanner connected to a Leica DMIRBE microscope (Leica). FM1-43 was excited by a 488 nm laser line and the resultant emission was collected using a Leica PL APO 100x/1.40 oil objective at 540-650 nm. FM1-43 fluorescence images were acquired at 37ºC. Quantification of intracellular FM1-43 fluorescence intensity was done using Leica Confocal Software (Leica).

3.12 ELECTROPHYSIOLOGY

RINm5F cells and mouse islet cells from $Ca_V1.2^{-/-}$ and $Ca_V1.2^{+/-}$ mice as well as $Ca_V1.3^{-/-}$ and $Ca_V1.3^{+/+}$ mice were subjected to different patch-clamp measurements. The cell-attached, conventional whole-cell and perforated whole-cell configurations of the patch-clamp technique were employed to register single Ca_V channel currents and whole-cell Ca_V channel currents, respectively. Recording electrodes were pulled from borosilicate glass capillaries

(Hilgenberg, Malsfeld, Germany), fire-polished on a horizontal programmable puller (DMZ Universal Puller, Zeitz-Instrumente, Augsburg, Germany) and then coated with Sylgard close to their tips. The electrode resistance ranged between 4 and 6 MΩ when electrodes were filled with electrode solutions and immersed in bath solutions. The electrode offset potential was corrected in bath solutions prior to gigaseal formation.

Cell-attached single Ca_V channel current recordings were made with an Axopatch 200B amplifier together with the software program pCLAMP 10 (Molecular Devices, Foster City, CA, USA). Electrodes were filled with a solution containing 110 mM $BaCl_2$, 10 mM tetraethylammonium chloride and 5 mM HEPES-$Ba(OH)_2$ (pH 7.4). Ba^{2+} was used as the charge carrier. A depolarizing external recording solution, containing 125 mM KCl, 30 mM KOH, 10 mM EGTA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-KOH (pH 7.15), was used to bring the intracellular potential to ~0 mV. Voltage pulses (200 ms) were applied at a frequency of 0.5 Hz to depolarize cells from a holding potential of -70 mV to a membrane potential of 0 mV. Resulting currents were filtered at 1 kHz and digitized at 5 kHz. All recordings were made at room temperature (about 22°C). Acquisition and analysis of data were done using the software program pCLAMP10 (Molecular Devices).

Whole-cell Ca_V channel current data were acquired with an Axopatch 200B amplifier together with the software program pCLAMP 10 (Molecular Devices) or an EPC-9 patch-clamp amplifier together with the software program PatchMaster (HEKA Elektronik, Lambrecht/Pfalz, Germany). For conventional whole-cell recordings, electrodes were filled with a solution consisting of 150 mM N-methyl-D-glucamine, 125 mM HCl, 10 mM EGTA, 1.2 mM $MgCl_2$, 3 mM MgATP, and 5 mM HEPES (pH 7.15). In perforated whole-cell patch-clamp experiments, the electrode solution contained 76 mM Cs_2SO_4, 1 mM $MgCl_2$, 10 mM KCl, 10 mM NaCl, and 5 mM HEPES (pH 7.35), as well as amphotericin B (0.24 mg/ml) to permeabilize the plasma membrane and allow low-resistance electrical access without breaking the patch. Cells used for both conventional and perforated whole-cell recordings were bathed in a solution containing 138 mM NaCl, 10 mM tetraethylammonium chloride, 10 mM $CaCl_2$, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES and 3 mM glucose (pH 7.4). After obtaining a seal, the holding potential was set to -70 mV during the course of an experiment. Depolarizing voltage pulses (100 ms) were made from a holding potential of -70 mV to several test potentials from -60 to 50 mV in 10 mV increments at 0.5 Hz. Resulting currents were filtered at 1 kHz and digitized at 5 kHz. All recordings were made at room temperature (about 22°C). Data analysis were done using pCLAMP10 (Molecular Devices) or FitMaster (HEKA). The amplitude of whole-cell currents was normalized to cell capacitance. To ensure exclusion of rapid transient Na^+ currents appearing at the initial period of depolarization during whole-cell Ca_V channel current recordings in RINm5F cells, we measured peak whole-cell Ca_V channel currents within a time window from 30 ms to 100 ms after start of depolarization.

3.13 STATISTICAL ANALYSIS

All data are presented as mean ± SEM. The non-parametric Kruskal-Wallis test or one-way ANOVA followed by least significant difference (LSD) test was used to compare multiple

groups. The non-parametric Mann-Whitney *U* test or unpaired Student's *t* test was employed to compare two groups. The significance level was set to 0.05.

4 RESULTS AND DISCUSSION

4.1 FUNCTIONAL $CA_V1.3$ CHANNELS ARE PRESENT IN A SUBGROUP OF MOUSE ISLET $CA_V1.2^{-/-}$ β CELLS (PAPER I)

$Ca_V1.3$ channels are expressed far more richly than $Ca_V1.2$ channels in human β cells [59, 145, 186]. $Ca_V1.3$ channel polymorphisms are associated with diabetes [145, 148]. However, it still remains elusive whether functional $Ca_V1.3$ channels reside in mouse pancreatic β cells [1-3]. In the present PhD work, we examined the presence of functional $Ca_V1.3$ channels in mouse islet $Ca_V1.2^{-/-}$ β cells by combining whole-cell patch-clamp analysis and single cell RT-PCR assay [187]. The $Ca_V1.3$ subunit-expressing cells possessed larger capacitance (> 7 pF) and typical Ca_V1 currents without superimposed voltage-gated Na^+ currents (Fig. 1a in Paper I). Importantly, they expressed insulin mRNA (Fig. 1b in Paper I). These electrophysiological criteria and molecular evidence ensure their β cell identity. Among 15 examined cells, 3 cells exhibited DHP-sensitive Ca_V1 currents that were blocked by nimodipine (Fig. 1a in Paper I). These data reveal that functional $Ca_V1.3$ channels are present in 20 % mouse islet $Ca_V1.2^{-/-}$ β cells [187]. Thus, the $Ca_V1.2^{-/-}$ mouse provides a convenient small animal model for understanding how T1D serum attacks β cells through $Ca_V1.3$ channels.

There has been considerable debate concerning the presence of functional $Ca_V1.3$ channels in the mouse islet β cell due to technical limitations [1, 2, 60, 70, 121, 188, 189]. Indeed, $Ca_V1.3$ channels differ appreciably from $Ca_V1.2$ channels in activation threshold and DHP sensitivity when heterologously expressed in *Xenopus* oocytes or mammalian cells [190, 191]. However, these electrophysiological and pharmacological differences are too small to be used for reliable discrimination between native $Ca_V1.2$ and $Ca_V1.3$ channels in islet β cells. Therefore, the presence and function of $Ca_V1.3$ channels in the mouse islet β cell remain controversial [1, 2]. The β cell-specific $Ca_V1.2$ subunit knockout causes a significant reduction in Ca_V channel currents and insensitivity of Ca_V channels to isradipine (2 μM) and Bay K8644 (1 μM) [60]. Genetic ablation of the DHP-sensitivity of $Ca_V1.2$ subunits renders Ca_V channel currents insensitive to isradipine (2 μM) and Bay K8644 (2 μM) in mouse islet β cells [188]. Hence, these results speak in favor of that only $Ca_V1.2$ subunits conduct Ca_V1 currents in mouse islet β cells.

However, a series of observations argue against the conclusion that only $Ca_V1.2$ subunits conduct Ca_V1 currents in mouse islet β cells [1, 2, 70, 121, 189]. The $Ca_V1.3$ subunit gene is more than 155 kb long and contains 49 exons. This multi-exon gene can undergo complex alternative splicing to produce numerous splice variants of the $Ca_V1.3$ subunit [192, 193]. The distinct splice variants of $Ca_V1.3$ channels possess different sensitivities to DHPs [190, 194]. This is explicitly exemplified in mouse hair cells where an isoform of $Ca_V1.3$ channels displays a rather low sensitivity to various Ca_V1 channel blockers [194]. Nifedipine at 10 μM blocks about 40 % of the Ca^{2+} current through this isoform. In striking contrast, nifedipine at 100 nM can completely ablate $Ca_V1.2$-mediated Ca^{2+} currents [194]. Furthermore, $Ca_V1.3$ subunit mRNAs and proteins have convincingly been detected in mouse islet β cells [70, 121, 189]. Genetic ablation of $Ca_V1.3$ subunits abrogates basal insulin secretion from mouse islets

[70]. Importantly, we now verify that a proportion of mouse islet $Ca_V1.2^{-/-}$ β cells express functional $Ca_V1.3$ channels [187]. These observations support the existence of functional $Ca_V1.3$ channels in mouse islet β cells.

4.2 T1D SERUM HYPERACTIVATES BOTH $CA_V1.2$ AND $CA_V1.3$ CHANNELS BY INCREASING THEIR CONDUCTIVITY AND NUMBER IN THE β CELL PLASMA MEMBRANE (PAPER I)

To characterize influences of T1D serum on β cell $Ca_V1.3$ channel behaviors, we performed cell-attached single-channel recordings in mouse islet $Ca_V1.2^{+/-}$ and $Ca_V1.2^{-/-}$ β cells [187]. In comparison to healthy serum, T1D serum not only made Ca_V1 channels spend more time in their open state, but also elevated the number of functional Ca_V1 channels, reflected by more unitary conductance levels (more layers of unitary Ba^{2+} currents) in both $Ca_V1.2^{+/-}$ and $Ca_V1.2^{-/-}$ β cells (Fig. 2a and c in Paper I). There are significant increase in the number, open probability and mean open time of Ca_V1 channels and significant decrease in the mean closed time of these channels in both $Ca_V1.2^{+/-}$ and $Ca_V1.2^{-/-}$ β cells exposed to T1D serum (Fig. 2b and d in Paper I).

Next, we characterized T1D serum-induced alterations of β cell $Ca_V1.2$ channels in mouse islet $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells by using perforated whole-cell and cell-attached single-channel patch-clamp techniques [187]. Whole-cell patch-clamp measurements revealed that T1D serum prominently increased whole-cell Ca_V channel currents in $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ cells leading to significant elevation of whole-cell Ca_V channel current density in comparison to healthy serum (Fig. 3 in Paper I). Cell-attached single-channel analysis showed that both $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ cells subjected to T1D treatment displayed more single Ca_V1 channels, manifested by more layers of unitary Ba^{2+} currents, and stayed open longer in comparison to those exposed to healthy serum (Fig. 4a and c in Paper I). The number, open probability and mean open time of single Ca_V1 channels following T1D serum treatment were significantly greater than those exposed to healthy serum. The mean closed time of single Ca_V1 channels exposed to T1D serum incubation was significantly shorter than that subjected to healthy serum treatment (Fig. 4b and d in Paper I).

A prerequisite for curing the polygenic metabolic disorder diabetes is a clear identification of individual genes involved [195-199]. We have previously revealed that hyperactivated β cell Ca_V1 channels by T1D serum mediate pathologically-exaggerated Ca^{2+} influx into pancreatic β cells leading to β cell apoptosis [5]. Two molecular subtypes of Ca_V1 channels, $Ca_V1.2$ and $Ca_V1.3$, are present in β cells [1-4]. It is important to know if T1D serum targets either or both of these two Ca_V1 subtypes in terms of prevention of Ca^{2+}-dependent β cell apoptosis. To gain such knowledge, we have carried out the present study. It turns out that T1D serum drives hyperactivation of both of these two Ca_V1 subtypes [187]. This finding pinpoints that $Ca_V1.2$ and $Ca_V1.3$ channels in the β cell are vulnerable to the attack of T1D serum, that their hyperactivation most likely underlies the molecular pathogenesis of T1D and that both $Ca_V1.2$ and $Ca_V1.3$ channels in the β cell should be considered to be target molecules for preventing and treating diabetes.

In our previous work, we revealed that T1D serum significantly elevates the amplitude of whole-cell and average unitary Ca_V1 currents in the β cell [5]. This could result from the enriched density and/or increased conductivity of these channels in the β cell plasma membrane. To determine which scenario occurred, the present work has now carefully characterized how T1D serum influences Ca_V1 channel behaviors. The obtained data on the biophysical properties of single Ca_V1 channels in $Ca_V1.2^{+/-}$, $Ca_V1.2^{-/-}$, $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells exposed to T1D serum enable us to mechanistically interpret T1D serum-induced hyperactivation of β cell Ca_V1 channels [187]. The Ca_V1 channel hyperactivation happens due to both the increased conductivity and elevated number of functional Ca_V1 channels in the recorded area of the β cell plasma membrane. The former is exhibited as an increased open probability attributed to the prolonged mean open time and shortened mean closed time. The latter is manifested as more levels of single Ca_V1 channel conductance [187]. These findings suggest that this T1D serum-induced pathological event likely involves multiple mechanisms to which attention should be drawn for effective intervention.

4.3 APOCIII IN T1D SERUM ACTS AS THE ACTUAL FACTOR ENHANCING β CELL CA_V CHANNEL CURRENTS

To electrophysiologically verify if elevated ApoCIII in T1D serum enhances Ca_V1 channel currents in the β cell, we examined the effect of immunoneutralization of ApoCIII with anti-ApoCIII antibodies on T1D serum-induced enhancement of Ca_V channel currents in mouse islet β cells by using perforated whole-cell patch-clamp analysis. It turned out that whole-cell Ca_V channel currents were larger in T1D serum-treated cells than in cells exposed to healthy serum (Fig. 1A). Importantly, whole-cell Ca_V channel currents in cells exposed to T1D serum pre-absorbed with anti-ApoCIII antibodies were similar to those appearing in healthy serum-treated cells (Fig. 1A). Whole-cell Ca_V channel current densities were significantly higher in T1D serum group than in healthy serum group (Fig. 1B). However, whole-cell Ca_V channel current densities measured in cells incubated with T1D serum pre-absorbed with anti-ApoCIII antibodies were not statistically different from those visualized in healthy serum-treated cells (Fig. 1B). It is clear that ApoCIII-depleted T1D serum could no longer enhance Ca_V channel currents in the β cell.

Indeed, our previous studies demonstrate that T1D serum contains elevated ApoCIII. Incubation with T1D serum significantly enhances $[Ca^{2+}]_i$ response to K^+ depolarization and thereby promotes β cell death [5, 6]. Pre-absorption of T1D serum with anti-ApoCIII antibodies efficiently prevents the enhanced $[Ca^{2+}]_i$ response and β cell death [6]. Exposure to either T1D serum or ApoCIII hyperactivates β cell Ca_V channels [5, 6]. These documented results indicate that ApoCIII in T1D serum is most likely to act as the actual player in hyperactivation of β cell Ca_V channels [5, 6, 200]. However, these findings did not electrophysiologically verify this indication [5, 6, 200]. We now show for the first time that ApoCIII-depleted T1D serum is incapable of enhancing Ca_V channel currents in the β cell. This confirms that ApoCIII indeed is the molecular entity in T1D serum responsible for hyperactivation of β cell Ca_V channels.

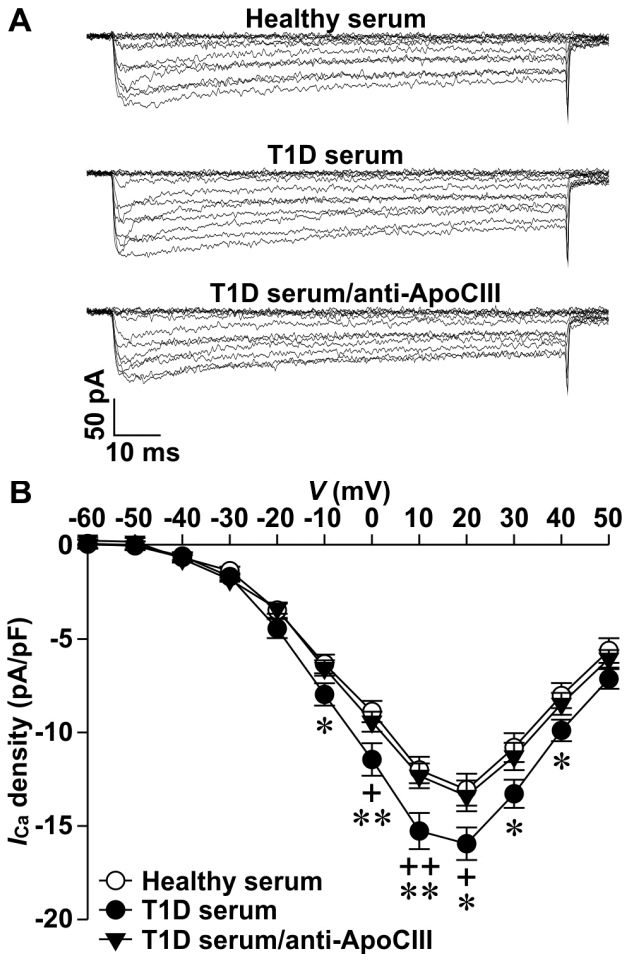

Figure 1. Immunoneutralization of ApoCIII blocks T1D serum-induced enhancement of Ca_V channel currents in the β cell. A: Sample whole-cell Ca_V channel current traces registered in mouse islet β cells following incubation with healthy serum (upper panel, cell capacitance: 5.22 pF), T1D serum (middle panel, cell capacitance: 5.29 pF) and T1D serum preabsorbed with anti-ApoCIII antibodies (lower panel, cell capacitance: 5.46 pF), respectively. B: Ca_V channel current density-voltage relationships in cells exposed to healthy serum (open circles, n = 32), T1D serum (filled circles, n = 32) or T1D serum preabsorbed with anti-ApoCIII antibodies (filled triangles, n = 32). *P < 0.05 and **P < 0.01 versus healthy serum. +P < 0.05 and ++P < 0.01 versus T1D serum plus anti-ApoCIII antibodies.

4.4 APOCIII SELECTIVELY HYPERACTIVATES β CELL CA_V1 CHANNELS (PAPER II)

To clarify what type of β cell Ca_V channels was altered in response to ApoCIII exposure, we selected RINm5F cells, which harbor the richest types of Ca_V channels among all available β cell types, for whole-cell patch-clamp analysis in combination with pharmacological discrimination [1-3, 200]. The obtained data show that ApoCIII in the absence of the Ca_V1 channel blocker nimodipine markedly increased whole-cell Ca_V channel currents in comparison to vehicle solution (Fig. 2a in Paper II), whereas this apolipoprotein could no longer produce such an effect in nimodipine-pretreated cells (Fig. 2c in Paper II). Whole-cell Ca_V channel current densities were significantly higher in the ApoCIII group than in the control group in the absence of nimodipine (Fig. 2b in Paper II) and very similar in the presence of nimodipine (Fig. 2d in Paper II). These data verify that ApoCIII selectively acts on β cell Ca_V1 channels [200]. These findings further confirm that the β cell Ca_V1 channel can be a target molecule for prevention of Ca^{2+}-dependent β cell destruction induced by the diabetogenic serum factor ApoCIII.

4.5 APOCIII ELEVATES BOTH THE CONDUCTIVITY AND NUMBER OF CA_V1 CHANNELS IN THE β CELL PLASMA MEMBRANE (PAPER II)

To determine whether the density and/or conductivity of β cell Ca_V channels changed in response to ApoCIII treatment, we analyzed unitary Ca_V1 channel currents, characterized by

a large unitary Ba^{2+} conductance with long-lasting openings, in mouse islet β cells and insulin-secrecting RINm5F cells following ApoCIII incubation (Fig. 1 in Paper II) [200]. We visualized that plasma membrane patches of ApoCIII-incubated cells accommodated more Ca_V1 channels than those of vehicle-treated cells (Fig. 1a and c in Paper II). Ca_V1 channels exposed to ApoCIII opened more frequently than those bathed in vehicle solution (Fig. 1a and c in Paper II). ApoCIII incubation significantly increased channel number, elevated open probability, prolonged mean open time and shortened mean closed time of Ca_V1 channels in comparison to exposure to control vehicle (Fig. 1b and d in Paper II). Apparently, both conductivity and number of β cell Ca_V1 channels undergo elevation in response to ApoCIII treatment [200]. We now reveal that ApoCIII treatment boosts both the density and conductivity of β cell Ca_V1 channels [200]. The aforementioned results demonstrate that ApoCIII indeed is the molecular entity in T1D serum responsible for selective hyperactivation of β cell Ca_V1 channels and that such hyperactivation occurs due to both the increased conductivity and elevated number of these channels in the plasma membrane [200].

4.6 APOCIII DOES NOT INFLUENCE β CELL CA_V1 CHANNEL EXPRESSION (PAPER II)

Appearance of ApoCIII-induced hyperactivation of β cell Ca_V channels requires overnight incubation. Hence, the effect might be attributed to an increase in Ca_V channel expression. To clarify such a possibility, we quantified β cell Ca_V1 channel expression in RINm5F cells following ApoCIII incubation [200]. Immunoblot analysis revealed that control and ApoCIII-treated samples gave similar intensities of $Ca_V1.2$, $Ca_V1.3$ and GAPDH immunoreactivities (Fig. 6a in Paper II). There was no significant difference in the relative abundance of $Ca_V1.2$ and $Ca_V1.3$ subunits in RINm5F cell homogenates subjected to ApoCIII incubation in comparison to vehicle incubation (Fig. 6b in Paper II). The data firmly exclude the possibility that ApoCIII elevates β cell Ca_V1 channel expression [200].

4.7 APOCIII HYPERACTIVATES β CELL CA_V CHANNELS THROUGH SR-BI AND β1 INTEGRIN (PAPER II)

Available evidence that β1 integrin signals not only downstream of ApoCIII but also upstream of PKA, PKC and Src kinase to upregulate Ca_V channels in other cell types made us postulate the possibility that β1 integrin may mediate ApoCIII-induced hyperactivation of β cell Ca_V channels [37, 165-168]. We tested this postulation by implementing RNA interference in combination with whole-cell Ca_V channel current analysis in RINm5F cells [200]. The obtained results show that transfection with two β1 integrin siRNAs significantly reduced β1 integrin protein expression (Fig. 7a and b in Paper II). Importantly, β1 integrin siRNA pretransfection efficiently counteracted ApoCIII-induced hyperactivation of β cell Ca_V channels (Fig. 7c and d in Paper II). Whole-cell Ca_V channel currents in β1 integrin siRNA-pretransfected cells incubated with ApoCIII (β1 integrin siRNA/ApoCIII) markedly decreased in comparison to those in NC siRNA-pretransfected cells exposed to ApoCIII (NC siRNA/apoCIII), but resembled those in three sets of control cells (Fig. 7c in Paper II). These control treatments include control vehicle incubation following mock (NO siRNA/Control),

NC siRNA (NC siRNA/Control) and β1 integrin siRNA pretransfection (β1 integrin siRNA/Control), respectively (Fig. 7c in Paper II). Ca_V channel current densities were significantly smaller in β1 integrin siRNA/ApoCIII group than in NC siRNA/apoCIII group (Fig. 7d in Paper II). The former manifested similar Ca_V channel current density, but the latter showed greater Ca_V channel current density as compared to NO siRNA/Control, NC siRNA/Control or β1 integrin siRNA/Control groups (Fig. 7d in Paper II). It is known that ApoCIII activates β1 integrin to promote PKC-mediated monocyte adhesion [37] and that β1 integrin activation stimulates PKA, PKC, Src kinase resulting in up-regulation of Ca_V channels in neurons, cardiomyocyte and vascular smooth muscle cells [165-168]. In line with these findings, our results clearly verify that β1 integrin conveys signals from ApoCIII to β cell Ca_V1 channels to mediate ApoCIII-induced hyperactivation of β cell Ca_V channels [200].

Existing documents illustrate no direct interaction of ApoCIII with β1 integrin [37, 39] and suggest that SR-BI should be a possible molecular bridge between ApoCIII and β1 integrin since this receptor physically associates with ApoCIII and interacts with β1 integrin [30, 164]. We have now clarified this suggestion in RINm5F cells by combining siRNA-mediated gene silencing and whole-cell Ca_V channel current analysis [200]. SR-BI siRNA transfection significantly reduced SR-BI at both mRNA and protein levels and sufficiently abolished enhancement of whole-cell Ca_V channel currents by ApoCIII (Fig. 8 in Paper II). Whole-cell Ca_V channel currents were smaller in SR-BI siRNA pretransfected cells incubated with ApoCIII (SR-BI siRNA/ApoCIII) than in those subjected to NC siRNA/apoCIII and similar to those in different control cells (Fig. 8e in Paper II). These control cells were exposed to NO siRNA/Control, NC siRNA/Control and SR-BI siRNA pretransfection followed by control vehicle treatment (SR-BI siRNA/Control), respectively (Fig. 8e in Paper II). However, whole-cell Ca_V channel currents were greater in NC siRNA/apoCIII-treated cells than in the aforementioned control cells (Fig. 8e in Paper II). SR-BI siRNA/ApoCIII group had significantly lower Ca_V channel current densities than NC siRNA/apoCIII group (Fig. 8f in Paper II). Ca_V channel current density in the former resembled, but in the latter appeared significantly higher than those in NO siRNA/Control, NC siRNA/Control or SR-BI siRNA/Control (Fig. 8f in Paper II). Undoubtedly, SR-BI gene silencing efficiently nullifies ApoCIII-induced hyperactivation of β cell Ca_V channels. In agreement with the previous findings that SR-BI not only physically associates with ApoCIII but also interacts with β1 integrin in other cell types [30, 164], our data verify that SR-BI serves as a molecular bridge between ApoCIII and β1 integrin with regard to β cell Ca_V channel hyperactivation [200].

4.8 APOCIII HYPERACTIVATES β CELL CA_V CHANNELS VIA COACTIVATION OF PKA AND SRC KINASE (PAPER II)

To clarify molecular signaling pathways in ApoCIII-induced hyperactivation of β cell Ca_V1 channels, we analyzed whole-cell Ca_V channel currents in RINm5F cells exposed to ApoCIII alone or together with the PKA inhibitors H-89 and PKI, the PKC inhibitor calphostin C (CalpC) or the Src kinase inhibitor PP2 [200]. The whole-cell Ca_V channel currents were larger in ApoCIII-treated cells than in control cells, whereas those in cells exposed to ApoCIII together with H-89, PKI or PP2 were in between (Fig. 3a, e and Fig. 4a in Paper II).

Whole-cell Ca_V channel currents in cells exposed to ApoCIII alone or together with CalpC appeared similar (Fig. 3c in Paper II). Ca_V channel current densities in ApoCIII group increased significantly in comparison to control group (Fig. 3b, d, f and Fig. 4b in Paper II). ApoCIII/H-89-, ApoCIII/PKI- or ApoCIII/PP2-cotreated groups manifested Ca_V channel current densities intermediate between those of ApoCIII and control groups (Fig. 3b, f and Fig. 4b in paper II). They were not significantly different from either ApoCIII or control groups in terms of Ca_V channel current densities. Ca_V channel current densities did not significantly differ between ApoCIII/CalpC-cotreated and ApoCIII-treated groups (Fig. 3d in Paper II). Furthermore, there was no significant difference between ApoCIII-untreated cells and those exposed to H-89, PKI, CalpC or PP2 (Supplementary Fig. S1 and S3 in Paper II). Undeniably, individual inhibition of PKA, PKC or Src kinase are incapable of effectively ablating ApoCIII-induced hyperactivation of β cell Ca_V channels. However, particularly noteworthy is that PKA or Src kinase inhibition has a tendency to counteract ApoCIII-induced hyperactivation of β cell Ca_V channels [200].

The question immediately arises as to what happens if combined inhibition of all these three kinases was applied. To answer this question, we characterized the effect of co-application of H-89, PKI, CalpC and PP2 on ApoCIII-induced hyperactivation of β cell Ca_V channels in RINm5F cells [200]. We observed that ApoCIII-treated cells displayed larger whole-cell Ca_V channel currents than control cells and cells treated with ApoCIII plus a protein kinase inhibitor cocktail of either PKI/CalpC/PP2 (Fig. 4a in Paper II) or H-89/CalpC/PP2 (Fig. 5a in Paper II). Control and ApoCIII/kinase inhibitor cocktail-cotreated cells exhibited similar whole-cell Ca_V channel currents (Fig. 4a and Fig. 5a in Paper II). Ca_V channel current densities were significantly greater in ApoCIII group than in control, ApoCIII/PKI/CalpC/PP2 and ApoCIII/H-89/CalpC/PP2 groups (Fig. 4b and Fig. 5b in Paper II), whereas Ca_V channel current densities did not differ significantly between control and ApoCIII/PKI/CalpC/PP2 or ApoCIII/H-89/CalpC/PP2 groups (Fig. 4b and Fig. 5b in Paper II). Moreover, PKI/CalpC/PP2 or H-89/CalpC/PP2 had no significant influence on whole-cell Ca_V channel currents in ApoCIII-unexposed cells (Supplementary Fig. S2 and S3 in Paper II). Obviously, combined inhibition of PKA, PKC and Src kinase effectively ablates ApoCIII-induced hyperactivation of β cell Ca_V channels [200].

The question subsequently arises as to whether coinhibition of PKA and Src kinase suffices to prevent ApoCIII-induced hyperactivation of β cell Ca_V channels. We circumvented the question by evaluating the influence of H-89/PP2 or PKI/PP2 cotreatments on ApoCIII-induced hyperactivation of β cell Ca_V channels in RINm5F cells [200]. We found that whole-cell Ca_V channel currents were larger in ApoCIII-treated cells than in control cells or cells treated with ApoCIII plus either H-89/PP2 or PKI/PP2 (Fig. 4a and Fig. 5c in Paper II). Similar whole-cell Ca_V channel currents occurred in the latter three cases (Fig. 4a and Fig. 5c in Paper II). Ca_V channel current densities in the ApoCIII group significantly increased in comparison to control, ApoCIII/H-89/PP2 and ApoCIII/PKI/PP2 groups (Fig. 4b and Fig. 5d in Paper II). These latter three groups displayed similar Ca_V channel current densities (Fig. 4b and Fig. 5d in Paper II). H-89/PP2- or PKI/PP2 did not significantly alter whole-cell Ca_V channel currents in ApoCIII-unexposed cells (Supplementary Fig. S2C, D, Fig. S3A and B in

Paper II). Undoubtedly, coinhibition of PKA and Src kinase suffices to prevent ApoCIII-induced hyperactivation of β cell Ca_V channels [200].

Phosphorylation of Ca_V channels is the foremost event in the regulation of these channels. We now demonstrate that complex inhibition of PKA, PKC and Src kinase ablates ApoCIII-induced hyperactivation of β cell Ca_V channels and that coinhibition of PKA and Src kinase suffices to execute this ablation [200]. However, individual inhibition of PKA, Src kinase or PKC only marginally or negligibly counteracts ApoCIII-induced hyperactivation of β cell Ca_V channels [200]. Evidently, ApoCIII relies on parallel PKA and Src pathways to upregulate β cell Ca_V channels [200]. This satisfactorily fills the critical gap between activated β1 integrin and hyperactivated Ca_V1 channels in β cells [200].

4.9 PCS1-MEDIATED ENDOCYTOSIS REGULATES THE NUMBER OF CA_V1 CHANNELS IN THE β CELL PLASMA MEMBRANE (PAPER III)

Endocytosis serves as a critical mechanism for density control of plasma membrane proteins including Ca_V channels [173-175, 201, 202]. It operates under direct control of a complex molecular network where the interaction of dynamin I with PCS1 takes center stage [174-178]. However, the β cell endocytic machinery is rarely understood and endocytic trafficking of β cell Ca_V1 channels is not known. We therefore examined the presence and endocytic activity of PCS1 in β cells as well as the involvement of PCS1 in endocytic trafficking of β cell Ca_V1 channels.

4.9.1 PCS1 is richly present in β cells (Paper III)

We explored if PCS1 is expressed in β cells. Immunoblot analysis of different mouse tissues detected a clear PCS1 immunoreactive band in both brain and pancreatic islets, but not in exocrine pancreas, heart, kidney, liver, lung, muscle and spleen (Figure 1A in Paper III). This immunoreactive band also appeared in HIT-T15, INS-1, MIN6-m9 and RINm5F cells (Figure 1B in Paper III). Furthermore, confocal double immunofluorescence images of isolated mouse (Figure 2A in Paper III) and rat islets (Figure 2B in Paper III) show that all endocrine cells were appreciably labeled by anti-PCS1 antibodies (Figure 2Aii and 2Bii in Paper III) and most of them exhibited intense insulin-like immunoreactivity (-LI) (Figure 2Ai and 2Bi in Paper III). The majority of cells were double-labeled, but some cells were only recognized by anti-PCS1 antibodies (Fig. 2Aiii and 2Biii in Paper III). In cultured mouse and rat islet cells, all clustered (Figure 2Av and 2Bv in Paper III) and single cells (Figure 2Aviii and 2Bviii in Paper III) showed intense PCS1-LI, whereas only some of these cells (Figure 2Aiv, 2Avii, 2Biv and 2Bvii in Paper III) exhibited intense insulin-LI. Both insulin-LI and PCS1-LI appeared in the cytoplasm with clear granule-like structures. PCS1-LI separated from insulin-LI (Figure 2Avi, 2Aix, 2Bvi and 2Bix in Paper III) with exception of a minor colocalization of PCS1-LI with insulin-LI in in the same subcellular structures (Figure 2Avi, 2Aix, 2Bvi and 2Bix in Paper III). Evidently, the key endocytic protein PCS1 is expressed in all mouse and rat pancreatic islet cells including insulin-secreting β cells as well as in HIT-T15, INS-1, MIN6-m9 and RINm5F cells. Subcellularly, it is situated in abundant granule-like structures that are smaller than insulin-LI profiles.

The highly specialized secretory β cell undergoes profound exocytosis. Such profound exocytosis is inevitably followed by effective endocytosis to preserve β cell integrity and functionality, including plasma membrane protein homeostasis. However, significant efforts have been devoted to the understanding of insulin exocytosis [2-4]. β Cell endocytosis has been mostly ignored. The molecular details of the endocytic machinery in the β cell remain under intensive investigation. We now show for the first time that mouse and rat islet β cells as well as insulin-secreting cell lines express PCS1 in the cytoplasm. This is intriguing since this protein has previously been considered to be exclusively present in neurons [179, 180, 203]. In addition, we also verify that PCS1 appears in abundant vesicular profiles distinct from insulin-containing granules. These PCS1-positive profiles are smaller than insulin-containing granules and appear to be endocytic, endosomal or lysosomal vesicles. However, the identity of the subcellular organelles where PCS1 resides remains to be clarified. These findings provide a key "cornerstone" towards an in-depth understanding of the β cell endocytic machinery.

4.9.2 PCS1 indispensably mediates β cell endocytosis (Paper III)

To verify the role of PCS1 in regulation of β cell endocytosis, we examined the effect of PCS1 knockdown on β cell endocytic activity by applying siRNA-mediated gene silencing and live-cell confocal imaging. Transfection with two PCS1 siRNAs significantly reduced PCS1 protein expression in RINm5F cells (Figure 3A and B in Paper III). Internalization of the endocytic marker FM1-43 was significantly less in cells transfected with PCS1 siRNAs than in non-transfected or NC siRNA-transfected cells following either extracellular application of 10 mM glyceraldehyde together with 2.8 mM glucose or stimulation with 30 mM KCl (Figure 3C-J in Paper III). Undoubtedly, downregulation of PCS1 expression dramatically dampens β cell endocytosis induced by a high carbohydrate challenge or high K^+ depolarization. These findings verify that PCS1 acts as a key endocytic protein to indispensably mediate β cells endocytosis (Figure 7A-C in Paper III) and add a new layer of complexity to our understanding of the β cell enocytotic machinery.

4.9.3 PCS1-mediated endocytosis operates as a homeostatic control system for the maintenance of Ca_V1 channel density in the β cell plasma membrane (Paper III)

To clarify the physiological importance of PCS1-mediated β cell endocytosis, we evaluated the effect of PCS1 knockdown on the density of Ca_V1 channels in the β cell plasma membrane following 1 h incubation with 10 mM glyceraldehyde plus 2.8 mM glucose. To maximally activate Ca_V1 channels in the recorded plasma membrane patches, 10 μM Bay K8644, a selective Ca_V1 channel activator, was included in both the electrode solution and the extracellular solution. This ensures reliable estimation of the density of Ca_V1 channels in the β cell plasma membrane. We found that Ca_V1 channel number, reflected by unitary Ba^{2+} current layer, increased in plasma membrane patches of PCS1 siRNA-transfected cells compared to cells transfected with NC siRNA (Figure 4A). The average number of unitary Ca_V1 channels were significantly greater in plasma membrane patches of PCS1 siRNA-

transfected cells than in those of cells transfected with NC siRNA (Figure 4B). Cells transfected with PCS1 siRNA did not significantly differ from NC siRNA-transfected cells in the open probability, mean open time and mean closed time of unitary Ca_V1 channels (Figure 4B). Indeed, the PCS1 knockdown-caused impairment in β cell endocytosis leads to the elevated density of β cell Ca_V1 channels. This strongly supports that PCS1-mediated endocytosis preserves the homeostasis of Ca_V1 channels in the β cell plasma membrane.

The integral membrane protein Ca_V1 channel is critical for β cell function and survival [1-4]. A diverse range of physiological scenarios from insulin secretion to β cell survival vitally rely on appropriate Ca^{2+} influx mediated by adequate numbers of Ca_V1 channels in the β cell plasma membrane [1-4]. Inappropriate increases or decreases of Ca_V1 channel density in the β cell plasma membrane bring about intracellular Ca^{2+} overload or deficiency and consequently β cell dysfunction and even destruction [1-4]. Ca_V1 channels as plasma membrane proteins critically depend on the endocytic machinery for their internalization to exquisitely control their density in the plasma membrane [173-175, 201, 202]. Our observation that knockdown of PCS1 expression significantly elevates Ca_V1 channel density in the β cell plasma membrane convincingly pinpoints that PCS1-mediated endocytosis operates as a homeostatic control system for the maintenance of Ca_V1 channel density in the β cell plasma membrane.

4.10 APOCIII ABROGATES PCS1-DEPENDENT ENDOCYTIC TRAFFICKING LEADING TO AN ELEVATION OF CA_V1 CHANNEL DENSITY IN THE β CELL PLASMA MEMBRANE (PAPER IV)

ApoCIII hyperactivates β cell Ca_V1 channels manifested as exaggerated elevation of both the conductivity and number of Ca_V1 channels in the β cell plasma membrane [200]. Such β cell Ca_V1 channel hyperactivation has been generally explained by SR-BI/β1 integrin-dependent coactivation of PKA and Src [200]. However, the specific mechanism whereby ApoCIII upregulates the number of Ca_V1 channels in the β cell plasma membrane remains elusive. To clarify such a mechanism, we characterized if ApoCIII interferes with endocytic activity to accumulate Ca_V1 channels in the β cell plasma membrane.

4.10.1 ApoCIII impairs β cell endocytosis without influencing PCS1 expression (Paper IV)

To examine if ApoCIII interferes with endocytic trafficking of β cell Ca_V1 channels, we first evaluated the effect of ApoCIII on constitutive and regulated endocytosis in the insulin-secreting RINm5F cells using live-cell confocal imaging of the endocytic marker FM1-43. ApoCIII-treated cells accumulated less FM1-43 than control cells bathed in extracellular solution containing neither glucose nor glyceraldehyde (Figure 1A and B in Paper IV). FM1-43 accumulation in ApoCIII-treated group was significantly impaired in comparison to control group (Figure 1C in Paper IV). Moreover, ApoCIII-treated cells internalized less FM1-43 than control cells following stimulation with 10 mM glyceraldehyde together with 2.8 mM glucose (Figure 1D and E in Paper IV). The fluorescence intensity of FM1-43 internalized into ApoCIII-treated cells significantly decreased as compared to that into

control cells (Figure 1F in Paper IV). It is clear that ApoCIII treatment not only dampens constitutive β cell endocytosis but also reduces regulated β cell endocytosis induced by a high carbohydrate challenge.

Endocytosis and exocytosis coordinately act to preserve protein homeostasis in the plasma membrane by internalizing and recruiting plasma membrane proteins including ion channels, respectively [173-175, 201, 202, 204]. Impaired endocytosis and consequent aberrations in plasma membrane protein homeostasis impair cell function and viability contributing to the pathogenesis of a series of diseases [205]. The aforementioned results show for the first time that ApoCIII impedes both constitutive and regulated β cell endocytosis. This undoubtedly provides a novel cellular mechanism underlying ApoCIII-induced pathological effects on β cells [6, 200].

The fact that knockdown of PCS1 effectively impairs β cell endocytosis, as presented in Paper III, made us wonder whether overnight ApoCIII exposure alters PCS1 expression to abrogate β cell endocytosis. Therefore, we examined PCS1 protein expression in RINm5F cells following ApoCIII incubation by using immunoblot analysis. We found that intensities of PCS1 and GAPDH immunoreactive bands were very similar between control and ApoCIII-treated samples (Figure 2A in Paper IV). The relative abundance of PCS1 protein in cell homogenates did not significantly differ between control vehicle incubation and ApoCIII exposure (Figure 2B in Paper IV). These results demonstrate that ApoCIII does not appreciably alter expression of β cell PCS1, but do not preclude the possibility that ApoCIII dampens PCS1 function.

4.10.2 ApoCIII retains more Ca_V1 channels in the β cell plasma membrane by abrogating PCS1-dependent endocytic trafficking (Paper IV)

Our findings that impaired β cell endocytosis occurs following either PCS1 knockdown or ApoCIII exposure (Figure 3 in Paper III and Figure 1 in Paper IV), the former inhibiting endocytic trafficking of β cell Ca_V1 channels (Figure 4 in Paper III), urged us to examine if the latter also does the same. We compared the effects of ApoCIII exposure and PCS1 knockdown on unitary Ca_V1 currents in RINm5F cells following 1 h incubation with 10 mM glyceraldehyde plus 2.8 mM glucose. Analysis of unitary Ca_V1 channel currents was performed in NC siRNA-pretransfected cells exposed to either control vehicle (NC siRNA/control) or ApoCIII (NC siRNA/ApoCIII) and cells pretransfected with PCS1 siRNAs followed by either control vehicle incubation (PCS1 siRNA/control) or ApoCIII exposure (PCS1 siRNA/ApoCIII). To ensure reliable estimation of the density of Ca_V1 channels in the β cell plasma membrane, 10 μM Bay K8644, a selective Ca_V1 channel activator [206, 207], was added to both the electrode solution and the extracellular solution for maximal activation of Ca_V1 channels in the recorded plasma membrane patches. We visualized that more Ca_V1 channels, reflected by more layers of unitary Ba^{2+} currents, resided in plasma membrane patches attached to NC siRNA/ApoCIII cells, cells subjected to PCS1 siRNA/control and PCS1 siRNA/ApoCIII cells compared to plasma membrane patches of NC siRNA/control cells (Figure 3A in Paper IV). Profiles of unitary Ca_V1 currents recorded in NC siRNA/ApoCIII, PCS1 siRNA/control and PCS1 siRNA/ApoCIII cells closely

resembled each other (Figure 3A in Paper IV). The average number of unitary Ca_V1 channels in NC siRNA/ApoCIII, PCS1 siRNA/control and PCS1 siRNA/ApoCIII groups was significantly increased in comparison to that in NC siRNA/control group (Figure 3B in Paper IV). This parameter did no significantly differ between NC siRNA/ApoCIII, PCS1 siRNA/control and PCS1 siRNA/ApoCIII groups (Figure 3B in Paper IV). Importantly, ApoCIII exposure could no longer increase the average number of unitary Ca_V1 channels in PCS1 siRNA-pretransfected cells (Figure 3B in Paper IV). Other parameters including the open probability, mean open time and mean closed time of unitary Ca_V1 channels were statistically similar between these groups (Figure 3B in Paper IV). This is most likely to be attributed to the scenario where maximal activation of Ca_V1 channels with 10 μM Bay K8644 masked the stimulatory effect of ApoCIII on Ca_V1 channel activity. The data reveal that ApoCIII accumulates Ca_V1 channels in the β cell plasma membrane by abrogating PCS1-dependent endocytic trafficking.

The above observation clearly demonstrates that PCS1 gene silencing and ApoCIII exposure produce similar effects on β cell Ca_V1 channels. Both significantly elevate the number of Ca_V1 channels in the β cell plasma membrane (Figure 4 in Paper III and Figure 3 in Paper IV). Particularly noteworthy is that ApoCIII exposure loses its ability to accumulate Ca_V1 channels in the β cell plasma membrane following knockdown of PCS1 (Figure 3 in Paper IV). This demonstrates that ApoCIII accumulates Ca_V1 channels in the β cell plasma membrane by abrogating the PCS1-dependent endocytic trafficking even though ApoCIII does not influence PCS1 expression. Ca_V1 channels excessively increase their density and conductivity to participate in a diverse range of pathological processes [5, 6, 53, 208]. Understanding what pathogens and how they accumulate Ca_V1 channels in the cell surface membrane to contribute development of diseases is a long-standing challenge. The present work deciphers an innovative mechanism whereby ApoCIII PCS1-dependently retains excessive Ca_V1 channels in the β cell plasma membrane to account for Ca^{2+}-dependent β cell destruction in diabetes development.

5 CONCLUSIONS

By satisfactorily accomplishing the proposed specific aims, this PhD work draws the following conclusions:

1) Functional $Ca_V1.3$ channels operate in 20 % mouse islet $Ca_V1.2^{-/-}$ β cells. They are characterized by a large unitary Ba^{2+} conductance with long-lasting openings in plasma membrane patches attached to islet cells exhibiting larger cell capacitance (> 7 pF) and insulin transcript positivity. This enables investigation of human β cell $Ca_V1.3$ channel-related disorders such as T1D serum-induced hyperactivation of β cell $Ca_V1.3$ channels to be performed in the β cell-specific $Ca_V1.2^{-/-}$ mice (Figure 2).

2) T1D serum hyperactivates both $Ca_V1.2$ and $Ca_V1.3$ channels by increasing their conductivity and number in the β cell plasma membrane. This pinpoints both $Ca_V1.2$ and $Ca_V1.3$ channels as potential druggable targets for counteraction of Ca^{2+} overload-induced β cell death (Figure 2).

3) ApoCIII in T1D serum is electrophysiologically confirmed to act as the actual factor enhancing Ca_V channel currents in the β cell. This suggests that neutralization or depletion of ApoCIII in T1D serum should be considered to be a new therapy against Ca_V channel hyperactivation-driven β cell destruction (Figure 2).

4) ApoCIII selectively hyperactivates β cell Ca_V1 channels through SR-BI/β1 integrin-dependent coactivation of PKA and Src kinase without influencing β cell Ca_V1 channel expression. ApoCIII-induced hyperactivation of β cell Ca_V1 channels occurs due to the enriched density and increased activity of functional Ca_V1 channels in the β cell plasma membrane. This novel signal-transduction pathway possesses great potential as an innovative drug discovery platform for the prevention of Ca^{2+}-dependent β cell death in association with diabetes (Figure 2).

5) PCS1 is richly expressed in β cells and indispensably mediates β cell endocytosis. PCS1-mediated endocytosis operates as a homeostatic control system to fine-tune the Ca_V1 channel density in the β cell plasma membrane. This adds a new layer of complexity to the mechanisms of β cell Ca_V1 channel regulation (Figure 2).

6) ApoCIII abrogates both constitutive and regulated β cell endocytosis without influencing PCS1 expression. Consequently, ApoCIII abrogates PCS1-dependent endocytic trafficking, thereby accumulating excessive Ca_V1 channels in the β cell plasma membrane. This provides new mechanistic explanation of Ca^{2+}-dependent β cell destruction in diabetes development and identifies a promising and attractive option to counteract the critical diabetogenic process Ca^{2+}-dependent β cell destruction (Figure 2).

This PhD work depicts a mechanistic picture of how ApoCIII renders Ca_V1 channels highly enriched and excessively activated in the β cell plasma membrane, thereby mediating pathologically exaggerated Ca^{2+} influx and driving Ca^{2+}-dependent β death. These findings lay the foundation for novel treatment strategies for diabetes.

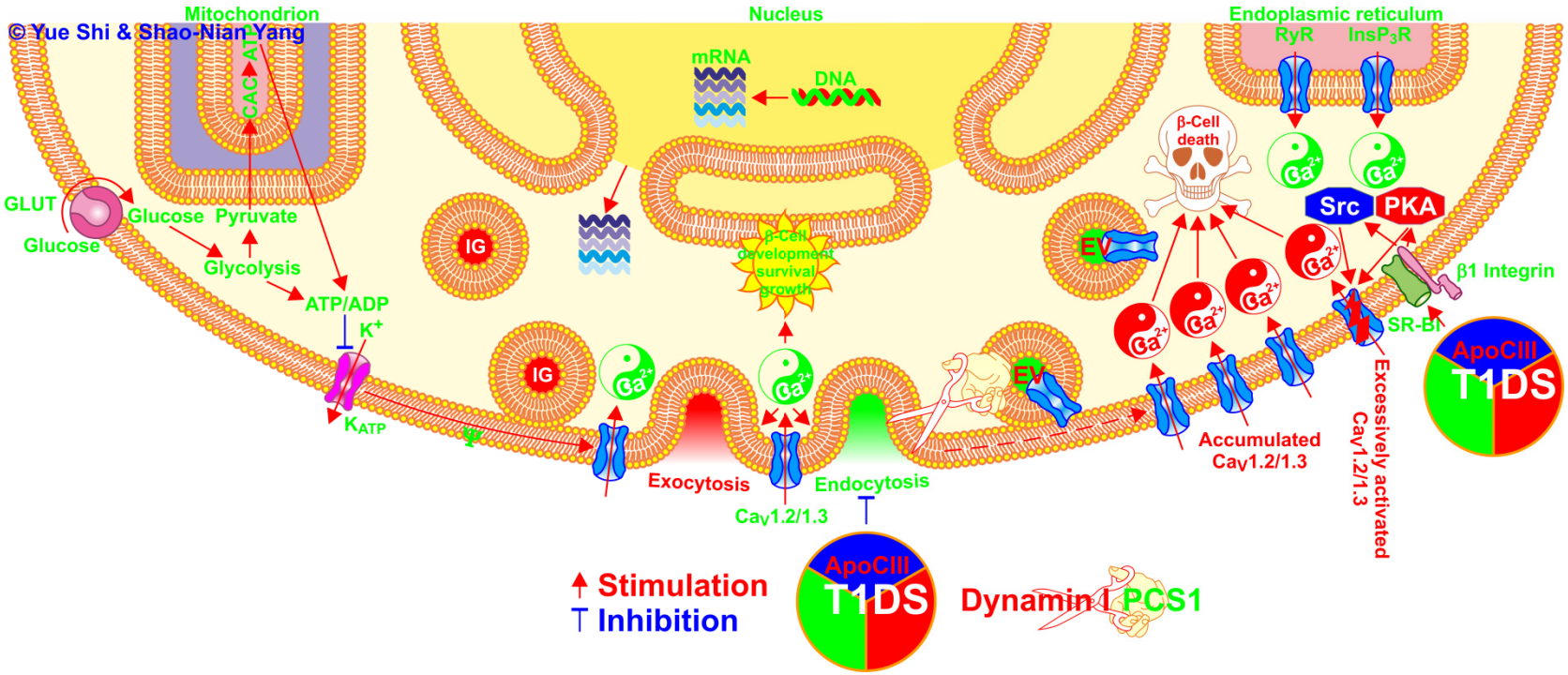

Figure 2. Scheme summarizing mechanisms of T1D serum-induced hyperactivation of Ca_V1 channels in the pancreatic β cell. The β cell mainly relies on $Ca_V1.2/1.3$ channel-mediated Ca^{2+} influx to govern glucose-stimulated insulin secretion and other Ca^{2+}-dependent cellular processes such as β cell development, survival and growth. The conductivity and density of β cell $Ca_V1.2/1.3$ channels appropriately alters to meet a wide range of physiological needs, whereas they aberrantly change in diabetes scenarios. T1D serum hyperactivates $Ca_V1.2/1.3$ channels by raising their open probability and number in the β cell plasma membrane. ApoCIII acts as the actual factor in T1D serum to selectively elevate Ca_V1 channel conductivity and density through SR-BI/β1 integrin-dependent coactivation of PKA and Src kinase. PCS1-mediated endocytosis serves as a homeostatic control system to fine-tune Ca_V1 channel density. ApoCIII abrogates PCS1-dependent β cell endocytic trafficking leading to accumulation of Ca_V1 channels in the β cell plasma membrane. The highly enriched and excessively activated Ca_V1 channels mediate pathologically exaggerated Ca^{2+} influx driving Ca^{2+}-dependent cell death and consequent diabetes.

ApoCIII, apolipoprotein CIII; CAC, citric acid cycle; $Ca_V1.2/1.3$, $Ca_V1.2$ and $Ca_V1.3$ channels; EV, endocytic vesicles; GLUT, glucose transporter; IG, insulin-containing granule; $InsP_3R$, $InsP_3$ receptor; PCS1, syndapin I/PACSIN 1; PKA, protein kinase A; RyR, ryanodine receptor; SR-BI, scavenger receptor class B type I; Src, Src kinase; T1DS, type 1 diabetic serum; ψ, depolarization.

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