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Doctoral Thesis

Studies on regulation of insulin secretion
in pancreatic β -cells

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2019

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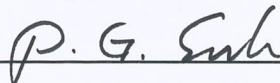
Studies on regulation of insulin secretion
in pancreatic β -cells

A thesis/dissertation
submitted to the Graduate School of UNIST
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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6/27/2019

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Studies on regulation of insulin secretion in pancreatic β -cells

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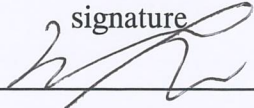
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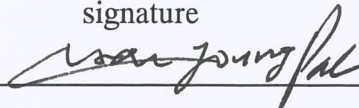
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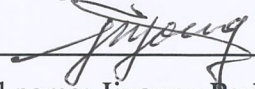
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Abstract

The type 2 diabetes mellitus (T2DM) was one of the most significant public health challenges in 21st century. The T2DM in most instances are characterized by alteration in insulin secretion and insulin sensitivity or a combination of both. For the last decade, a lot of attention has been focused on insulin resistance as a critical cause on development of T2DM. However, after many years from diagnosis of the T2DM, individuals with the disease were largely accompanied by continuous decline in β -cell function. Indeed, pancreatic β -cells have vital role in pathogenesis of T2DM and understanding β -cells physiology can reliably alleviates the disease. Thus, in the future, cure for T2DM will require genetic or pharmacological approaches to be beneficial to β -cells. Here, I focused the phospholipase C β (PLC β) for elucidation of molecular aetiology for T2DM and zafirlukast drug for effective interventions to combat T2DM epidemic.

In the first chapter of this dissertation, I reviewed overall regulatory mechanism of insulin secretion, especially focused on PLC β s and high-throughput tools for discovering regulator of insulin secretion. In the second chapter, I described the PLC β 1-mediated insulin secretion. Phospholipase C β (PLC β) exerts biological influences through G protein-coupled receptors (GPCRs). GPCRs are involved in regulating glucose-stimulated insulin secretion (GSIS). Previous studies have suggested that PLC β s might play an important role in pancreatic β -cells. However, due to a lack of the specific inhibitors of PLC β isozymes and appropriate genetic models, the *in vivo* function of specific PLC β isozymes in pancreatic β -cells and their physiological relevance in the regulation of insulin secretion has not been studied so far. The present study showed that PLC β 1 was crucial for β -cell function by generation of each PLC β conditional knockout mice. Mice lacking PLC β 1 in β -cells exhibited a marked defect in GSIS, leading to glucose intolerance. In *ex vivo* studies, the secreted insulin level and Ca²⁺ response in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets was lower than those in the *Plcb1^{fl/fl}* islets under the high glucose condition. PLC β 1 led to potentiate insulin secretion via stimulation of particular G_q protein-coupled receptors. *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a high fat diet developed more severe glucose intolerance due to a defect in insulin secretion.

In the last chapter, I reported a novel function of zafirlukast as insulin secretagogue. The zafirlukast has been reported to be anti-inflammatory and widely used to alleviate the symptoms of asthma. However, its influence on insulin secretion in pancreatic β -cells has not been investigated. Herein, we examined the effects of zafirlukast on insulin secretion and the potential underlying mechanisms. Among the cysteinyl leukotriene receptor 1 antagonists, zafirlukast, pranlukast, and montelukast, only zafirlukast enhanced insulin secretion in a concentration-dependent manner in both

low and high glucose conditions and elevated the level of $[Ca^{2+}]_i$, further activating Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), protein kinase B (AKT), and extracellular signal-regulated kinase (ERK) signaling. These effects were nearly abolished by the L-type Ca^{2+} channel antagonist nifedipine, while treatment with thapsigargin, a sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor, did not have the same effect, suggesting that zafirlukast primarily induces the entry of extracellular Ca^{2+} rather than intracellular Ca^{2+} from the endoplasmic reticulum. Zafirlukast treatment resulting in a significant drop in glucose levels and increased insulin secretion in C57BL/6J mice.

The present study identified PLC β 1 and zafirlukast as an important factor that regulates β -cell insulin secretion. These findings will contribute to an improved understanding of the β -cell pathophysiology and can be considered as candidate for therapeutic intervention in diabetes mellitus.

Contents

Abstract	i
Contents	iv
List of Figures	vi
List of Tables	viii
Abbreviations	ix
Chapter I. Background	1
1.1. Diabetes mellitus – Type II diabetes	1
1.2. Mechanism of insulin secretion	1
1.3. PLC β -mediated regulation of GSIS	3
1.4. Overview of G $_q$ PCRs which potentiates GSIS through PLC β signaling	5
1.5. High-throughput luminescent reporter of insulin secretion	8
1.6 Reference	17
Chapter II. Phospholipase Cβ1 potentiates glucose-stimulated insulin secretion	25
2.1. Introduction	25
2.2. Materials and methods	26
2.3. Results	31
2.4. Conclusion and Discussion	35
2.5. Reference	54
Chapter III. Zafirlukast promotes insulin secretion by increasing calcium influx through L-type calcium channels	59
3.1. Introduction	59
3.2. Materials and methods	60
3.3. Results	62
3.4. Conclusion and Discussion	65
3.5. Reference	77

Chapter IV. Acknowledgements **80**

List of Figures

- Figure 1.1. Vascularization and innervation of Langerhans islets
- Figure 1.2. Structure of mammalian PLC isotypes and detailed action of PLCβs
- Figure 1.3. PLCβ as key molecule of GPCR signaling
- Figure 1.4. Overview of phospholipase Cβ-mediated insulin secretion pathways
- Figure 1.5. Schematic diagram of a proinsulin-luciferase fusion protein and validation of luciferase activity as a proxy for secreted insulin
- Figure 2.1. Expression profile of PLCβ isozymes in pancreatic β-cells
- Figure 2.2. Inducible ablation of PLCβ isozymes in adult pancreatic β-cells
- Figure 2.3. Impaired glucose tolerance in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice
- Figure 2.4. Defect in GSIS in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice
- Figure 2.5. Normal insulin production and islet morphology
- Figure 2.6. Decreased selective G_qPCR-induced insulin release in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets
- Figure 2.7. Impaired insulin secretion in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a HFD
- Figure 2.8. Similar body weight and fasting and re-feeding glucose levels between control and PLCβ cKO mice following tamoxifen treatment
- Figure 2.9. Normal insulin sensitivity in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a normal chow diet and HFD
- Figure 2.10. PLCβ1 protein expression in α-cell and δ-cells in pancreatic islets from control and *Plcb1^{fl/fl}; Pdx1-CreERT2* islets
- Figure 2.11. Reduced PLCβ1 expression in human β-cells from type 2 diabetes patients compared to normal subjects
- Figure 2.12. Reduced PLCβ1 expression in hypothalamus results in no alteration in food intake
- Figure 2.13. No change in β-cell proliferation and β-cell turnover in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets
- Figure 2.14. PLCβ isozyme expression in each PLCβ isozyme conditional knockout islets
- Figure 2.15. Reconstitution of PLCβ1 expression to rescue cells from abnormal insulin secretion
- Figure 3.1. Zafirlukast-dependent potentiation of insulin secretion in MIN6 cells.
- Figure 3.2. Distinct effect of zafirlukast on insulin secretion among cysteinyl leukotriene receptor 1 (CysLTR1) antagonists.
- Figure 3.3. Zafirlukast-induced increase in intracellular calcium ([Ca²⁺]_i) in MIN6 cells.
- Figure 3.4. Increase in insulin secretion and [Ca²⁺]_i following stimulation with zafirlukast in primary pancreatic islets.
- Figure 3.5. Zafirlukast-induced phosphorylation of CaMKII, AKT, and ERK.

Figure 3.6. Effect of zafirlukast on insulin secretion elicited by extracellular Ca^{2+} influx through L-type voltage dependent calcium channels (L-VDCCs).

Figure 3.7. Glucose-lowering effect of zafirlukast as a result of increased insulin secretion *in vivo*.

Figure 3.8. Pranlukast- and montelukast-induced phosphorylation of CaMKII, AKT, and ERK.

Figure 3.9. Effect of zafirlukast on $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free Krebs' buffer (KRB) medium.

List of Tables

Table 1.1. GPCRs highly relevant $G_q/PLC\beta$ signaling for stimulating insulin secretion

Table 2.1. Sequences of quantitative RT-PCR primer upstream and downstream

Abbreviations

AC	Adenylyl cyclase
AKT	Protein kinase B
AMS	α -methyl serotonin maleate salt
AT1	Angiotensin II receptor 1
AVP	Vasopressin
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine 3',5'- monophosphate
CCK	Cholecystokinin-8
CNS	Central nervous system
CysLTR1	Cysteinyl leukotriene receptor 1
DAG	Diacylglycerol
DHP	Dihydropyridine
DM	Diabetes mellitus
EC50	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
E_{max}	Maximum effect concentration
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FFA1	Free fatty acid receptor 1
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GLUT2	Glucose transporter 2
GPCR	G protein-coupled receptor
GSIS	Glucose-stimulated insulin secretion
HFD	High-fat diet
HTR2B	Serotonin receptor 2B
IHC	Immunohistochemistry
IL-6R	Interleukin (IL)-6 receptor
IP₃	Inositol triphosphate
IP₃R	inositol trisphosphate receptors
IP₄	Inositol 1,3,4,5-tetrakisphosphate

K_{ATP} channels	ATP-sensitive K ⁺ channels
KP	Kisspeptin-10
L-VDCC	L-type voltage dependent Ca ²⁺ channel
MCP-1	Monocyte chemotactic protein 1
NSAID	Non-steroidal anti-inflammatory drug
OA	Oleic acid
OLFR15	Olfactory receptor
OXO-M	Oxotremorine-M
Pdx1	Pancreas/duodenum homeobox protein 1
PDZ	Postsynaptic density protein 95, disc large, zona occludens-1
PH	Pleckstrin homology
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLCβ	Phospholipase C β
PPAR-γ	Proliferator-activated receptor- γ
SOC	Store-operated channel
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
TRPC	Transient receptor potential channel
5-HT	Serotonin
[Ca²⁺]_i	Intracellular calcium level

Chapter I

Background

1.1. Diabetes mellitus – Type II diabetes

The Diabetes mellitus (DM) is chronic condition in which individuals gradually lose capacity to regulate blood sugar (glucose), referred to as hyperglycemia. It causes high risk for micro- and macrovascular complications such as retinopathy, nephropathy, and cardiovascular comorbidities [1]. In a recent decades, this disease have been globally epidemic that the International Diabetes Federation reported 425 million adults have diabetes and expected to rise to 629 million by 2045 [2]. In 1936, the distinct definition between type 1 diabetes (T1DM) and type 2 diabetes (T2DM) was clearly described [3]. T1DM is primarily developed by autoimmune response against pancreatic β -cells, leading to absolute insulin deficiency. Individuals with T1DM are totally dependent on exogenous insulin to control blood glucose levels. T2DM, far more common type of diabetes, which accounts for more than 90% of all diabetes cases, is due to insulin resistance and/or abnormal insulin secretion. T2DM is heterogeneous disease resulting from genetic and environmental risk factors. Patients with T2DM require change in life style or hypoglycemic agents [4]. Epidemiological studies show that β -cells function start to decline after several years from clinical diagnosis and β -cells lose capacity to hypersecrete insulin to overcome insulin resistance [5]. The β -cell dysfunction includes reduction in insulin secretion to glucose [6] and non-glucose secretagogues [7, 8], change in pulsatile and oscillatory trends of insulin secretion [9, 10], alteration in proinsulin to insulin conversion [11, 12]. With systemic understanding for insulin resistance of glucose recipient tissues such as muscle, adipose tissue and liver, comprehensive studies about β -cell function in progression of T2DM are essential to true cure for T2DM.

1.2. Mechanism of insulin secretion

The main pathophysiology of T2DM is the inability of pancreatic β -cells to properly secrete enough insulin in response to various stimuli, including high glucose [13, 14]. In addition to glucose, hormonal factors, such as vasopressin, glucagon-like peptide 1, cholecystokinin, and neuronal factors, such as acetylcholine and serotonin, plus non-glucose nutrients, such as free fatty acids (FFAs), have been reported to participate in potentiating glucose-stimulated insulin secretion (GSIS) [15]. These mediators exert their biological effects through G-protein coupled receptors (GPCRs), which have been reported to stimulate insulin secretion by increasing insulin exocytosis [16, 17]. This stimulation

includes an increase in cytosolic Ca^{2+} levels and the number of readily releasable granules as well as the activation of exocytotic machinery. The phospholipase $\text{C}\beta$ ($\text{PLC}\beta$) that preferentially couples to the $\text{G}\alpha_q$ protein of GPCRs has been thought to play an important role in fine-tuning the regulation of insulin secretion [18]. Using potent inhibitors and small interfering RNAs, several studies have described $\text{PLC}\beta$ s as important regulators in the pathway for insulin release. This review provides a comprehensive understanding of the potential regulatory modes of $\text{PLC}\beta$ s in regulating insulin secretion in pancreatic β -cells.

In pancreatic β -cells, glucose is the primary regulator of insulin secretion. Glucose is transported into β -cells via glucose transporter 2 (GLUT2) and metabolized, which leads to a rise in the ATP/ADP ratio. The relative increase in ATP levels causes closure of ATP-sensitive K^+ channels (K_{ATP}). Recently, it has been reported that exposure of high glucose induced K_{ATP} channel endocytosis and reduced surface K_{ATP} channels, which contributed to increased glucose responses rather than to ATP-dependent gating of K_{ATP} channels [19]. Blocking the efflux of K^+ results in the depolarization of membrane potentials and the opening of L-type voltage-dependent Ca^{2+} channels (L-VDC), inducing an influx of extracellular Ca^{2+} . The elevated intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) are involved in the activation of Ca^{2+} -related signaling molecules and such components of exocytotic machinery as Ca^{2+} /calmodulin-dependent protein kinase II [20, 21] and SNARE proteins [22]. This process is known as GSIS.

In addition to triggering insulin secretion by glucose, GSIS can be augmented by non-glucose metabolites, neuronal and hormonal ligands from insulin vesicles, nerve endings, and blood capillaries surrounding pancreatic β -cells in an autocrine and paracrine manner in complex Langerhans islets (Figure 1.1). These mediators primarily act on surface GPCRs, which activate intracellular signaling. The types of GPCRs and their natural ligands that affect the potentiation of insulin secretion have been identified (Table 1.1). However, as there may be other GPCRs highly expressed in mouse islets, further studies are required to completely understand their putative effects on insulin secretion. The activated GPCRs induce dissociation of heterotrimeric G proteins, which propagate intracellular signaling. The $\text{G}\alpha_s$ and $\text{G}\alpha_i$ proteins couple to adenylyl cyclase (AC), and activation of $\text{G}\alpha_s$ proteins stimulates AC and insulin secretion, but activation of $\text{G}\alpha_i$ proteins inhibits this process. The AC catalyzes ATP to 3',5'-cyclic AMP (cAMP), which results in subsequent activation of protein kinase A (PKA). The PKA induces increased insulin secretion by directly phosphorylating exocytotic proteins, such as snapin and synaptotagmin [23, 24], increasing the total number of insulin granules available for release [25], inactivating K_{ATP} channels (phosphorylation site S^{1448}), and activating Cav1.2 channels [26]. The $\text{G}\alpha_q$ proteins promote lipid-derived signaling through $\text{PLC}\beta$ s, which hydrolyze membrane-bound phosphatidylinositides and induce increased $[\text{Ca}^{2+}]_i$ mobilization. Also, pancreatic β -cells contains a

range of nuclear receptors including peroxisome proliferator-activated receptor- γ (PPAR- γ) [27], liver X receptor [28] and androgen receptor [29] activated by corresponding ligands such as fatty acids, prostaglandins, oxysterols and androgen, respectively. However, phospholipid such as phosphoinositides can regulate activity of nuclear receptors [30]. Through PLC β -mediated generation of second messenger phospholipids, GPCRs can mediate activation of nuclear receptors which induce gene expression as transcription factor or regulate metabolic function [31, 32], glucose or lipid metabolism [28], for proper insulin secretion. Additional details of G_qPCRs-PLC β signaling are described below.

1.3. The PLC β -mediated regulation of GSIS

Distinct structures and expression patterns of PLC β s in pancreatic β -cells

To date, 13 mammalian PLC isozymes have been characterized and classified into six different subtypes: PLC β 1-4, PLC γ 1,2, PLC δ 1,3,4, PLC ϵ , PLC ζ , and PLC η 1,2 [33]. All isozymes share a highly conserved domain. The core domain includes a pleckstrin homology (PH) site, four tandem EF hands, a X-Y linker, and a C2 domain [34]. Using the PH domain, PLC anchors phosphatidylinositol 4,5-bisphosphate (PIP₂) and $\beta\gamma$ -subunits of G proteins. The EF hand is a known Ca²⁺-binding region, which promotes G α_q -mediated GTP hydrolysis. The X-Y linker contains the catalytic activity. The C2 domain regulates PLC activity involving Ca²⁺-dependent membrane targeting. Additionally, there are other unique domains specific to each subtype. Notably, PLC β s contain a postsynaptic density protein 95, disc large, zona occludens-1 (PDZ)-binding motif at the C-terminus which interacts with different PDZ proteins (Figure 1.2) [35]. Approximately half of G_qPCRs also have a PDZ-binding motif at the long C-terminal region. Each PLC β isozyme has different PDZ-binding motifs, enabling each PLC β to interact with specific G_qPCRs via particular PDZ proteins [36, 37].

The expression patterns and regulation of PLC β s, PLC β 1-4, are somewhat different. PLC β 1 and PLC β 4 are enriched in neural tissue, PLC β 2 is limited to hematopoietic cells, and PLC β 3 is more widely expressed [38]. In pancreatic β -cells, the expression of PLC β isozymes has been shown to differ according to species, specimen type, and detection method. In rat insulinoma cell lines, such as INS-1 and betaG 40/110 cells, immunoblotting has shown that PLC β 1, PLC β 2, and PLC β 3 are detectable, but the expression levels of these isozymes are slightly reduced in rat islets [39]. The PLC β 1 is especially localized in the nucleus as well as the cytoplasm in the mouse insulinoma cell line, MIN6 [40]. Regarding rat primary tissue, all PLC β isozymes have been detected in the islets of Langerhans by western blot analyses. Using immunohistochemistry (IHC) of the rat pancreas, PLC β 1 and PLC β 4 have

been found in endocrine tissues. PLC β 2 and PLC β 3 are mainly expressed in the periphery of the islets [41]. The use of monoclonal antibodies revealed high levels of PLC β 4 and moderate levels of PLC β 3 in the pancreatic islets in the IHC of the mouse pancreas. PLC β 1 was mainly found in exocrine tissue [42]. However, there is other evidence that both rat and mouse islets express PLC β 1. [43]. Recently, we found that PLC β 1, PLC β 3, and PLC β 4 were expressed in the MIN6 cell line and mouse primary islets (unpublished data). As the physiological function of each PLC β isozyme in pancreatic β -cells remains largely unknown, it is important to clearly define the regulatory mode of each PLC β during insulin secretion.

Activation of PLC β s by glucose in pancreatic β -cells

Glucose is the most potent component that regulates β -cell functions. In addition to metabolic functions, glucose can modulate expression of several genes such as PLC β s. As a result of liquid chromatography-mass spectrometry/mass spectrometry-based proteomics of human islets after 24 h of high glucose exposure, PLC β 4 was characterized as a differentially abundant protein, with PLC β 4 downregulated under conditions of high glucose levels [44]. The exposure to high glucose levels for several days induced expression of PLC β 1 and endoplasmic reticulum (ER) stress markers, such as GRP78, sXBP1, ATF4, and Grp78. Losartan, a selective angiotensin II type 1 receptor blocker, inhibits the PLC β -IP $_3$ -Ca $^{2+}$ pathway and prevents glucose-induced expression of PLC β 1 and ER stress markers, leading to alleviated glucose toxicity conditions [45]. Several proteins, including PLC β s, have been found to be altered during conditions of high glucose levels, but further studies are required to determine how glucose is involved in the regulation of PLC β expression.

Insulin secretion is followed by the rapid breakdown of polyphosphoinositides hydrolyzed by PLC, and the glucose transported into β -cells is coupled to phosphoinositide-specific phospholipase C (PI-PLC) activity [46]. The use of [3 H]inositol-labeled phospholipids in islets and high-performance anion exchange chromatography revealed that high glucose levels (20 mM) induced early increases in inositol 1,4,5-triphosphate (IP $_3$) and inositol 1,3,4,5-tetrakisphosphate (IP $_4$) resulting from the breakdown of PIP $_2$ [47]. Additionally, PLC β 1-mediated PIP $_2$ hydrolysis might be critical for insulin secretion. Mouse islets respond less to glucose stimulation when compared with rat islets in the second phase of potentiation during insulin secretion, showing insufficient increments in PI hydrolysis. This is because of the underexpression of PLC β 1 in mouse islets compared to rat islets [43].

However, the effect of PI-PLC on insulin secretion is not related to glucose metabolism because treatment with glycolytic intermediates, such as glucose 6-phosphate did not alter PLC activity. This

might be due to an increase of $[Ca^{2+}]_i$ in the submicromolar range. The glucose-induced $[Ca^{2+}]_i$ levels lead to activation of PLC in pancreatic β -cells [48, 49]. However, little is known about how glucose-induced oscillations of $[Ca^{2+}]_i$ might be related to periodic activations of PLC β s.

PLC β -mediated downstream pathway for amplifying GSIS

Recently, several reports have described the stimulatory function of newly discovered G_qPCRs involved in the regulation of insulin secretion (Table 1.1). The activated G_qPCR generates G α_q and, in turn, activates membrane-associated PLC β s. The PLC β s hydrolyze PIP₂ into inositol triphosphate (IP₃) and diacylglycerol (DAG), which serve as important second messengers in signal transduction [50]. IP₃ is targeted to inositol trisphosphate receptors (IP₃Rs) on the ER, inducing Ca²⁺ mobilization from the ER and rapid elevation of cytosolic Ca²⁺ levels [51] (Figure 1.3). The generation of DAG results in subsequent activation of protein kinase C (PKC), increasing insulin granules in the readily-releasable pool [52] and activating transient receptor potential (TRP) channels, such as TRPM4/5 [53] and TRPC3 [54]. The DAG itself may be engaged in insulin secretion. DAG binds to the H567 site of Munc13-1, which is a DAG receptor, leading to insulin vesicle priming in pancreatic β -cells [55]. Locally reduced PIP₂ and increased DAG directly activate TRPC3 [56]. The release of the G $\beta\gamma$ complex also plays a role in insulin secretion. When the $\beta\gamma$ subunit-binding domains, such as the G α subunit of the retinal G-protein transducing or the PH domain of G-protein-coupled receptor kinase 2, were expressed, the G $\beta\gamma$ -mediated signaling was masked, and insulin secretion was inhibited [57]. Also, the G $\beta\gamma$ -dependent pathway is involved in the kisspeptin-10-dependent increase in insulin secretion [58]. However, the role of the G $\beta\gamma$ subunit in regulation of insulin secretion is still not definitively known because it serves as a negative regulator of voltage-gated calcium channels in neuronal and non-neuronal cells [59]. Besides the IP₃-mediated release of Ca²⁺ from internal stores, PLC β can facilitate a further increase in $[Ca^{2+}]_i$ by activation of store-operated channels (SOC) [60]. The PLC β -IP₃-induced depletion of Ca²⁺ in the ER enables STIM proteins to oligomerize and translocate to the plasma membrane, where they organize SOC with ORAI proteins and trigger store-operated Ca²⁺ entry. In addition to the GPCR-regulatory mode, the PLC β 1 in the nucleus participates in modulating transcription of PPAR- γ , which is critical for maintaining β -cell function [40] (Figure 1.4).

1.4. Overview of G_qPCRs which potentiates GSIS through PLC β signaling.

Muscarinic receptor (M₃)

The parasympathetic nervous system contributes to promoting GSIS by releasing acetylcholine, a major parasympathetic neurotransmitter, in pancreatic islets. The acetylcholine from nerve endings exerts its effects through muscarinic type 1 and 3 (M1 and M3) receptors on pancreatic β -cells [61-63]. The activation of muscarinic receptors and insulin secretion is directly related to PLC β activity. Carbachol and oxotremorin M increase PLC β -induced IP₃ production and transient [Ca²⁺]_i and insulin secretion, and their effects are abolished by aminosteroid U-73122, a PLC inhibitor [64]. Muscarinic receptor stimulation leads to rapid and sustained PLC β activity, and the periodic activation of PLC is tightly regulated by dynamic [Ca²⁺]_i oscillations, whereas sustained PLC β activity is enhanced by increased cytosolic Ca²⁺ levels from intracellular stores and SOC channels [65]. Cholinergic regulation is also involved in insulin-containing granule movement toward proximal regions of the plasma membrane. IP₃-mediated Ca²⁺ mobilization by PLC β s during cholinergic activation causes Ca²⁺/calmodulin-dependent phosphorylation of myosin light chains. The phosphorylated myosin facilitates recruitment of insulin vesicles to the readily releasable pool [66]. Chronic exposure to high glucose or carbachol levels causes β -cell desensitization, reducing the activation of insulin secretion and the PLC response following high glucose stimulation [67].

FFA receptor 1 (FFA1)

Free fatty acid receptor 1 (FFA1) is preferentially expressed in pancreatic β -cells and participates in amplifying GSIS [68]. FFA1 can be activated by FFAs including saturated, mono- and polyunsaturated medium-to-long-chain fatty acids [69]. PLC β signaling has an essential role in FFA-induced insulin secretion [70]. Additionally, treatment with an inhibitor for G α_q , YM-254890, and inhibitor for PLC, U73122 and neomycin, results in reduced insulin secretion and FFA1-mediated Ca²⁺ efflux from the ER [71, 72]. The increase in [Ca²⁺]_i induced by stimulation of FFA1 is attenuated by nitrendipine, a LTCC blocker, which indicates that not only G α_q -PLC β -mediated intracellular Ca²⁺ efflux from the ER but also extracellular Ca²⁺ influx via L-VDCC is also involved in FFA1-induced insulin secretion [73]. DAG also exerts synergistic effects on insulin secretion as an activator of L-VDCC following activation of FFA1 [74]. Additionally, nonselective cation ion channels, such as TRPC3 channels, also contribute to the FFA1-PLC β pathway to augment insulin secretion, showing that inhibition of PLC and PKC activities results in reduced NSCC current and decreased GSIS induced by FFA1 stimulation [54]. FFA1 also activates the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway, which is a downstream pathway of G α_q -PLC β [75]. However, whether activation of the ERK1/2 pathway correlates with induction of insulin secretion remains controversial [76].

Serotonin receptor (HTR2B)

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter, and its action on the central nervous system (CNS) in the regulation of mood and behavior is well-known. However, 5-HT is also found outside the CNS, such as in the enterochromaffin cells of the gut and in the platelet and pancreatic β -cells [77, 78]. The pancreatic β -cells are able to synthesize 5-HT from 5-hydroxytryptophan via tryptophan hydroxylase 1 (TPH1), and the expression of TPH1 and the synthesis of 5-HT are dramatically increased under conditions in which the ability to secrete insulin has been enhanced, such as during pregnancy and the insulin-resistant state [79-81]. The 5-HT is stored in insulin vesicles and co-secreted with insulin. These act on surface receptors, primarily Htr2b and Htr3a, in an autocrine and paracrine manner [82], leading to potentiating GSIS [83, 84]. The Htr3a is a ligand-gated ion channel, and the Htr2b receptor is coupled to the G_{α_q} protein, which activates the PLC β -IP $_3$ pathway to promote an increase in $[Ca^{2+}]_i$. Treatment of cells with 5-HT plus α -methyl serotonin maleate salt (AMS) and BW723C86, a Htr2b agonist, results in increased $[Ca^{2+}]_i$ [85]. Also, the effects of AMS on GSIS disappears upon exposure to the IP $_3$ receptor inhibitor, Xestospongin C [83]. Use of a rapid and sensitive method for determining PLC activity revealed that 5-HT induced a robust and rapid depletion in [3 H]phosphatidylinositol, a prelabeled substrate of PLC, and increased levels of [3 H]inositol phosphate [86]. Together, these findings suggest that 5-HT receptors, which are G_q PCRs, are functionally coupled to PLC β -mediated signaling.

Angiotensin II receptor 1 (AT1)

Angiotensin II (Ang II) induces exocytosis of insulin vesicles through Ang II receptors. The angiotensin system has been classically linked to the regulation of blood pressure and fluid balance. However, there is an angiotensin-generating system in the pancreas [87], and Ang II receptor 1 (AT1) is also expressed in pancreatic β -cells [88]. Ang II induces an increase in $[Ca^{2+}]_i$ by promoting both the influx of extracellular Ca^{2+} through L-VDCC and the PLC β -IP $_3$ -mediated efflux of Ca^{2+} from the ER [89]. PLC β signaling has been shown to be involved in the Ang II-mediated regulation of GSIS. Future studies should therefore characterize the role of PLC β signaling in the Ang II pathway that potentiates GSIS.

Interleukin (IL)-6 receptor (IL-6R)

The proinflammatory cytokine, IL-6, affects nonimmune events, such as the induction of glucose

metabolism [90]. IL-6R has been localized to pancreatic β -cells, and circulating hepatic IL-6 potentiates insulin secretion [91]. Various inhibitors, such as U-73122 and neomycin for PLC and xestospondin C for the IP₃ receptor, have been used to show that IL-6 enhances GSIS in PLC β -IP₃-dependent pathways. Among the isozymes of PLC, PLC β 1 knockdown in MIN6 cells abolishes the stimulatory effect of IL-6 on GSIS, implying that PLC β 1 plays an important role in the IL-6-induced enhancement of GSIS [92].

Olfactory receptor (OLFR15)

Olfactory receptors have important functions in olfactory chemo-sensation, by detecting different odorants [93]. OLFR15 and OLFR21 are expressed in pancreatic β -cells (OLFR15 \gg OLFR21) [94]. As a ligand for OLFR15, octanoic acid (OA) mediates GSIS potentiation [94]. In the presence of an inhibitor for PLC and IP₃, neomycin and Xestospongin C, OA-enhanced GSIS is attenuated. Additionally, among primary PLCs (PLC β and PLC γ), inhibition of PLC β 1 results in attenuated enhancement of GSIS in response to OA [95], suggesting that the G_q-PLC β 1-IP₃ pathway is involved in OLFR15-dependent increases in GSIS.

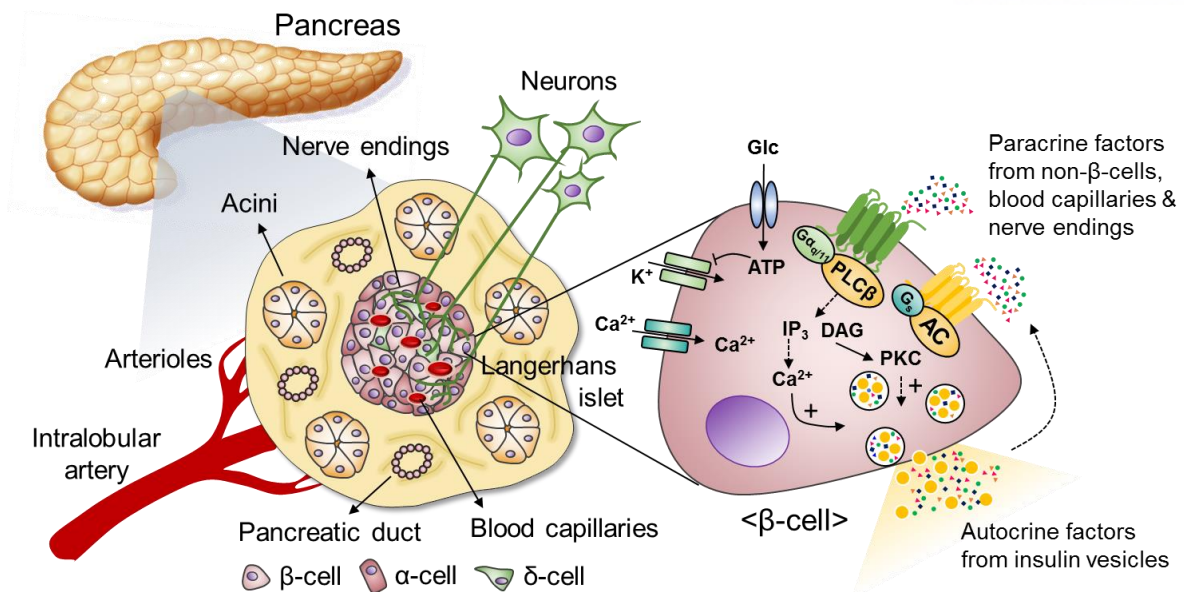
1.5. High-throughput luminescent reporter of insulin secretion

Up to date, measuring secreted insulin level have relied on the enzyme-linked immunosorbent assay (ELISA). The ELISA have been most widely used detection platform for assays of β -cell function. However, it was required multiple-liquid transferring steps, limited detection range, and high cost (> \$2~3 per sample), making it challenging for application of high-throughput screening. Degorce et al. [96] and Bielefeld-Sevigny et al. [97] pioneered notably in high-throughput screening applications based on fluorescence resonance energy transfer (FRET). No-wash assays such as time-resolved FRET (TR-FRET) and AlphaLISA immunoassay platform exhibit improved sensitivity, extensive dynamic range alternative to conventional ELISA. While these assays suited for larger format experiments, they still are comparably expensive (\$0.6~1.7 per sample) and require advanced hardware compatible with these assays.

There have been many attempts to replace the ELISA using surrogate marker such as green fluorescent protein (GFP) or luciferase cloned to the end of the insulin propeptide and co-secreted with insulin [98, 99]. While intensity of GFP and luciferase was increased in high glucose condition, they were highly correlated with insulin level measured by ELISA. Moreover, constructs including surrogate markers have possibility to be misfolded in cells, leading to detrimental effects on cell function [100]. Indeed, more than 95% of a proinsulin-firefly luciferase fusion construct was retained in the ER and

cells expressing proinsulin-Gaussia luciferase fusion protein had less potency to secrete insulin upon high glucose stimulation than control cells.

Burns SM et al. developed a luminescent insulin secretion assay for tracking insulin secretion in rapid and inexpensive way, enabling high-throughput screening application (Figure 1.5). Gaussia luciferase was inserted in replace of C-peptide portion of proinsulin, flanked by cleave sites for proinsulin convertase (Figure 1.5A). Luciferase was co-localized with insulin in cells stably expressing the construct, insulin secretion was highly correlated with luciferase, and fusion protein was not retained in the ER. We created stable cell lines expressing reporter construct using lentivirus. Upon stimulation with high glucose and oxo-m, muscarinic receptor agonist, cells potentiated insulin secretion and effects were disappeared with treatment of diazoxide, ATP sensitive- K^+ channel activator. The assay requires \$0.05 per sample and > 40-fold less time than the conventional ELISA. Also, they exhibit remarkable sensitivity and wide dynamic detection range. Using this assay, we screened FDA-approved drug to discover a putative candidate which augments insulin secretion. Among, zafirlukast, asthma drug as cysteinyl leukotriene receptor 1 antagonist, was selected as novel factor which have not reported as insulin secretagogues. We firstly demonstrated function of zafirlukast in pancreatic β -cells focused on insulin secretion.



This image was revised from figure in mini-review, Pénicaud L. *Biochimie.*(2017) 143:29-32

Figure 1.1. Vascularization and innervation of Langerhans islets

The endocrine pancreas is highly entangled by vascularization and innervation, which are critical for communication between cells and tissues. The pancreatic islets are comprised of α -cells, β -cells, and δ -cells. The autocrine factors are released from insulin-containing vesicles in β -cells, and the paracrine mediators are secreted from non- β -cells, blood capillaries, and nerve endings. They exert their biological effects mainly by acting on G-protein coupled receptors (GPCRs). In pancreatic β -cells, phospholipase C β (PLC β) and adenylyl cyclase (AC) generate important second messengers to enhance glucose-stimulated insulin secretion (GSIS). The more detailed pathway and abbreviations are listed in the Figure 2 legend. This image was revised from figure in mini-review, *Biochimie.*(2017) 143:29-32 [85].

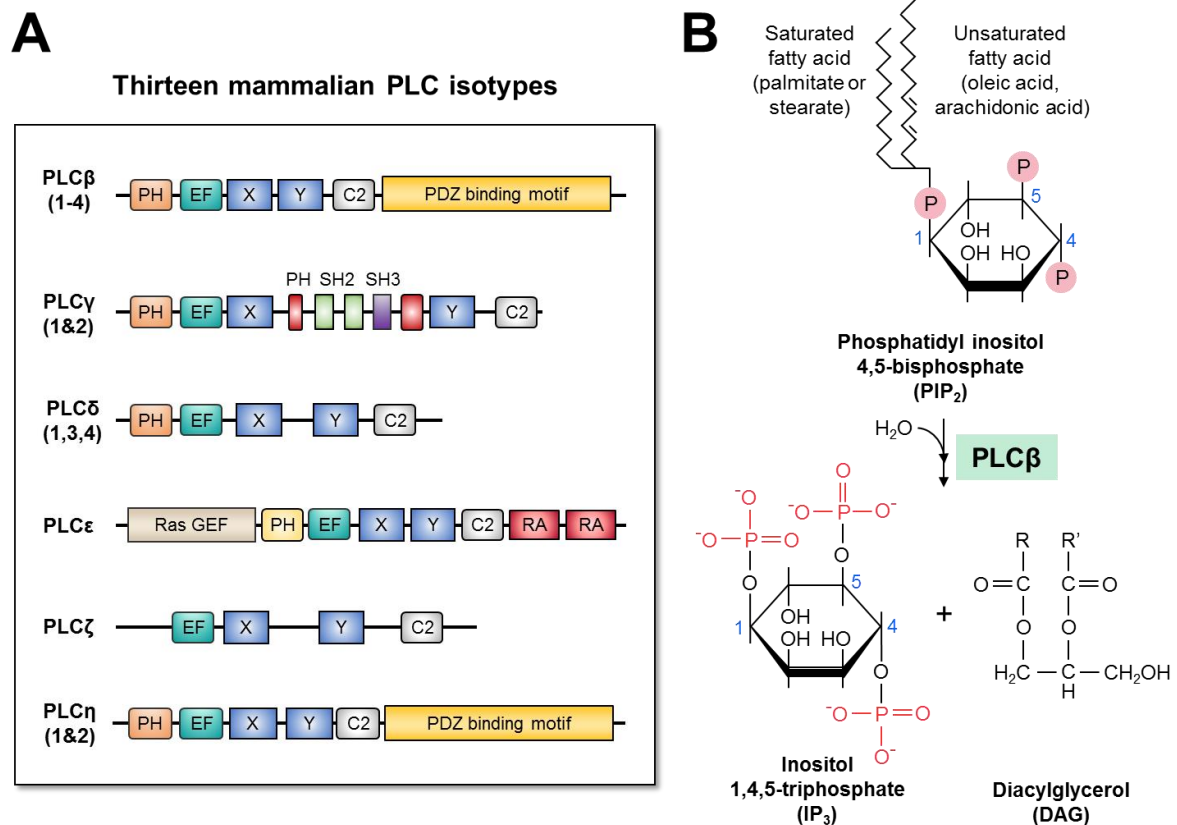
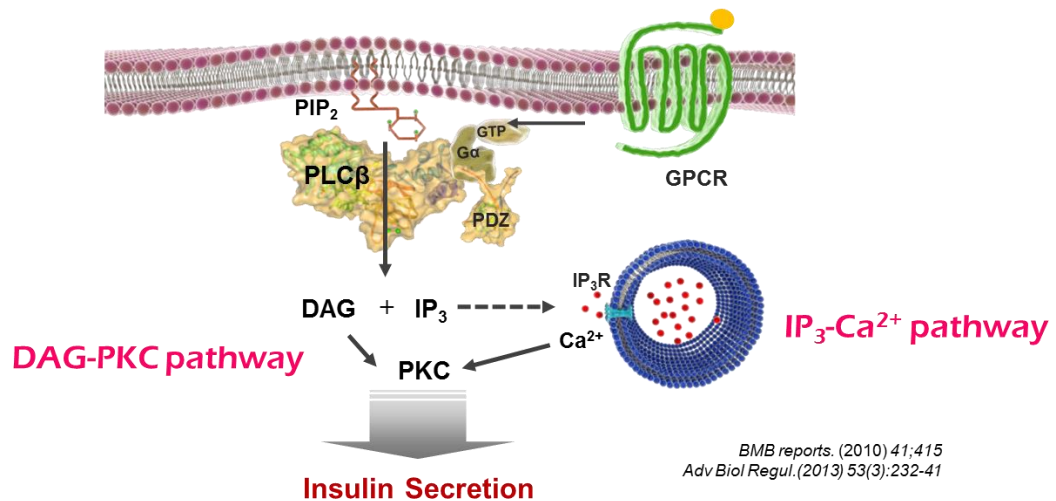


Figure 1.2. Structure of mammalian PLC isotypes and detailed action of PLCβs

(A) The thirteen kinds of mammalian phospholipase C (PLC) isotypes are classified into six isotypes ((β, γ, δ, ε, ζ, η) according to their structure. The PLC isotypes have a highly conserved domain. The PH domain enables PLC to anchor plasma membrane, C2 domain could regulate PLC activity, XY domain has catalytic activity and EF hand is Ca²⁺-binding region which triggers GTP hydrolysis of Gα_q. The PLCβ isozymes contain different PDZ binding motif which binds to particular PDZ scaffolding proteins. (B) The PLCβs selectively catalyze the hydrolysis of phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP₂) on the glycerol portion of the phosphodiester bond. It results in the release of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). PH; pleckstrin homology, SH2; SRC homology 2, GEF; guanine nucleotide exchange factor, PDZ; PSD-95/Discs-large/ZO-1.

- The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂)
- Generation of second messenger (DAG and IP₃)
- Four PLCβ isozymes (PLCβ_{1~4})
Temporal and spatial expression, intracellular distribution, biological effect and regulatory properties



PLCβ-mediated GPCR signaling might be involved in insulin secretion

Figure 1.3. PLCβ as key molecule of GPCR signaling

The PLCβ is one of the enzymes of the six subtypes (β, γ, δ, ε, ζ, and η) that plays a crucial role in GPCR-mediated signaling. PLCβ hydrolyzes membrane-bound PIP₂ into IP₃ and DAG, which are important second messengers in the downstream signal pathway, regulating Ca²⁺ mobilization and Protein kinase C (PKC) activation, respectively. The family of PLCβ consists of four isozymes, PLCβ₁ to PLCβ₄, sharing their primary structure. However, each isozyme differs in temporal and spatial expression, intracellular distribution, biological and regulatory properties. The PLCβ isozymes contain different PDZ binding motifs that recognize specific PDZ scaffolding proteins. Various PDZ proteins enable PLCβ to be coupled to specific GPCR signaling pathway. The GPCR-PLCβ signaling is involved in amplifying GSIS through mainly IP₃-Ca²⁺ pathway and DAG-PKC pathway. PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PDZ, PSD-95/Discs-large/ZO-1.

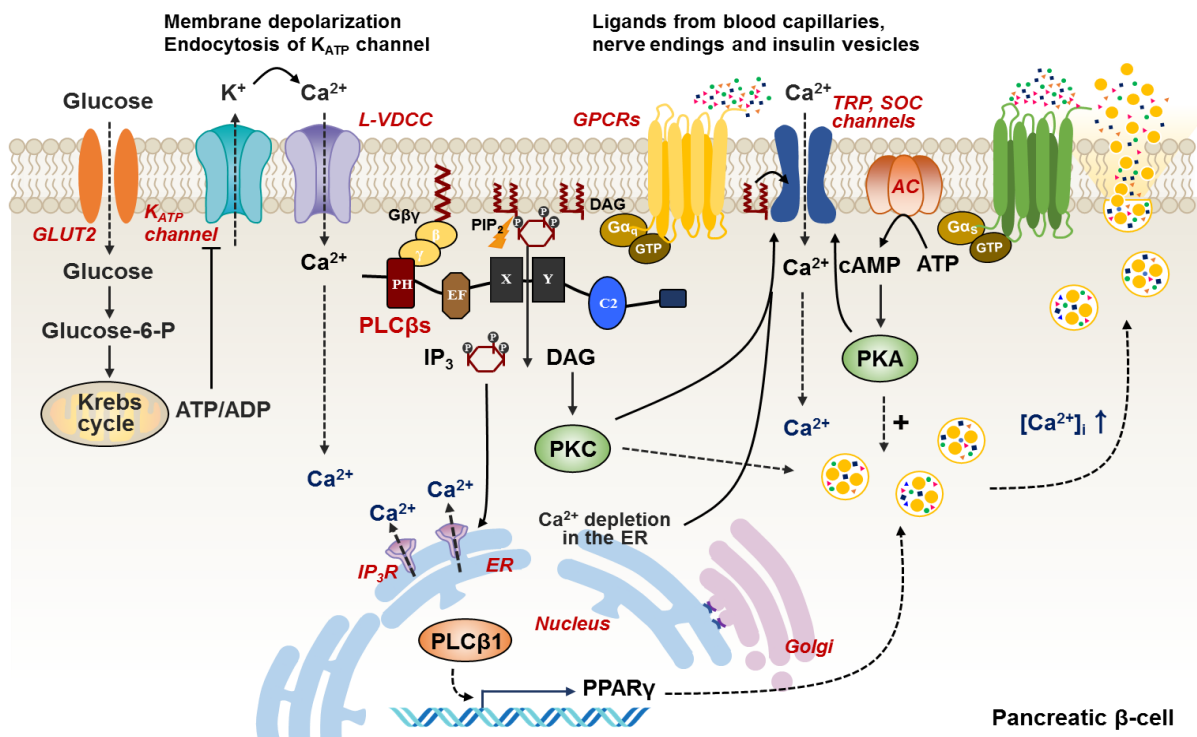


Figure 1.4. Overview of phospholipase C β -mediated insulin secretion pathways

As the main regulator of insulin secretion, glucose is transported into the β -cells through GLUT2 and metabolized, increasing the ATP/ADP ratio. This results in closure of K_{ATP} channels and endocytosis of K_{ATP} channels, inducing membrane depolarization and activation of L-VDCC. The extracellular influx of Ca^{2+} through L-VDCC induces a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$), triggering insulin secretion. This process is called glucose-stimulated insulin secretion (GSIS). There are amplifying pathways for potentiating GSIS. GPCR signaling is mainly involved in the augmentation of GSIS by different ligands. Each PLC β and AC is coupled to $G_{\alpha q}$ and $G_{\alpha s}$ proteins of GPCRs, respectively. PLC β hydrolyzes membrane-bound PIP $_2$ into IP $_3$ and DAG, and AC generates cAMP, which activates PKA. The IP $_3$ binds to IP $_3$ R on the ER and induces Ca^{2+} efflux from internal stores. Ca^{2+} depletion in the ER activates SOC channels on plasma membranes (PMs), inducing the influx of extracellular Ca^{2+} . The increased DAG and activated PKC lead to activation of TRP channels. The PKC and PKA directly activate components of exocytotic machinery, increasing in RRP s adjacent to the PM. The nuclear PLC β 1 facilitates induction of PPAR- γ expression and an increase in insulin secretion.

GLUT2, glucose transporter 2; K_{ATP} , ATP-sensitive potassium channel; L-VDCC, L-type voltage-dependent Ca^{2+} channel; GPCR, G protein-coupled receptor; TRP, transient receptor potential channel; SOC, store-operated channels; RRP, readily-released pool; PLC β , phospholipase C β ; AC, adenylyl cyclase; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; ER, endoplasmic reticulum; IP₃R, IP₃ receptor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PPAR- γ , peroxisome proliferator-activated receptor- γ .

Table 1.1. GPCRs highly relevant Gq/PLC β signaling for stimulating insulin secretion

<i>Receptor name (alias)</i>	<i>Natural ligand</i>	<i>G protein</i>	<i>References</i>
<i>Muscarinic acetylcholine M₃ receptor (M₃)</i>	<i>Acetylcholine</i>	<i>G_q, (G_s)</i>	<i>Proc Natl Acad Sci U S A.(2010) 107:21181-6 Diabetes.(2001) 50(11):2505-13</i>
<i>Cholecystokinin A receptor (CCK₁)</i>	<i>Cholecystokinin-8</i>	<i>G_q, (G_s)</i>	<i>Br J Pharmacol. (2015) 172:5050-67</i>
<i>Free fatty acid receptor 1 (GPR40, FFA1)</i>	<i>Mid- and long FFAs</i>	<i>G_q</i>	<i>Sci Rep. (2016) 6:25912</i>
<i>KiSS1-derived peptide receptor (GPR54)</i>	<i>Kisspeptin-10</i>	<i>G_q</i>	<i>Diabetologia (2009) 52:855–862</i>
<i>Vasopressin receptor 1B (V_{1B})</i>	<i>Vasopressin</i>	<i>G_q</i>	<i>J Biol Chem.(1990) 265, 15724-15730</i>
<i>Serotonin receptor (HTR2B)</i>	<i>Serotonin</i>	<i>G_q</i>	<i>Diabetologia. (2016) 59:744-54</i>
<i>Angiotensin II receptor type 1 (AT₁)</i>	<i>Angiotensin II</i>	<i>G_q</i>	<i>Diabetologia. (2006) 49:321-31</i>
<i>Interleukin-6 receptor (IL-6R, CD126)</i>	<i>Interleukin-6</i>	<i>G_q</i>	<i>Diabetes. (2011) 60:537-47</i>
<i>Olfactory receptors 15 (OLFR15)</i>	<i>Octanoic acid</i>	<i>G_q</i>	<i>Sci Rep. (2018) 8:1499</i>

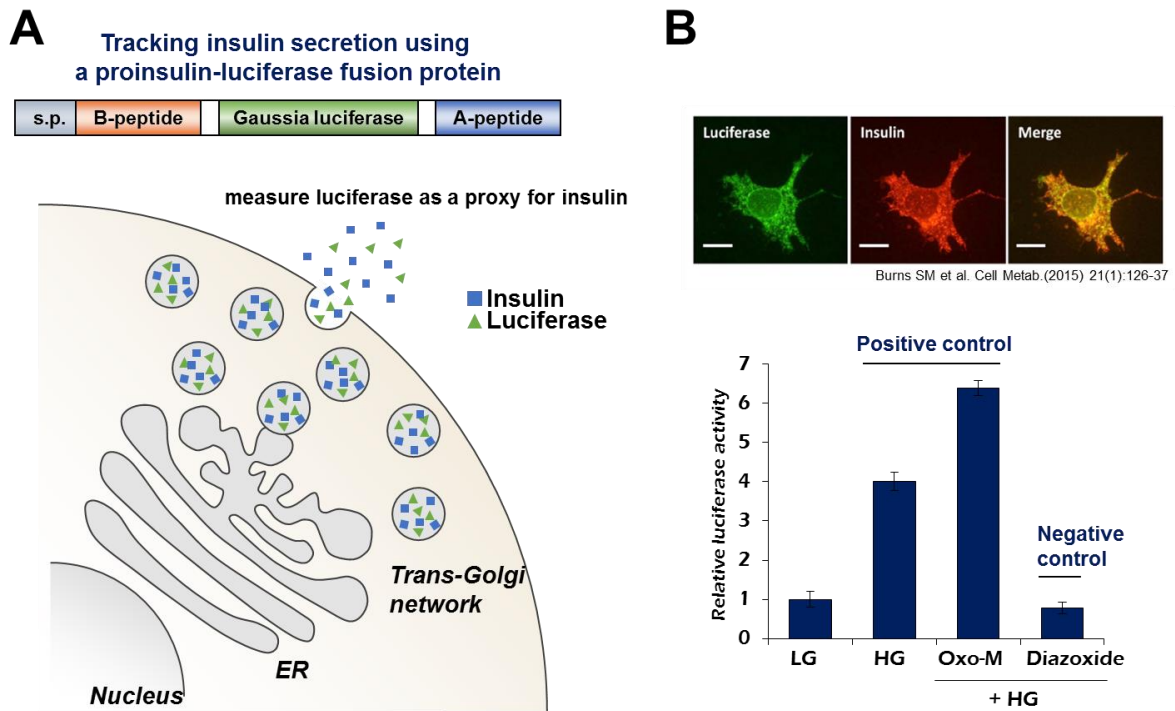


Figure 1.5. Schematic diagram of a proinsulin-luciferase fusion protein and validation of luciferase activity as a proxy for secreted insulin

(A) Diagram of proinsulin-luciferase fusion construct, showing *Gaussia* luciferase was inserted in C-peptide portion of proinsulin which was co-secreted with insulin. (B) Co-localization of luciferase and insulin. Luciferase activity upon high glucose with Oxo-M, positive control, and diazoxide, negative control.

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Chapter II

Phospholipase C β 1 potentiates glucose-stimulated insulin secretion

2.1. Introduction

Diabetes mellitus has grown to epidemic proportions and is considered a leading cause of death worldwide [1]. The etiology provides that a defect in pancreatic β -cell function significantly contributes to the pathogenesis of diabetes [2, 3]. Therefore, recent studies have focused on the improvement of β -cell function via descriptions of new secretagogue in insulin secretion and the molecular mechanisms underlying these effects [4].

Glucose-stimulated insulin secretion (GSIS) in the pancreatic β -cell is mainly regulated by the glucose-stimulated triggering and amplifying pathways by a variety of factors [5]. The sequence of events that occurs as part of the triggering pathway are well established, i.e., glucose metabolism, closure of ATP-sensitive K^+ channels, membrane depolarization, and Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Increased intracellular Ca^{2+} levels induce exocytosis of the insulin vesicle via activation of the exocytotic machinery [6]. The amplifying pathway is involved in both phases of GSIS and its potentiation in response to secretagogues such as non-glucose nutrients (e.g., free fatty acids and amino acids), hormones, and neuronal factors. A variety of substances such as serotonin (5-HT), nucleotides, amylin, or bivalent cations can also co-localize with insulin in secretory granules. These mediators are co-secreted with insulin upon glucose stimulation, primarily acting on surface receptors in both a paracrine and autocrine manner [7-10]. The majority of these extracellular signals are mediated via G protein-coupled receptors (GPCRs), which activate intracellular signaling by generating inositol triphosphate (IP_3), diacylglycerols (DAGs), and cyclic adenosine 3',5'-monophosphate (cAMP).

Studies have shown that mice with GPCR-deficient in β -cells exhibit glucose intolerance and are unable to potentiate GSIS in response to corresponding ligands [11, 12]. Recently, these islet GPCRs have been regarded as potential targets for regulating insulin release, with several drugs already approved for the treatment of diabetes mellitus [13]. However, the exact mechanism underlying the amplifying effect has remained elusive and further studies are necessary to identify additional targets and mechanisms that involve the regulation of insulin secretion.

Phospholipase C β (PLC β) is one of the six enzyme subtypes (β , γ , δ , ϵ , ζ , and η) that play a principal role in GPCR-mediated signaling. PLC β hydrolyzes membrane-bound phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG, which are important secondary messengers in the downstream

signal pathway, regulating Ca^{2+} mobilization and protein kinase C (PKC) activation, respectively. The PLC β family consists of four isozymes, PLC β 1 to β 4, which share their primary structure; however, each isozyme differs in temporal and spatial expression, intracellular distribution, biological effect, and regulatory properties [14, 15].

Emerging evidence has shown that PLC β s are considered crucial effectors in GPCR-mediated signaling transduction for potentiating insulin secretion. β -cell-specific ablation of G protein α -subunits $\text{G}\alpha_q$ and $\text{G}\alpha_{11}$, which activate PLC β s, results in glucose intolerance and impaired insulin secretion. This indicates that PLC β s also contribute to the regulation of insulin secretion [16, 17]. However, previous studies have not focused on the roles of the specific PLC β isozymes, and have only demonstrated the putative role of PLC β s in pancreatic β -cells, showing that blockade by non-selective PLC inhibitors, PKC inhibitors, or Ca^{2+} chelators leads to diminished insulin release [18, 19]. Additionally, it remains unclear which PLC β regulates insulin secretion via which GPCR.

To address the physiological role of PLC β s, we generated PLC β isozyme-deficient mice in pancreatic β -cells. β -cell-specific PLC β 1 conditional knockout (cKO) mice (*Plcb1*^{fl/fl}; *Pdx1-CreERT2*) showed impaired glucose tolerance due to deficiency in insulin secretion, with more severe phenotypes seen in *Plcb1*^{fl/fl}; *Pdx1-CreERT2* mice fed high-fat diets (HFDs). Furthermore, we discovered that particular GPCR-induced insulin release was PLC β 1-dependent. Taken together, the data presented here show that PLC β 1 is an important factor in the regulation of insulin secretion.

2.2. Materials and Methods

Mice

Plcb1, 2, 3, and 4 cKO mice were generated by crossing *Plcb1*, 2, 3, or 4^{loxP/loxP} mice (C57BL6/J background) with *Pdx1-CreERT2* transgenic mice (C57BL6/J background). The LoxP sites of *Plcb1*^{loxP/loxP} mice were flanked with exon 11, the LoxP sites of *Plcb2*^{loxP/loxP} mice were flanked with exons 8–12, the LoxP sites of *Plcb3*^{loxP/loxP} mice were flanked with exons 9–10, and the LoxP sites of *Plcb4*^{loxP/loxP} mice were flanked with exon 6. Animals were backcrossed for at least eight generations onto a C57BL6/J background prior to use in these experiments. To induce the ablation of each *Plcb* isozyme gene, 4-week-old mice were injected with 2 mg of tamoxifen (Sigma, St. Louis, MO) intraperitoneally twice weekly for 2 weeks (four injections in total). Tamoxifen-treated male mice were used for all analyses. The genotypes of each group were confirmed by PCR. Genomic DNA from mice tails were used as templates for primers specific to the *Plcb* isozyme. To simulate a western diet, mice

were fed a HFD (60% kcal fat; #D12492; Research Diets, New Brunswick, NJ) from 6 weeks of age. The HFD study was performed with mice fed HFD for 10 weeks. Animals were maintained in a specific pathogen-free animal facility under a 12 h light/12 h dark cycle at a temperature of 21°C. The mice were allowed free access to water and food. All procedures were performed in accordance with the UNIST Guide for the Care and Use of Laboratory Animals (UNISTIACUC-14-013, -014, -015 and -016).

Animal experiments

For the glucose tolerance test, animals were fasted overnight (16 h) and injected intraperitoneally with 2 mg/g of glucose. For the insulin tolerance test, animals were fasted for 6 h and intraperitoneally injected with 0.3 U/kg insulin. Blood glucose levels were assessed before injection and at 15, 30, 60, and 120 min after injection. Animals were treated intraperitoneally with a single dose of 5-HT (0.3 mg per mouse; Tocris Bioscience, Bristol, UK) and a single dose of bethanechol (2 µg/g; Sigma). Plasma glucose and insulin levels were measured at the indicated time points. Blood glucose levels were measured with a glucometer (Roche, Basel, Switzerland) and plasma insulin levels were estimated by ELISA using a mouse insulin ELISA kit (Shibayagi, Gunma, Japan).

Western blotting

Isolated islets were lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA) containing a cocktail of proteinase inhibitors and phosphatase inhibitors according to the manufacturer's recommendation. The total protein concentration was determined with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Total protein was separated with 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA), blocked with 5% (w/v) fat-free milk, and incubated with primary antibodies overnight at 4°C. After washing with Tris-buffered saline containing Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody and visualized using enhanced chemiluminescence reagents (GE Healthcare Life Sciences, Marlborough, MA). Anti-PLCB1 (sc-205), anti-PLCB2 (sc-206), anti-PLCB3 (sc-403), and anti-PLCB4 (sc-404) were obtained from Santa Cruz Biotechnology (Dallas, TX); anti-β-actin (GTX109639) was obtained from GeneTex (Irvine, CA). In apoptotic marker, anti-caspase3 and anti-PARP1 antibody were obtained from Abcam (Cambridge, UK) and Cell Signaling (Danvers, MA).

Islet isolation

The pancreas was directly injected with 3 mL of 1 mg/mL collagenase (Roche) in Hank's buffered saline solution (HBSS; Welgene, Gyeongsan-si, Republic of Korea) modified with 25 mM HEPES and adjusted to pH 7.4 with NaOH before addition of 0.25% (w/v) bovine serum albumin. The inflated pancreas was then dissected and incubated at 37°C for 15 min with manual shaking every 5 min. Samples were then filtered and washed with ice-cold modified HBSS three times, after which islets were separated by density gradient in Biocoll separating solution (Biochrom, Cambridge, UK). Islets were picked from the resulting layers and washed at least three times with ice-cold modified HBSS. Islets were then hand-selected under a dissecting microscope (SZ2-ST; Olympus, Tokyo, Japan), and cultured in RPMI-1640 medium at 37°C in a cell CO₂ incubator overnight before the beginning of the experiments.

Islet secretion assay

After overnight recovery, size-matched medium islets and MIN6 cells were equilibrated with Krebs buffer solution (KRB; 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 10 mM HEPES, and adjusted to pH 7.4 with NaOH before addition of 0.5 g/L bovine serum albumin) plus 2.8 mM glucose for 1 h. The islets were transferred to a 48-well plate and stimulated in 300 µL of Krebs buffer plus 2.8 mM and 16.7 mM glucose for 1 h (10–20 islets/well) containing ligands such as kisspeptin-10 (KP; Tocris Bioscience), [arg8][9]-vasopressin (AVP) acetate, 5-HT, cholecystokinin-8 (CCK; Tyr[SO₃]¹)CCK-8), oxotremorine-M (OXO-M; DiscoverRx), and oleic acid (OA; Sigma). The supernatant was then collected, and insulin levels were normalized to the total protein level. The amount of insulin was quantified by ELISA using a mouse insulin ELISA kit (Shibayagi). All steps were performed in a CO₂ cell incubator.

Determination of intracellular Ca²⁺

Islets were washed twice, after which 10–20 islets per well were seeded onto 96-well optical-bottom plates (Thermo Fisher Scientific) and loaded with fura-2-acetoxymethyl ester (Fura-2AM; final concentration 4 µM) in KRB for 45 min. Loaded islets were washed twice and used after a 15-min stabilization period. The plate was inserted into a microscope holder in contact with 100 µL of buffer at

37°C. Glucose (16.7 mM) and ligands (50 μ M 5-HT and 20 μ M OXO-M) were applied on top of the imaged islets, as indicated in the figure legends. The excitation signals were estimated using a fluorimeter to monitor fura-2AM fluorescence (motorized inverted microscope IX81; Olympus).

Total insulin content measurement

The total pancreas was placed in an acid-ethanol solution (1.5% HCl in 70% EtOH) and incubated overnight at -20°C . The pancreas was then homogenized and again incubated overnight at -20°C . The homogenized pancreas was centrifuged at 2,000 rpm for 15 min at 4°C , after which the aqueous solution was transferred and neutralized in 100 μ L of acid-ethanol extract with 100 μ L of 1M Tris (pH 7.5). The neutralized solution was measured by ELISA and protein content level was used as the internal control.

Islet morphometry and immunohistochemistry

Isolated pancreases were fixed in 10% neutral buffered formalin (Sigma) at 4°C overnight. Tissues were then embedded in paraffin, and 2 μ m sections were sliced and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) using standard procedures or immunostained with PLC β 1 (ab185724; Abcam), insulin antibody (ab6995; Abcam), glucagon antibody (G2654; Sigma), Somatostatin antibody (#13-2366; American Research Products Inc., Waltham, MA) and Ki-67 antibody (ab15580; Abcam). Sections were revealed using HRP- or Alexa dye-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). For insulin positivity, the islet insulin immunoreactive cross-sectional area were analyzed and calculated using ImageJ software (NIH).

Quantitative real-time PCR

RNA was isolated with TRIzol reagent and cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was performed using Prime Q-Mastermix (Genet Bio, Daejeon, Republic of Korea) and the LightCycler 480 II Real-Time PCR system (Roche). Relative gene expression was calculated using the $\Delta\Delta\text{Ct}$ method using 36B4 as an internal control. The primer sets used are listed in Table 2.1.

Brain immunohistochemistry

Mice were transcardially perfused with a 4% paraformaldehyde and post-fixed overnight at 4°C. Fixed brains were placed in 30% sucrose in phosphate buffered saline (PBS) overnight and the brain was sliced into 40µm thickness using vibrating blade microtome. Freely floating sections were rinsed with PBS and permeabilized in PBS containing 0.2% Triton X-100 for 1 h at room temperature (RT). After permeabilization, brain sections were blocked with 10% normal goat serum in PBS for 1 h. For investigation of PLCβ1 expression, brain sections were incubated with anti-PLCβ1 primary antibody (1:200, sc-5291; Santa Cruz Biotechnology) at 4°C for 12 h followed by incubation for 1 h at RT with goat anti-mouse Alexa-488 conjugated IgG (1:500, Invitrogen). All stained sections were counterstained with Hoechst 33258 (#94403; Sigma) and mounted with mounting medium (F4680; Sigma). Images were acquired with Confocal microscopy (FV1000, Olympus).

Metabolic analysis

Food intake were determined using Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA). Mice were individually placed in each cages of the calorimetric system which were kept at room temperature, 21°C. Measurements were proceeded for 72 h. The mice were free to access to water and foods.

Generation of stable cell lines

Plcb1-deleted MIN6 cell lines were generated by transfection of DNA construct expressing sgRNA (ex30; CCGATCGTACATCCAGGAGGTGG) plus Cas9 (ToolGen, Seoul, Republic of Korea) using lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the protocol from manufacturer. The cells were selected with hygromycin (200µg/mL, Sigma) in DMEM medium containing 10% FBS for 4 days. The PLCβ1 reconstitution was achieved using lentiviral vector expressing PLCβ1 (EX-Mm20972-Lv157 plasmid; GeneCopoeia, Rockville, MD) in *Plcb1*-deleted MIN6 cells. Loss and reconstitution of PLCβ1 expression was determined by Western blot.

Statistical analysis

Data were analyzed using a two-tailed unpaired sample *t*-test, with ****p* < 0.001, ***p* < 0.01, and

* $p < 0.05$ considered as statistically significant.

2.3. Results

PLC β 1, 3, and 4 isozymes are moderately expressed in pancreatic β -cells

PLC β s have been shown to be major effector molecules in the pancreatic β -cell signaling cascade and have been implicated in the potentiation of insulin release [20, 21]. The four PLC β isozymes, PLC β 1 to β 4, differ in expression pattern and regulation. However, studies of PLC β isozyme expression patterns have been controversial [22-24], with additional investigations necessary to determine the expression levels of individual PLC β isozymes. First, to confirm the PLC β isozyme expressed in pancreatic β -cells, we assessed PLC β isozyme expression patterns in MIN6 cells, a mouse pancreatic β -cell line, and in mouse primary pancreatic islets in which β -cells are the major cell type by Western blotting (Figure 2.1A) and qRT-PCR (Figure 2.1B, C). PLC β 1 is enriched in the cerebral cortex and hippocampus [25], whereas PLC β 4 is highly expressed in the cerebellum [26]. Expression of PLC β 2 is largely restricted to hematopoietic cells [27], while PLC β 3 is ubiquitously expressed [28]. Indeed, PLC β 1 and PLC β 4 were highly expressed in the brain, while PLC β 2 was limited to the thymus enriched origin of hematopoietic cells. In the case of mouse pancreatic β -cells in the present study, PLC β 1, β 3, and β 4 expressions were apparent; however, PLC β 2 was detected at only trace levels in MIN6 cells and pancreatic islets. Taken together, these results led us to further investigate PLC β isozymes as a potential regulator of β -cell function.

Each PLC β isozyme was selectively ablated in adult pancreatic β -cells

To gain insight into the physiological role of each PLC β isozyme in adult pancreatic β -cells, we employed the conditional gene knockout system to eliminate each *Plcb* isozyme gene in mature β -cells. After embryonic day 16.5, pancreas/duodenum homeobox protein 1 (Pdx1) is specifically expressed in the β -cell compartment in pancreatic islets [29]. Thus, we generated PLC β isozyme cKO mice in adults by mating floxed/floxed mice for each PLC β isozyme with mice carrying the *Pdx1-creERt2* transgene, in which tamoxifen induced activation of Cre recombinase via the Pdx1 promoter (Figure 2.2A). Cre activation was induced in 4-week-old male mice via twice weekly intraperitoneal injections of tamoxifen for 2 weeks. After 1 week of tamoxifen injection, reduced expression of each PLC β isozyme was confirmed in protein extracts from islets prepared from control and PLC β isozyme cKO mice

(Figure 2.2B). This finding indicated that PLC β isozyme deletion was restricted to the β -cell area and any alteration of phenotype in PLC β cKO mice was derived from the deletion of the PLC β isozyme in pancreatic β -cells.

Loss of PLC β 1 in adult pancreatic β -cells resulted in impaired glucose tolerance

Next, we assessed glucose homeostasis in the PLC β cKO mice. Before and after tamoxifen injection, all mice carrying *Pdx1-creERT2* showed similar body weights (Figure 2.8A), as well as fasting and re-feeding blood glucose levels, as the control group (Figure 2.8B). A glucose tolerance test was used to measure the dynamics of glucose response in mice (Figure 2.3A–D). Following intraperitoneal injection of glucose, only the *Plcb1^{fl/fl}; Pdx1-CreERT2* mice exhibited a lower ability to clear glucose from the blood than the *Plcb1^{fl/fl}* mice, which resulted in glucose intolerance (Figure 2.3A); the other groups showed similar glucose tolerances to that of the control group (Figure 2.3B–D). Despite glucose intolerance, *Plcb1^{fl/fl}; Pdx1-CreERT2* mice showed similar insulin sensitivity to that of the *Plcb1^{fl/fl}* mice, as shown by the insulin tolerance test, which suggested that impaired glucose tolerance was not caused by insulin sensitivity (Figure 2.9A). Thus, the *Plcb1* gene is required for normal glycemic control.

***Plcb1^{fl/fl}; Pdx1-CreERT2* mice displayed altered insulin secretion to glucose stimulation**

To test the underlying cause of glucose intolerance, we measured plasma insulin levels in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice after overnight fasting as well as 15 and 30 min after intraperitoneal glucose challenge. Plasma insulin levels of *Plcb1^{fl/fl}; Pdx1-CreERT2* mice were lower than that of *Plcb1^{fl/fl}* mice, leading to increased plasma glucose levels (Figure 2.4A, B).

The defect in GSIS likely underlies the impaired glucose tolerance. To study the β -cell autonomous effect, and to determine whether *Plcb1^{fl/fl}; Pdx1-CreERT2* islets showed a corresponding decrease in GSIS, we performed GSIS assays using islets isolated from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice. At basal glucose levels (2.8 mM), insulin levels secreted from *Plcb1^{fl/fl}; Pdx1-CreERT2* islets were similar to *Plcb1^{fl/fl}* islets. After high glucose stimulation (16.7 mM) for 1 h, *Plcb1^{fl/fl}; Pdx1-CreERT2* islets secreted lower insulin than that of the *Plcb1^{fl/fl}* islets (Figure 2.4C). An increase in intracellular calcium ($[Ca^{2+}]_i$) is critical for regulated insulin exocytosis [30]. We measured the $[Ca^{2+}]_i$ level in primary islets isolated from the *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice using the fluorescent Ca^{2+} indicator Fura-2AM. Under high glucose stimulation, $[Ca^{2+}]_i$ levels were dramatically increased in primary islets; however, the elevation of $[Ca^{2+}]_i$ by glucose was markedly lower in primary islets

from *Plcb1^{fl/fl}; Pdx1-CreERT2* mice than in islets from the *Plcb1^{fl/fl}* mice (Figure 2.4D). This abnormal Ca^{2+} response in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice islets may have caused the impaired GSIS. These results suggest that PLC β 1 affects pancreatic β -cell function, with a potential role in mediating insulin secretion.

Insulin production and islet morphological characteristics were normal in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice

Impaired insulin secretion was associated with abnormal insulin synthesis or pancreatic β -cell architecture [31, 32]. First, we confirmed the expression of PLC β 1 by performing immunohistochemistry analysis. Consistent with the previously published data [22, 33], PLC β 1 was localized in both nucleus and cytosol in islet cells and was also detected in the nucleus in exocrine cells. However, α -cells and δ -cells were either negative or weakly positive for PLC β 1 staining (Figure 2.10). Double staining for insulin and PLC β 1 on the pancreas sections indicated that PLC β 1 expression was selectively eliminated in the β -cell compartment in the pancreas of tamoxifen-treated mouse (Figure 5A). Also, mRNA levels of PLC β 1 were diminished in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets relative to *Plcb1^{fl/fl}* islets (Figure 2.5B). We then examined whether reduced total insulin content in the pancreas might cause glucose intolerance and impaired insulin secretion. The total insulin content in the pancreas from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice was measured by ELISA and the insulin mRNA levels were determined by qRT-PCR from islets isolated from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice (Figure 2.5B, C). The total insulin content was similar in the pancreases of the *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice and there were no differences in insulin mRNA levels (*Ins1* and *Ins2*) between *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* islets in which the *Plcb1* gene was stably ablated. In H&E-stained sections, *Plcb1^{fl/fl}; Pdx1-CreERT2* islets appeared similar in shape, size, and distribution in the pancreas compared to *Plcb1^{fl/fl}* islets (Figure 2.5D). Furthermore, pancreatic β -cells from the *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice had similar areas (Figure 2.5E). Histological analysis revealed no pathological clues in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets. These results indicate that insulin secretion dysfunction is not caused by defects in insulin synthesis and β -cell architecture.

GPCR-dependent augmentation of GSIS was selectively diminished in *Plcb1*-deficient pancreatic islets

In pancreatic β -cells, G_q -protein coupled receptors (G_q PCRs) are involved in regulating potentiation of GSIS, with PLC β s acting as principal effector molecules downstream of GPCRs [16,

34]. Our initial findings indicated that PLC β 1 was engaged in the regulation of GSIS. To examine whether PLC β 1 was also associated with G $_q$ PCR-dependent potentiation of GSIS, we performed an insulin secretion assay using islets isolated from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice. Isolated islets were incubated with ligands known to augment insulin release via G $_q$ PCRs, including AVP, 5-HT, KP, CCK, OXO-M, and OA, in the presence of high glucose concentrations (16.7 mM) [13]. Treatment with these ligands led to a significant enhancement of GSIS in islets from *Plcb1^{fl/fl}* mice; however, these potentiation effects were diminished in the *Plcb1^{fl/fl}; Pdx1-CreERT2* islets for AVP, 5-HT, and KP (Figure 2.6A). These results indicated that the elimination of *Plcb1* in pancreatic β -cells caused defects in the potentiation of specific G $_q$ PCR-dependent insulin secretion.

PLC β 1 transduces intracellular signaling from specific 5-HT receptors in the superior temporal gyrus, leading to the pathogenesis of schizophrenia [35, 36]. In pancreatic β -cells, 5-HT potentiates GSIS by increasing the glucose-evoked Ca $^{2+}$ response [37]. We further explored 5-HT-mediated insulin secretion in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice. 5-HT induced an increase in [Ca $^{2+}$] $_i$ that led to potentiation of insulin secretion; however, the 5-HT-dependent effect was attenuated in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets compared to *Plcb1^{fl/fl}* islets, which resulted in altered insulin secretion (Figure 2.6B). In contrast, exposure to OXO-M, a muscarinic receptor agonist, triggered similar Ca $^{2+}$ peaks in *Plcb1^{fl/fl}; Pdx1-CreERT2* and *Plcb1^{fl/fl}* islets (Figure 2.6C). Next, we intraperitoneally injected 5-HT into *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice and measured the changes in plasma insulin and glucose at serial time points. Following 5-HT injection, there was a clear trend toward increased plasma insulin levels with decreased plasma glucose levels; however, the augmentation of 5-HT-induced insulin secretion disappeared in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice (Figure 2.6D). Additionally, we performed intraperitoneal injections of bethanechol, a nonsubtype-selective muscarinic agonist, in *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice. After bethanechol treatment, no significant change in plasma insulin and glucose levels were observed between groups, consistent with our *ex vivo* results (Figure 2.6E). These findings indicate that PLC β 1 is required for GSIS in a selective G $_q$ PCR-dependent manner, particularly as a 5-HT receptor, in pancreatic β -cells.

High-fat diet caused severe defects in glucose homeostasis in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice

Microarray analysis revealed that PLC β 1 expression in pancreatic β -cells was significantly lower in patients with type 2 diabetes than in non-diabetic subjects (Figure 2.11, $p = 0.01$), implying that PLC β 1 might contribute to the pathophysiology of type 2 diabetes [38]. To test these effects, *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice were challenged with a HFD that mimicked the status of the human

metabolic syndrome known to cause an increased functional response in pancreatic β -cells. HFD induced a significant, progressive increase in body weight in both *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice, although no significant differences in weight gain were observed between groups (Figure 2.7A). In addition, islet morphology and β -cell proportion were not distinguishable between the two groups (Figure 2.7B). Next, we performed the glucose tolerance test to analyze glucose homeostasis in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice during HFD intake. *Plcb1^{fl/fl}; Pdx1-CreERT2* mice receiving the HFD exhibited marked defects in glucose clearance in the blood relative to *Plcb1^{fl/fl}* mice (Figure 2.7C), with no change in insulin sensitivity (Figure 2.9B) or total insulin content (Figure 2.7D) between groups. This pronounced deficiency in glucose tolerance was correlated with reduced plasma insulin levels, which led to elevated glucose levels in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice (Figure 2.7E). Correspondingly, the GSIS assay in cultured islets from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a HFD showed that the stimulatory effect of high glucose was lower in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets than in those of *Plcb1^{fl/fl}* islets (Figure 2.7F). Thus, PLC β 1 is necessary to maintain normal insulin secretion from β -cells and this mechanism is more prominent in a HFD-induced state.

2.4. Conclusion and Discussion

It has been shown that progressive β -cell dysfunction, defined as the inability to secrete insulin in response to high glucose levels, is a leading pathophysiological sign of type 2 diabetes. The β -cell mechanism for sensing glucose and releasing insulin is mediated by specialized transporters and sequential enzymes [6]. While these mechanisms for GSIS is well established, the underlying factors that mediate GSIS remain poorly understood.

A recent study showed that PLC β 1 levels in β -cells of patients with type 2 diabetes was lower than in those of non-diabetic subjects. This finding suggests a possible function for PLC β 1 in the alteration of insulin secretion and pathogenesis of type 2 diabetes [38]. Interestingly, the potency of the insulin secretory response of mouse islets is lower than that of rat islets due to the lower expression of PLC β 1 and PLC δ 1 in mice, indicating a critical role of PLC β 1 in β -cell function [24]. Furthermore, the silencing of nuclear PLC β 1 results in reduced GSIS with decreased mRNA expression of peroxisome proliferator-activated receptor- γ in the INS-1 rat insulinoma cell line [33]. However, further studies exploring the function of the PLC β isozyme in pancreatic β -cells *ex vivo* and *in vivo* are required to understand the precise mechanisms controlling insulin secretion in response to different secretagogues. Here, we demonstrated that PLC β 1 enabled β -cells to manage their highly specialized function, particularly insulin secretion. We demonstrated that the conditional ablation of PLC β 1 in pancreatic β -

cells of adult mice led to glucose intolerance due to decreased insulin secretion without altering insulin content or affecting β -cell architecture. In addition, the *Pdx1* promoter showed restricted Cre activity primarily within the hypothalamus and Cre-mediated recombination in hypothalamus region could be involved in regulation of food intake or body weight [39]. Although PLC β 1 expression was decreased in hypothalamus, *Plcb1^{fl/fl}; Pdx1-CreERT2* mice showed no alteration in food intake or body weight (Figure 2.12A, B). The impaired insulin secretion in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice was attributed to an intrinsic defect in β -cells, as isolated islets from these mice exhibited lower GSIS than those of *Plcb1^{fl/fl}* mice. Our data showed that PLC β 1 exerted its effects exclusively via GSIS augmentation, making it important to determine the mechanism through which glucose induces PLC β 1-mediated insulin secretion. Glucose can stimulate the production of inositol phosphates in pancreatic islets, and PLC β is a class of enzymes involved in phosphoinositide hydrolysis. Therefore, glucose is likely to stimulate PLC activity, leading to the accumulation of inositol trisphosphate and inositol tetrakisphosphate [40, 41]. Despite a lack of evidence supporting the involvement of mediators, glucose may couple the PLC β -mediated production of inositol phosphates and insulin secretion [17, 42].

Here, we confirmed that PLC β 1 contributed to 5-HT-, VAP-, and KP-dependent insulin secretion. In pancreatic islets, innervation and vascularization are important mechanisms in the regulation of insulin secretion. Notably, modulators such as 5-HT, released from pancreatic nerve endings, and vasopressin or kisspeptin, released from blood capillaries, activate PLC β -coupled G proteins and augment insulin release under high glucose conditions. Moreover, β -cells co-secrete various substances, including 5-HT, with insulin under high glucose stimulation. These mediators from blood, nerve, and insulin vesicles could activate GPCR/PLC β 1 signaling in a paracrine and autocrine manner, leading to increased insulin secretion [7-10]. In particular, 5-HT-dependent insulin secretion is more prominent during pregnancy. Pregnancy results in increased β -cell proliferation and GSIS to compensate for the enhanced insulin demand through 5-HT signaling [37, 43]. Indeed, maternal lactogens significantly induce the synthesis and secretion of 5-HT, which activates the Htr2b receptor on β -cells to augment GSIS [44]. In male adult *Plcb1^{fl/fl}; Pdx1-CreERT2* mice, there was no remarkable change in β -cell proliferation and β -cell turnover (Figure 2.13A, B). However, PLC β 1 signaling that regulates GSIS through serotonergic pathways might also be active during pregnancy, and PLC β 1 could be the genetic cause of gestational diabetes, which has great potential for developing into type 2 diabetes mellitus [45].

GPCRs can bind specific PLC β s and modulate intracellular signaling by binding distinct partners, such as A-kinase anchoring proteins and postsynaptic density disc-large ZO-1 proteins [46, 47]. However, further studies are needed to clarify which binding partners are engaged in G_qPCR/PLC β 1 signaling.

In this study, alterations to specific ligands, such as CCK, OXO-M, and OA, did not produce any differences in insulin secretion between pancreatic islets from *Plcb1^{fl/fl}; Pdx1-CreERT2* and *Plcb1^{fl/fl}* mice. One possible explanation for this distinction is functional compensation. Although there was no compensatory upregulation of PLC β isozymes (Figure 2.14A, B), due to a shared primary structure and similar enzymatic activity, other still expressed PLC β isozymes might be engaged with these G $_q$ PCRs. In addition, this finding indicates the involvement of G protein families other than G $_q$, such as the G $_s$ family [48, 49]. Indeed, treatment of pancreatic islets with carbamylcholine, a muscarinic receptor agonist, and CCK results increased cAMP levels, which can enhance insulin secretion, indicating that they could control insulin secretion in a PLC β 1-independent manner [50]. In fact, CCK $_1$ receptors have been reported to couple to both G $_q$ and G $_s$. The CCK $_1$ -G $_s$ pathway has been shown to regulate insulin secretion under high glucose conditions, while CCK $_1$ -G $_q$ signaling is able to regulate insulin secretion under low glucose conditions [51].

The glucose-stimulated [Ca $^{2+}$] $_i$ increase in β -cells requires Ca $^{2+}$ influx via membrane depolarization and activation of L-type calcium channels. Besides IP $_3$ -mediated Ca $^{2+}$ efflux from the endoplasmic reticulum (ER), PLC β can contribute to further [Ca $^{2+}$] $_i$ increases via its function as an important upstream signaling molecule for activation of store-operated channels (SOCs) and transient receptor potential channels (TRPCs) [52]. Depletion of ER Ca $^{2+}$ stores via the IP $_3$ receptor leads to activation of SOCs on the plasma membrane. Local reduction of PIP $_2$ and increased DAG directly facilitate activation of TRPC3 [53], with PKC also activating nonselective cation channels, increasing [Ca $^{2+}$] $_i$ [18]. However, despite these observations, further studies are required about a direct association between the PLC β pathway and activation of Ca $^{2+}$ channels.

Normal pancreatic β -cells have the capacity to secrete adequate levels of insulin necessary to compensate for increased insulin demands under conditions of HFD-associated insulin resistance [54]. Indeed, in islets isolated from mice fed HFD, pancreatic islets were expanded and secreted insulin levels were augmented, about 0.5 ng/ μ g protein/h, as compared to those fed normal chow diet (NCD), about 0.1 ng/ μ g protein/h. Also, basal insulin levels were increased in HFD-fed mice, around 0.5 ng/ml, as compared to NCD-fed mice, around 0.2 ng/ml. In *Plcb1^{fl/fl}; Pdx1-CreERT2* mice, HFD-feeding induced defects in glucose homeostasis. Similar to the role of PLC β 1 in regulating insulin secretion in normal *Plcb1^{fl/fl}; Pdx1-CreERT2* mice, the HFD-fed *Plcb1^{fl/fl}; Pdx1-CreERT2* mice also developed severe glucose intolerance due to a deficiency in insulin secretion. In addition, we confirmed that reduced insulin secretion was recovered in adding PLC β 1 back in MIN6 cell lines (Figure 2.15A, B). These result strongly support that effect on reduced insulin secretion was due to the PLC β 1 deletion and PLC β 1 can be considered a candidate to improve therapeutic intervention related to diabetes mellitus [55].

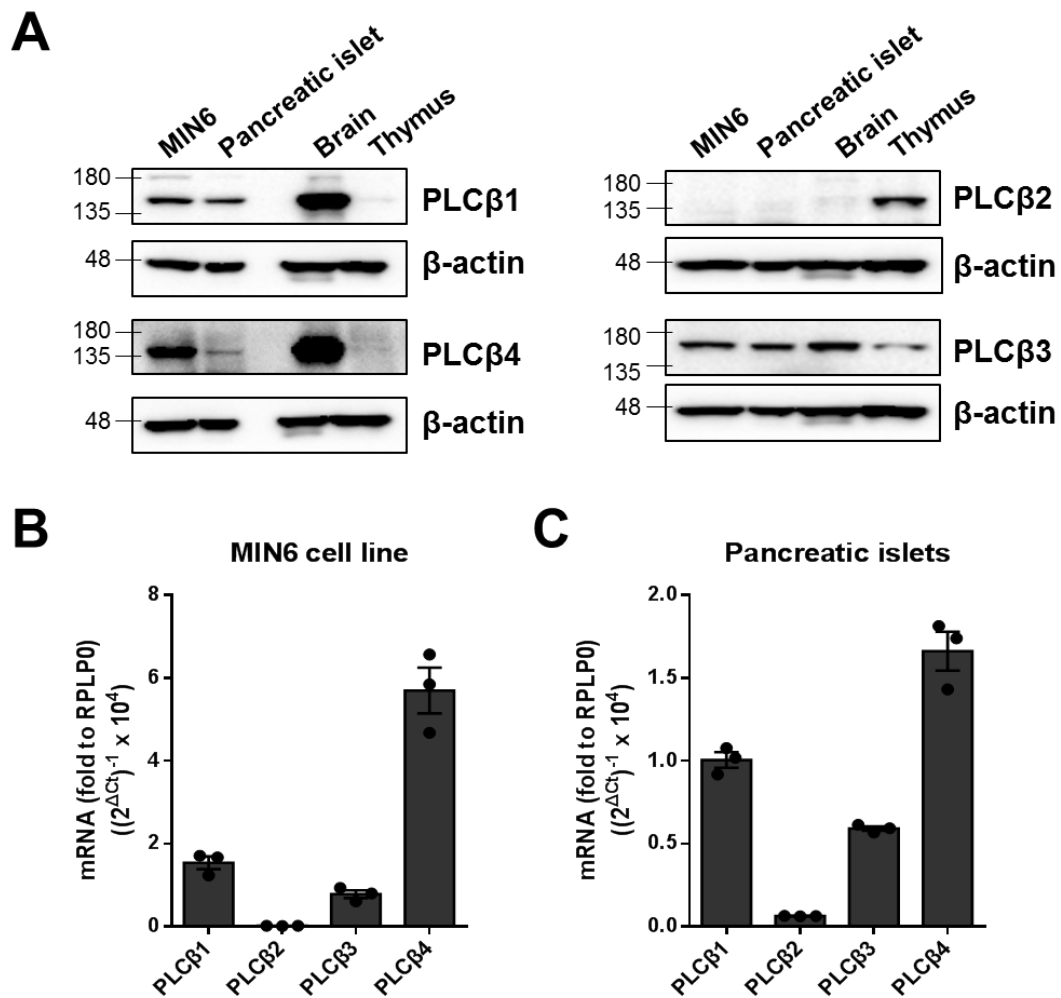


Figure 2.1. Expression profile of PLCβ isoforms in pancreatic β-cells

(A) Western blot analysis showing PLCβ isoform proteins from extracts of MIN6 cells and isolated islets from wild type mice (C57BL6/J, 8 weeks old). (B, C) qRT-PCR analysis showing relative PLCβ isoform mRNA levels in total RNA from MIN6 cells and islets isolated from wild type mice. Lysates from the brain and thymus were used as positive controls.

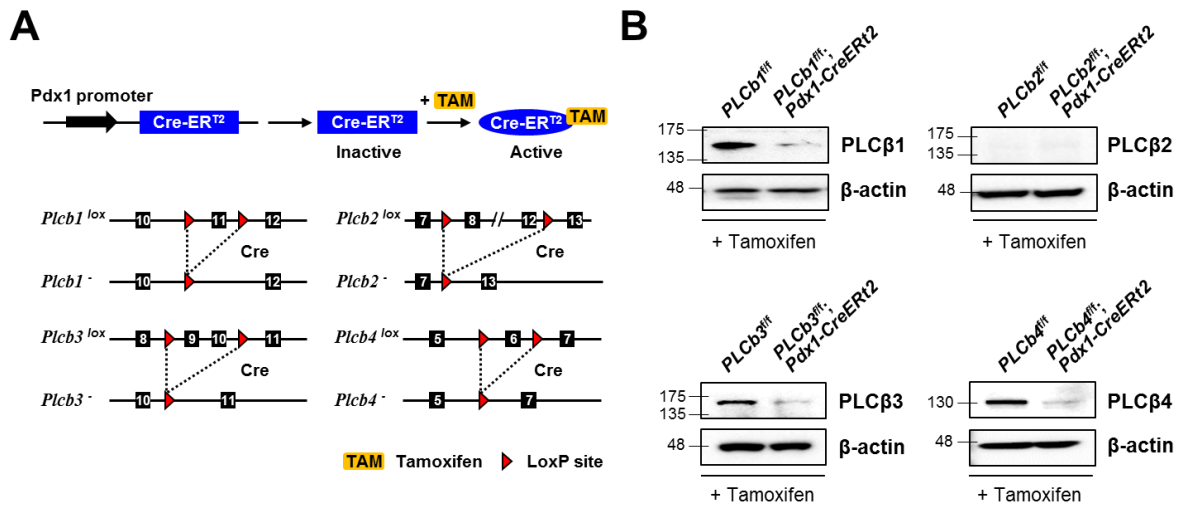


Figure 2.2. Inducible ablation of PLC β isozymes in adult pancreatic β -cells

(A) Graphical diagram of adult *Plcb1*, *2*, *3*, or *4*^{fl/fl} mice carrying the *Pdx1-creERT2* transgene injected with tamoxifen to induce β -cell-specific deletion of PLC β 1, 2, 3, or 4 isozymes. (B) Western blot analysis showing the reduction in PLC β isozyme protein contents from extracts of isolated islets from each *Plcb*^{fl/fl} and *Plcb*^{fl/fl}; *Pdx1-creERT2* mice following tamoxifen treatment.

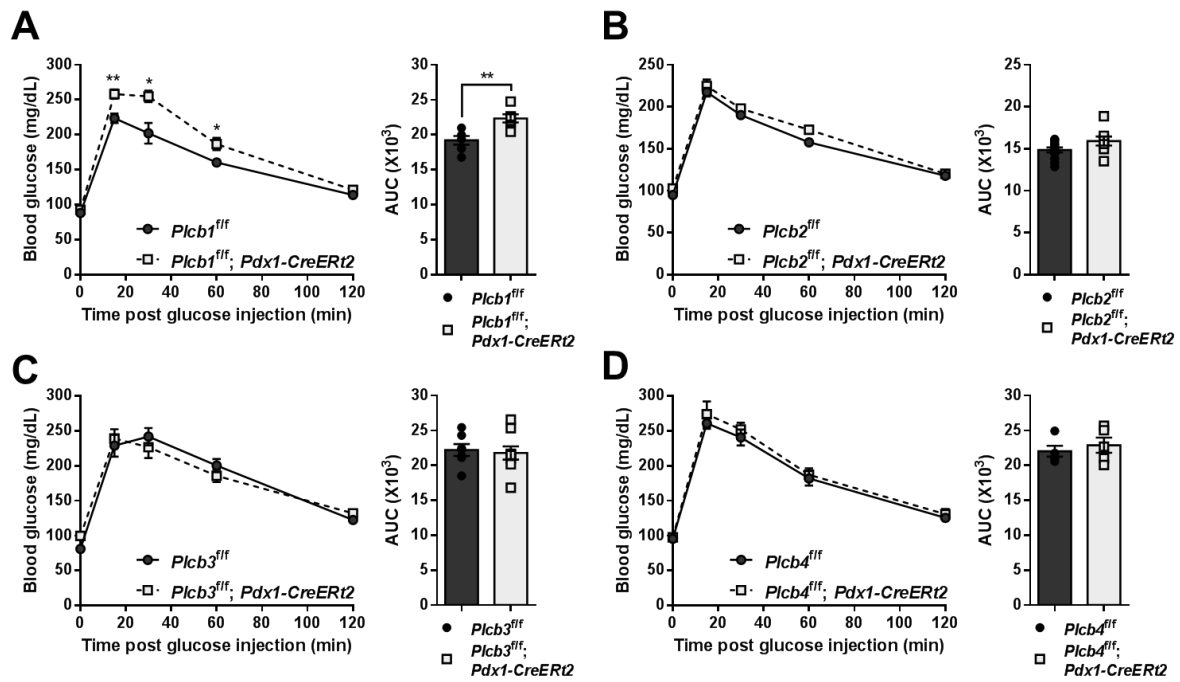


Figure 2.3. Impaired glucose tolerance in *Plcb1^{f/f}; Pdx1-CreERT2* mice

(A) Glucose tolerance test (2 mg glucose/g, intraperitoneal injection) for tamoxifen-treated *Plcb1^{f/f}* and *Plcb1^{f/f}; Pdx1-CreERT2* mice (n = 6 per group), (B) *Plcb2^{f/f}* and *Plcb2^{f/f}; Pdx1-CreERT2* mice (*Plcb2^{f/f}*, n = 12 vs. *Plcb2^{f/f}; Pdx1-CreERT2*, n = 8), (C) *Plcb3^{f/f}* and *Plcb3^{f/f}; Pdx1-CreERT2* mice (*Plcb3^{f/f}*, n = 7 vs. *Plcb3^{f/f}; Pdx1-CreERT2*, n = 10), and (D) *Plcb4^{f/f}* and *Plcb4^{f/f}; Pdx1-CreERT2* mice (n = 5 per group). Error bars represent \pm SEM. ** $p < 0.01$ and * $p < 0.05$.

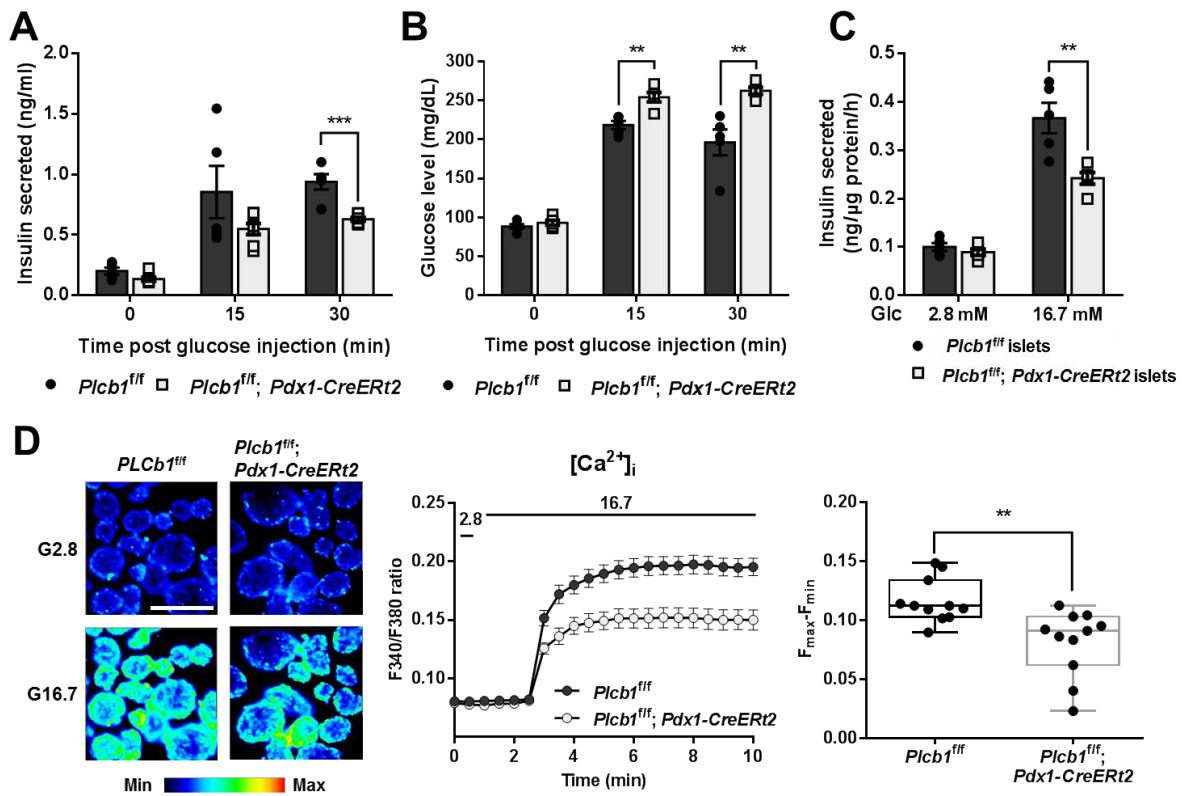


Figure 2.4. Defect in GSIS in *Plcb1^{flf}; Pdx1-CreERT2* mice

(A) Plasma insulin and (B) glucose levels after overnight fasting (16 h), 15 min, and 30 min post glucose injection in *Plcb1^{flf}* and *Plcb1^{flf}; Pdx1-CreERT2* mice (*Plcb1^{flf}*, n = 5 vs *Plcb1^{flf}; Pdx1-CreERT2*, n=7). (C) Secreted insulin levels from *Plcb1^{flf}* and *Plcb1^{flf}; Pdx1-CreERT2* islets. The results represent five independent experiments with islets from three mice per genotype. (D) Intracellular Ca^{2+} influx in isolated mouse islets from *Plcb1^{flf}* and *Plcb1^{flf}; Pdx1-CreERT2* mice. Pseudo-colored image of the fura-2AM fluorescence ratio (F340/F380) following high glucose

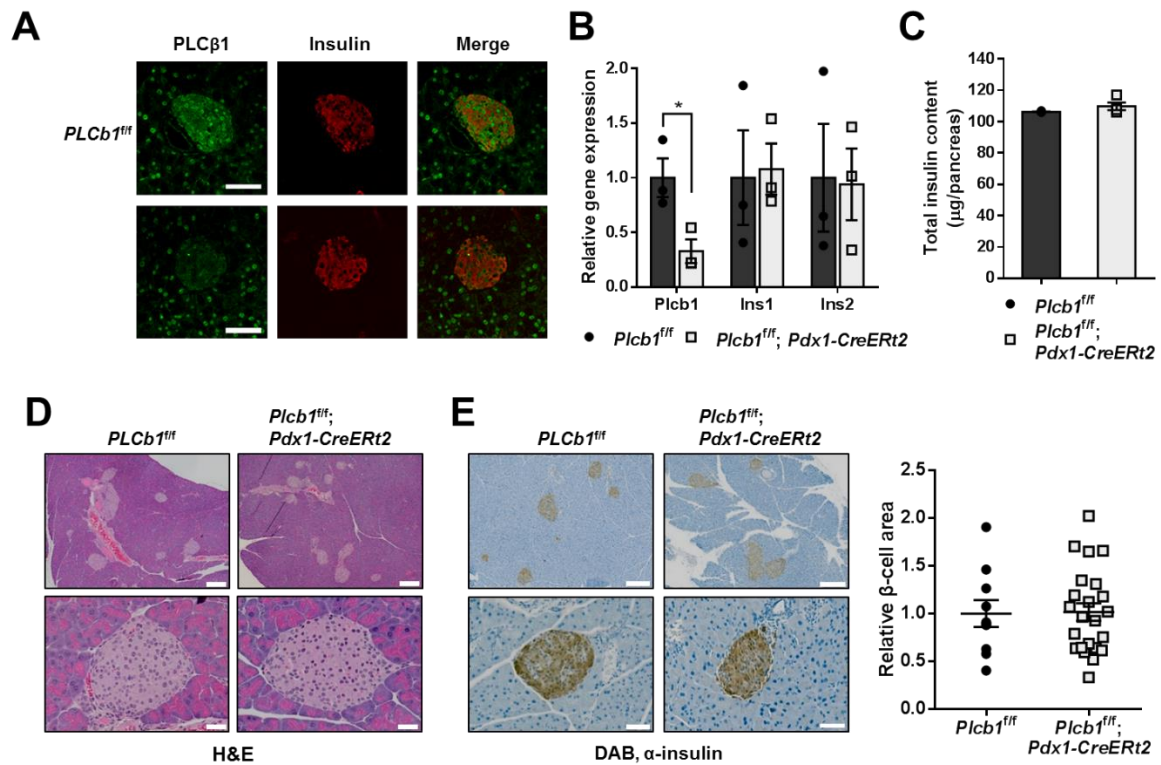


Figure 2.5. Normal insulin production and islet morphology

(A) Pancreatic sections stained with insulin and PLCβ1 antibodies. (B) mRNA expression of insulin genes from *Plcb1*^{fl/fl} and *Plcb1*^{fl/fl}; *Pdx1-CreERT2* islets (three independent experiments with pooled islets from three mice per group). (C) Total insulin content of the pancreas (n = 4 per group). (D) Pancreatic sections from *Plcb1*^{fl/fl} and *Plcb1*^{fl/fl}; *Pdx1-CreERT2* mice stained with H&E. (E) Insulin staining of pancreatic sections and quantification of β-cell area in *Plcb1*^{fl/fl} and *Plcb1*^{fl/fl}; *Pdx1-CreERT2* mice (four to five islets were analyzed from the *Plcb1*^{fl/fl}, n = 4 vs. *Plcb1*^{fl/fl}; *Pdx1-CreERT2*, n = 5). Scale bars, low magnification: 200 μm, high magnification: 50 μm. Error bars represent ± SEM. **p* < 0.05.

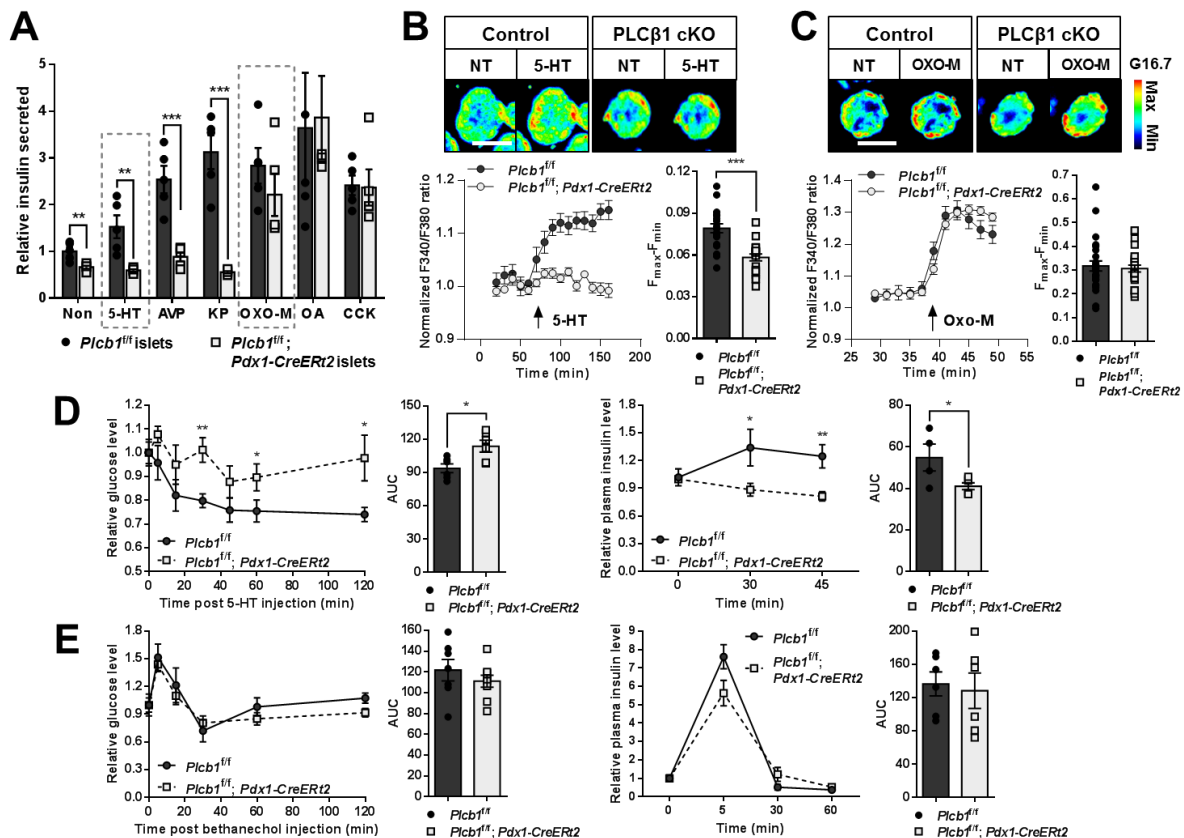


Figure 2.6. Decreased selective G_q PCR-induced insulin release in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets

(A) Selective augmentation of insulin secretion in pancreatic islets. Isolated pancreatic islets prepared from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* were incubated for 1 h at 37°C in Krebs solution containing 16.7 mM glucose. In addition, the incubation medium contained one of the following ligands: AVP (5 μ M), 5-HT (50 μ M), KP (1 μ M), CCK (500 nM), OXO-M (20 μ M), and OA (10 μ M). Supernatants were analyzed by ELISA. Data shown are representative of five independent experiments with islets from six to eight mice per genotype. (B) Pseudo-colored image of the fura-2AM fluorescence ratio (F340/F380, upper panel), the change in F340/F380 (left panel), and the maximum change in the F340/F380 response (right panel) to 5-HT treatment (50 μ M, n = 20 per group) and (C) to OXO-M treatment (20 μ M, n = 25 per group). Blood glucose and insulin levels of *Plcb1^{fl/fl}; Pdx1-CreERT2* mice and *Plcb1^{fl/fl}* littermates after receiving (D) a single dose of 5-HT (0.3 mg per mouse, *Plcb1^{fl/fl}*, n = 5 vs. *Plcb1^{fl/fl}; Pdx1-CreERT2*, n = 6) and (E) a single dose of bethanechol (2 μ g/g, intraperitoneal injection, *Plcb1^{fl/fl}*, n = 6 vs. *Plcb1^{fl/fl}; Pdx1-CreERT2*, n = 7). Plasma glucose and insulin levels were measured at the indicated time points. Scale bar: 400 μ m. Error bars represent \pm SEM. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

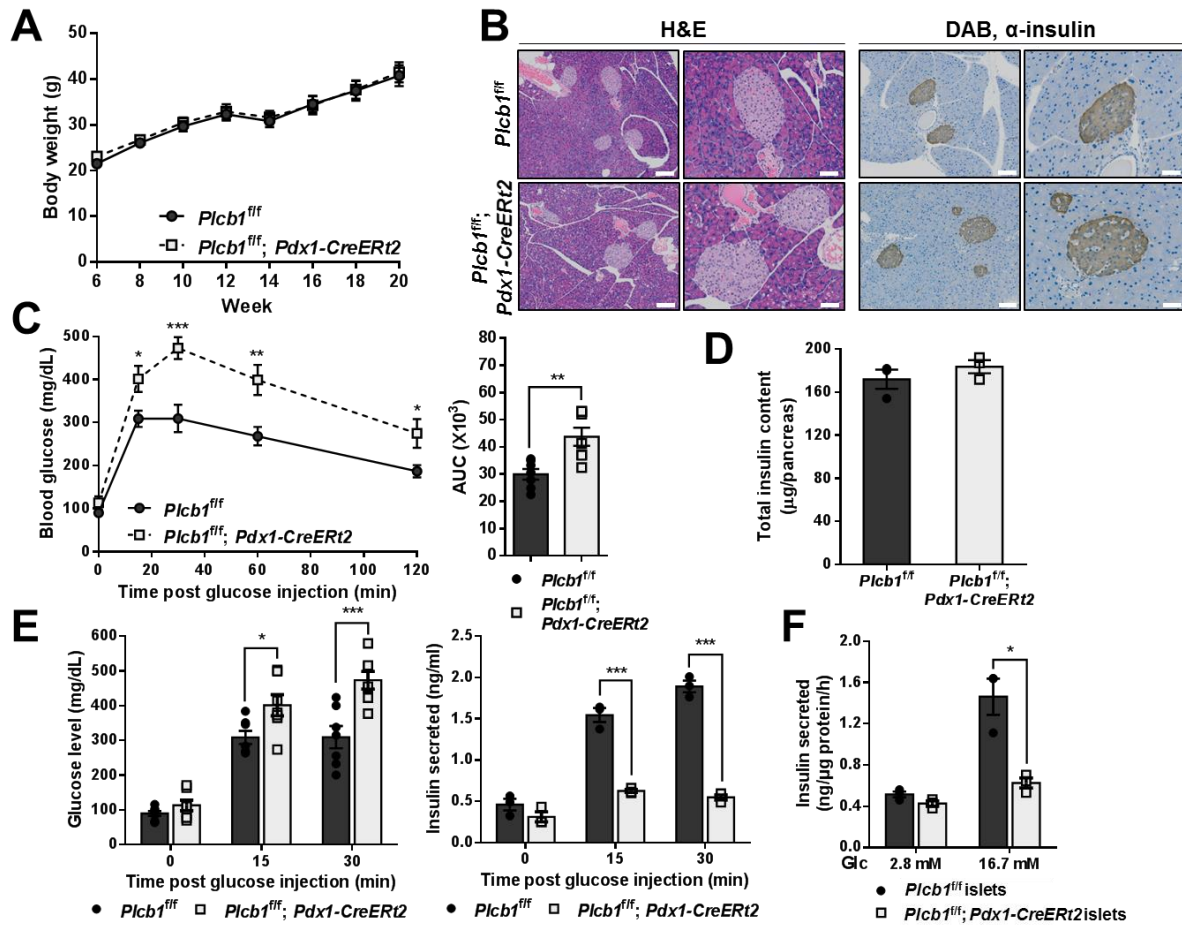


Figure 2.7. Impaired insulin secretion in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a HFD

(A) Body weight during HFD feeding (*Plcb1^{fl/fl}*, n = 5 vs. *Plcb1^{fl/fl}; Pdx1-CreERT2*, n = 4). (B) Pancreatic sections from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice stained with H&E and insulin staining of pancreatic sections (20 weeks old mice in figure 7A). (C) Glucose tolerance test (2 mg glucose/g, intraperitoneal injection, n = 7 per group). (D) Total insulin content of pancreas (n = 4 per group). (E) Plasma glucose and insulin levels from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice (n = 5–7 per group) post glucose injection. (F) Secreted insulin levels from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* islets (three independent experiments with pooled islets from three mice per group). Scale bars, low magnification: 200 μm, high magnification: 50 μm. Error bars represent ± SEM. ****p* < 0.001, ***p* < 0.01, and **p* < 0.05.

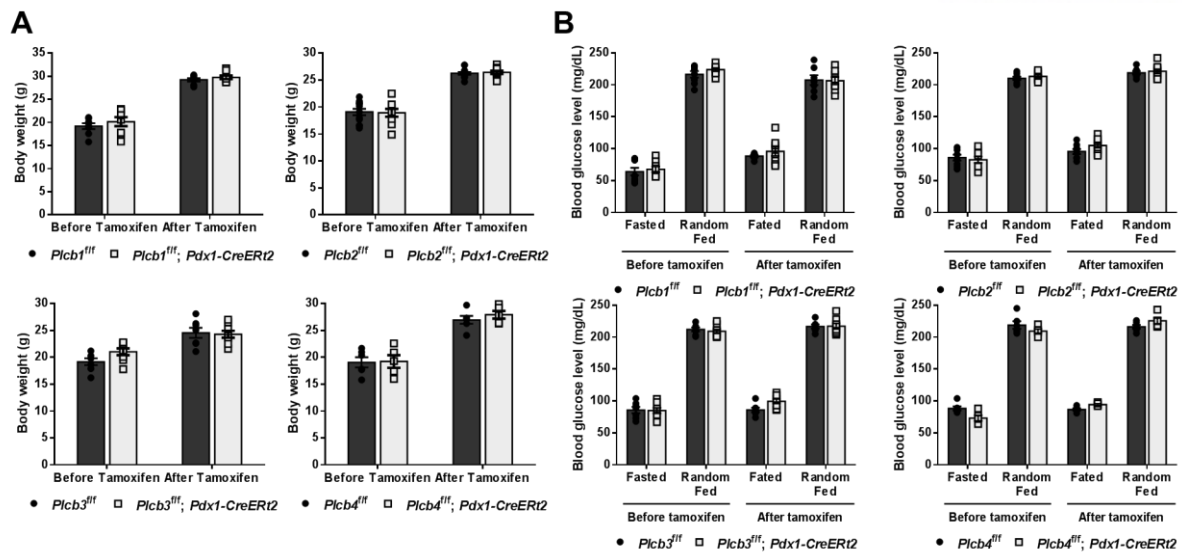


Figure 2.8. Similar body weight and fasting and re-feeding glucose levels between control and PLC β cKO mice following tamoxifen treatment

(A) Body weight of *Plcb1^{f/f}* and *Plcb1^{f/f}; Pdx1-CreERT2* mice (*Plcb1^{f/f}*, n = 7 vs. *Plcb1^{f/f}; Pdx1-CreERT2*, n = 8), *Plcb2^{f/f}* and *Plcb2^{f/f}; Pdx1-CreERT2* mice (*Plcb2^{f/f}*, n = 12 vs. *Plcb2^{f/f}; Pdx1-CreERT2*, n = 9), *Plcb3^{f/f}* and *Plcb3^{f/f}; Pdx1-CreERT2* mice (*Plcb3^{f/f}*, n = 6 vs. *Plcb3^{f/f}; Pdx1-CreERT2*, n = 7), and *Plcb4^{f/f}* and *Plcb4^{f/f}; Pdx1-CreERT2* mice (*Plcb4^{f/f}*, n = 6 vs. *Plcb4^{f/f}; Pdx1-CreERT2*, n = 5) mice. (B) Fasting and re-feeding glucose levels of *Plcb1^{f/f}* and *Plcb1^{f/f}; Pdx1-CreERT2* mice (*Plcb1^{f/f}*, n = 7 vs. *Plcb1^{f/f}; Pdx1-CreERT2*, n = 8), *Plcb2^{f/f}* and *Plcb2^{f/f}; Pdx1-CreERT2* mice (*Plcb2^{f/f}*, n = 8 vs. *Plcb2^{f/f}; Pdx1-CreERT2*, n = 9), *Plcb3^{f/f}* and *Plcb3^{f/f}; Pdx1-CreERT2* mice (*Plcb3^{f/f}*, n = 7 vs. *Plcb3^{f/f}; Pdx1-CreERT2*, n = 8), and *Plcb4^{f/f}* and *Plcb4^{f/f}; Pdx1-CreERT2* mice (*Plcb4^{f/f}*, n = 6 vs. *Plcb4^{f/f}; Pdx1-CreERT2*, n = 5) mice before and after tamoxifen.

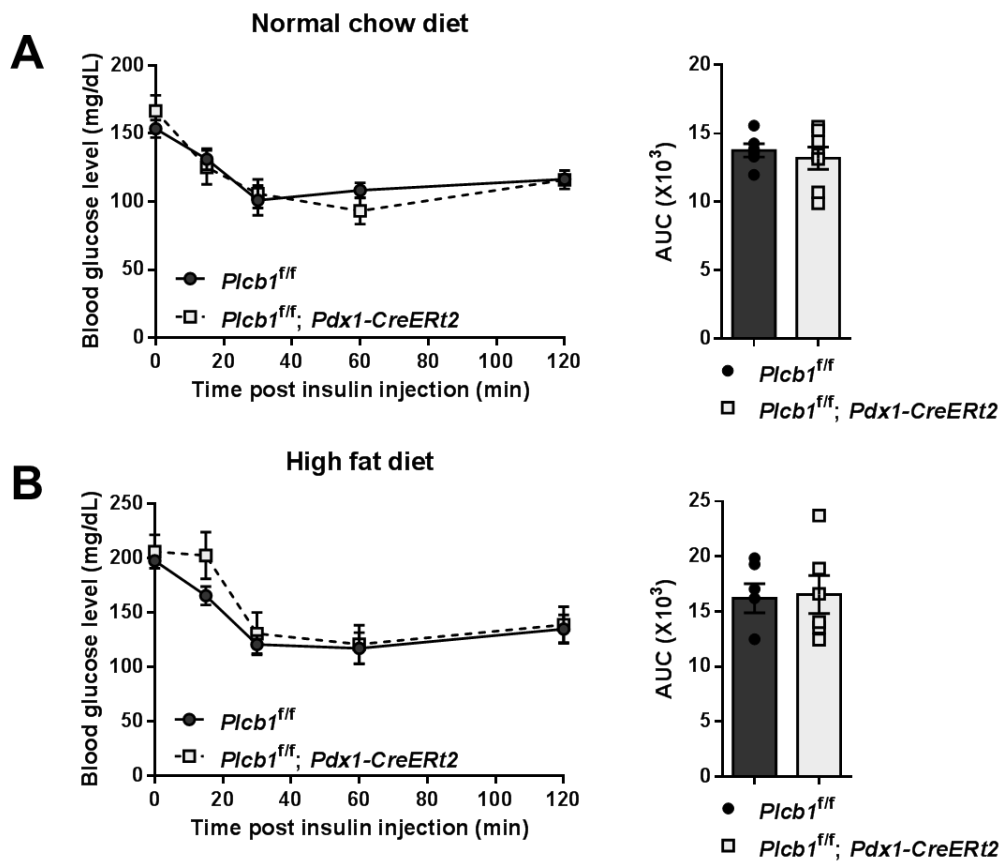


Figure 2.9. Normal insulin sensitivity in *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a normal chow diet and HFD

(A) Insulin tolerance test (0.3 U/kg) for control and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a normal chow diet (*Plcb1^{fl/fl}*, n = 6 vs. *Plcb1^{fl/fl}; Pdx1-CreERT2*, n = 7) and (B) *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice fed a HFD (n = 6 per group).

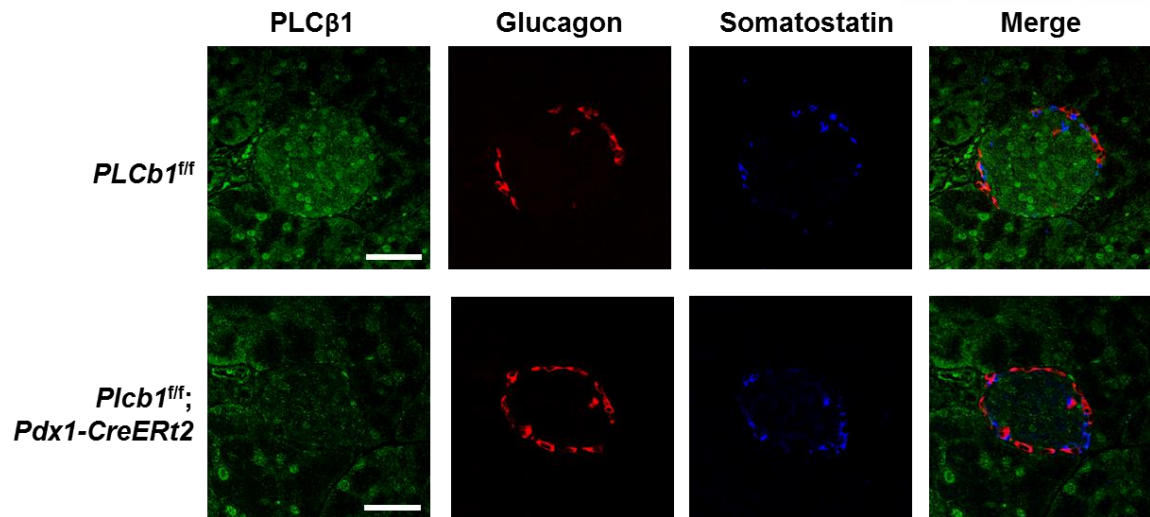


Figure 2.10. PLCβ1 protein expression in α -cell and δ -cells in pancreatic islets from control and *Plcb1^{fl/fl}; Pdx1-CreERT2* islets

Pancreatic sections stained with PLCβ1, glucagon and somatostatin antibodies. Scale bars, 50 μ m.

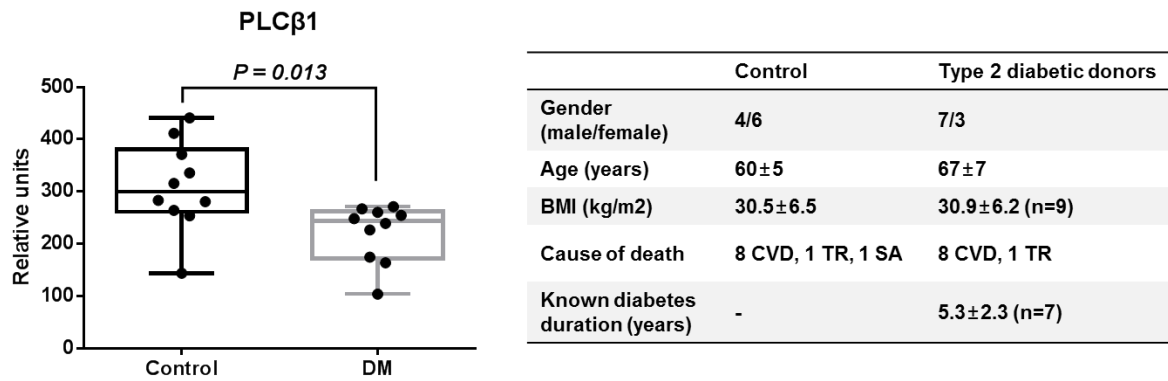


Figure 2.11. Reduced PLCβ1 expression in human β-cells from type 2 diabetes patients compared to normal subjects

PLCβ1 expression in human β-cells from normal and type 2 diabetes patients (DM) plotted as relative units (Gene Expression Omnibus public repository, accession no. GSE20966).

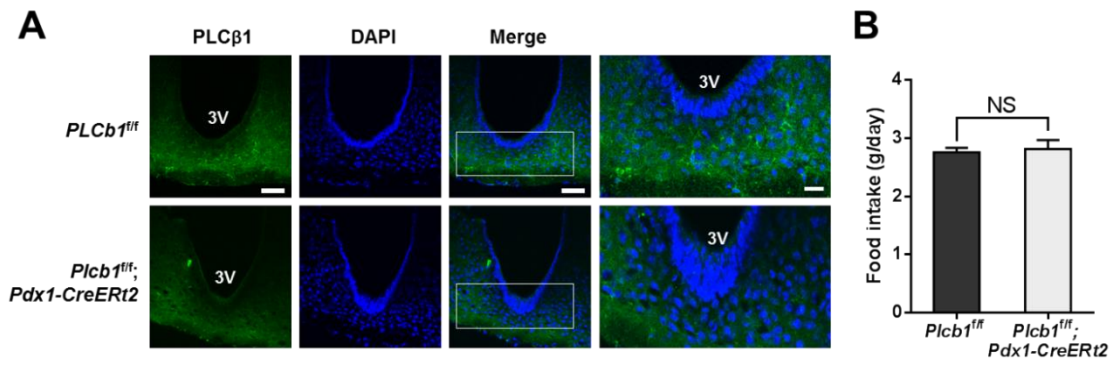


Figure 2.12. Reduced PLC β 1 expression in hypothalamus results in no alteration in food intake

(A) Brain sections stained with PLC β 1 antibodies in hypothalamus. (B) Absolute food intake (n = 6 per group). 3V; Third ventricle. Scale bars, low magnification: 50 μ m, high magnification: 100 μ m. Error bars represent \pm SEM.

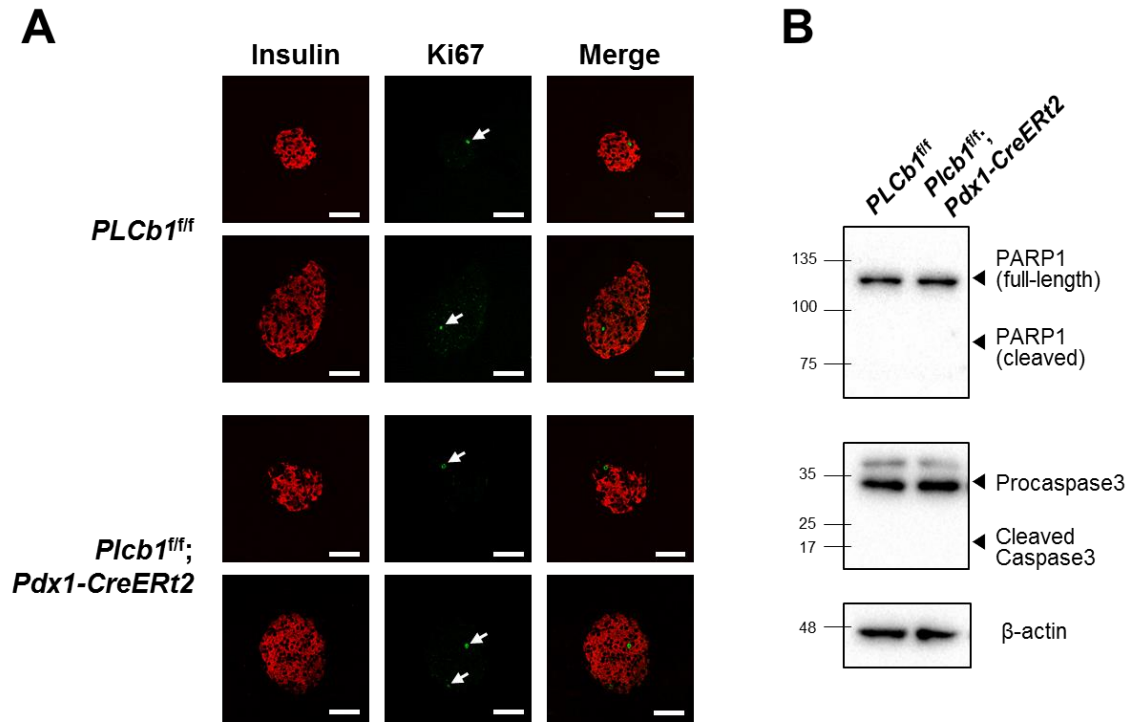


Figure 2.13. No change in β -cell proliferation and β -cell turnover in *Plcb1^{fl/fl}; Pdx1-CreERT2* islets

(A) Pancreatic sections stained with insulin and Ki-67 antibodies. (B) Western blot analysis showing apoptotic protein contents from extracts of isolated islets from *Plcb1^{fl/fl}* and *Plcb1^{fl/fl}; Pdx1-CreERT2* mice. Scale bars, 50 μ m.

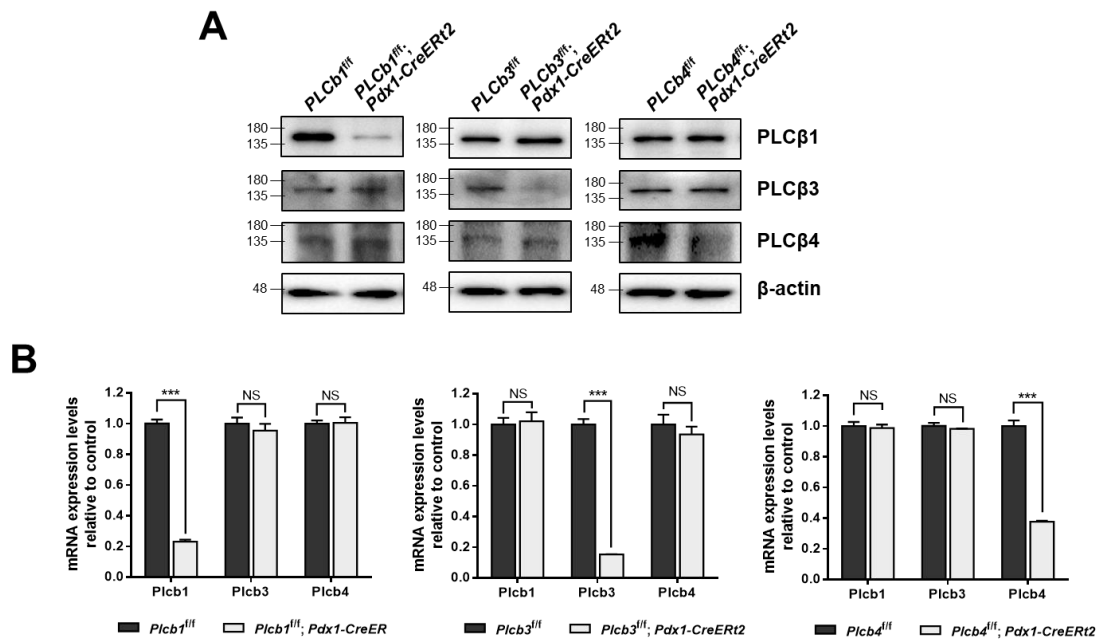


Figure 2.14. PLCβ isozyme expression in each PLCβ isozyme conditional knockout islets

(A) Western blot and (B) qRT-PCR analysis showing all PLCβ isozyme contents from extracts of isolated islets from each PLCβ isozyme conditional knockout mice following tamoxifen treatment. Error bars represent \pm SEM. *** $p < 0.001$.

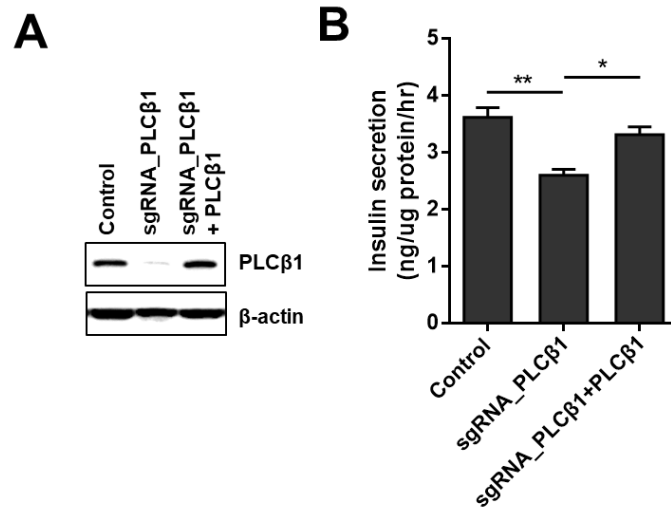


Figure 2.15. Reconstitution of PLCβ1 expression to rescue cells from abnormal insulin secretion

(A) Western blot analysis showing the PLCβ1 protein contents and (B) Secreted insulin level from PLCβ1-rescued MIN6 cell lines under high (16.7mM) glucose condition (n = 3). Error bars represent \pm SEM. $**p < 0.01$ and $*p < 0.05$.

Table 2.1. Sequences of quantitative RT-PCR primer upstream and downstream

Gene	Sequence (5'-3')
Plcb1-F	AGA TCA GCG AGG ACA GCA AT
Plcb1-R	GCC CAG GCA GTG ATA TTT GT
Plcb3-F	TTC GCC CTG ATG AGT TTC CC
Plcb3-R	AGC ACT TCG TTG AGT CTC GG
Plcb4-F	CCA CCG ACA CCA TAC GGA AA
Plcb4-R	GGA GAT GTG TCG GTA GCC T
Ins-1-F	GAC CAG CTA TAA TCA GAG ACC ATC
Ins-1-R	GTA GGA AGT GCA CCA ACA GG
Ins-2-F	GGC TTC TTC TAC ACA CCC AT
Ins-2-R	CCA AGG TCT GAA GGT CAC CT
36b4-F	TGG CCA ATA AGG TGC CAG CTG CTG
36b4-R	CTT GTC TCC AGT CTT TAT CAG CTG CAC

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Chapter III

Zafirlukast promotes insulin secretion by increasing calcium influx through L-type calcium channels

3.1. Introduction

Zafirlukast is a prescription drug used for the treatment of asthma. Zafirlukast blocks the action of leukotrienes, especially leukotriene type D₄ and E₄, on cysteinyl leukotriene receptor 1 (CysLTR1), alleviating breathing passage inflammatory responses such as airway constriction and mucus secretion in the lungs [1, 2]. Pranlukast and montelukast, two other leukotriene antagonists, also selectively inhibit activation of CysLTR1 [3]. CysLTR1 antagonists are considered safe and their anti-inflammatory properties have been well documented [4-6]. However, patient with mild asthma who received zafirlukast treatment (20 mg twice daily) showed adverse hypoglycemic effects compared with the placebo control [7]. The mechanism underlying the hypoglycemia is unclear and the functional role of zafirlukast in pancreatic β -cells has not been elucidated.

Insulin is the major hormone that lowers glucose levels in the body and insulin secretion is tightly regulated to manage blood glucose levels [8]. Glucose is transported into β -cells by glucose transporters and metabolized to produce ATP. An increase in the ATP:ADP ratio results in closure of ATP-sensitive K⁺ channels (K_{ATP} channels), membrane depolarization, and activation of voltage-dependent Ca²⁺ channels. An increase in the intracellular calcium ([Ca²⁺]_i) level induces exocytosis of insulin vesicles by a Ca²⁺-binding membrane protein located in the membrane of the docked granule [9]. Insulin secretion can be increased in the presence of hormonal and neuronal factors such as glucagon-like peptide and serotonin [10].

The effects of zafirlukast on insulin secretion have not been examined. Here, we investigated the effect of zafirlukast on insulin release in the regulation of [Ca²⁺]_i levels in pancreatic β -cells. Among montelukast, pranlukast, and zafirlukast, only zafirlukast treatment induced increases in insulin secretion and [Ca²⁺]_i in MIN6 cells, triggering phosphorylation of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), protein kinase B (AKT), and extracellular signal-regulated kinase (ERK). This zafirlukast-dependent stimulation in insulin release was also observed in mouse primary pancreatic islets. However, pranlukast had no effect on the [Ca²⁺]_i level or insulin secretion, and montelukast inhibited insulin secretion and the phosphorylation of related signaling molecules. In pancreatic β -cells, the zafirlukast-dependent stimulation of insulin release was suppressed by nifedipine, a dihydropyridine (DHP) L-type Ca²⁺ channel (L-VDCC) antagonist, suggesting that L-VDCCs are involved in the

underlying mechanism. Moreover, treatment of mice with zafirlukast resulted in a glucose-lowering response followed by promotion of insulin secretion *in vivo*. These findings reveal a novel function of zafirlukast in pancreatic β -cells [11].

3.2. Materials and Methods

Animals

All animal experiments were performed in accordance with Ulsan National Institute for Science and Technology Guide for the Care and Use of Laboratory Animals. C57BL/6J mice were maintained in animal facility on a pathogen-free facility under a 12-h light/dark cycles at temperature of 21. They were free to water and food. After the 8-week-old male C57BL/6J mice were fasted for 6 h, animals intraperitoneally injected 0.1 mg/g zafirlukast or vehicle and blood glucose and insulin levels were monitored. For glucose tolerance test, animals were fasted for overnight (16 h) and administrated 0.1 mg/g zafirlukast plus 2 mg/g D-glucose. Blood glucose level were assessed at indicated times. The blood glucose levels were determined by tail blood on glucometer and serum insulin level were estimated by ELISA (Shibayagi).

Insulin secretion assay

MIN6 cells were plated in 96-well plates (4×10^5 cells/well) and cultured for 3 days. Before experiment, cells were washed in phosphate-buffered saline (PBS) and equilibrated with 2.8 mM glucose in Kreb's buffer (KRB; 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 25 mM NaHCO_3 , and 10 mM HEPES, adjusted to pH 7.4 with NaOH before the addition of 3 g/L bovine serum albumin [BSA]) for 1 h. Size-matched medium islets were equilibrated in KRB with 2.8 mM glucose for 1 h and transferred to a 48-well plate (20 islets/well). MIN6 cells and pancreatic islets were stimulated with zafirlukast (Z4152; Sigma), pranlukast (Sigma), or montelukast (Sigma) in 300 μL of KRB plus 1 mM glucose and 20 mM glucose for 1 h. The supernatant was collected and the amount of insulin was quantified with an insulin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi). All steps were performed in a CO_2 cell incubator.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Following chemical stimulation for 24 h, MIN6 cells were supplemented with MTT and

incubated for 4 h. The cells were then incubated with dimethyl sulfoxide to dissolve the formazan by shaking for 5 min and the absorbance was measured.

Total insulin content

Following treatment with zafirlukast, pranlukast and montelukast, MIN6 cells were washed in PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (#89900; Thermo Fisher Scientific). The total insulin content of the lysed extracts was measured by ELISA.

Determination of intracellular Ca²⁺

After MIN6 cells were grown on glass coverslips coated with poly-L-lysine for 2 days, the cells were washed twice and loaded with fura-2-acetoxymethyl ester (Fura-2AM; final concentration 2 μ M) in KRB for 1 h. The coverslips were then washed three times and inserted into a microscope holder in contact with 500 μ L of buffer at 37°C. The chemicals (50 μ M zafirlukast, montelukast, or pranlukast; 1 μ M nifedipine; 1 μ M thapsigargin) were applied on top of the imaged cells, as indicated in the figure legends. The excitation signals were measured using a fluorimeter to monitor Fura-2AM fluorescence (Olympus motorized inverted microscope IX81).

Islet isolation

The pancreas of 8-week-old male C57BL/6J mice was inflated with 3 mL of 1 mg/mL collagenase (Roche) in Hank's buffered saline solution (HBSS; Welgene), which was modified with 25 mM HEPES and adjusted to pH 7.4 with NaOH, and 0.25% (w/v) BSA was added. The collagenase-injected pancreas was incubated in a 37°C water bath for 15 min and manually shaken every 5 min. The digested pancreas was washed with modified HBSS on ice three times. Islets were separated by density gradient in Bicol solution (Biochrom) and handpicked under a dissection microscope. Islets were cultured in RPMI-1640 medium in a CO₂ incubator overnight.

Western blotting

MIN6 cells treated with leukotriene receptor antagonists were lysed in RIPA buffer (Thermo Fisher Scientific), which included a cocktail of protease and phosphatase inhibitors according to the manufacturer's instructions. Total protein concentration was estimated with the Pierce BCA Protein

Assay Kit (Thermo Fisher Scientific). Total protein was separated by gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). The membrane was blocked with 5% (w/v) fat-free milk and incubated with primary antibodies overnight at 4°C. After washing with Tris Buffered Saline with Tween 20 buffer, the membrane was incubated with horseradish peroxidase-labeled secondary antibody and proteins were visualized with enhanced chemiluminescence reagents (GE Healthcare Life Sciences). Anti-phospho-CaMKII (#3361), anti-phospho-AKT (#9271), and anti-phospho-ERK (#9101) antibodies were obtained from Cell Signaling. Anti-CaMKII (sc-5306), anti-AKT (sc-1619), and anti-ERK (sc-154) antibodies were obtained from Santa Cruz Biotechnology and the anti- β -actin (GTX109639) antibody was obtained from GeneTex.

3.3. Results

Zafirlukast increased insulin secretion in both low and high glucose conditions

To investigate the physiological role of zafirlukast on the function of pancreatic β -cells, MIN6 cells were treated with zafirlukast for 1 h and the level of secreted insulin was measured in low (2.8 mM) and high (16.7 mM) glucose conditions. Interestingly, we found that zafirlukast markedly increased insulin secretion in the basal condition (2.8 mM glucose; approximately 4.4-fold increase compared with the control) and glucose-induced insulin secretion (16.7 mM glucose, approximately 1.7 fold-increase compared with the control) at approximately 50 μ M zafirlukast which is the maximum effect concentration (E_{max}). In the dose-response curve, the half maximal effective concentration (EC_{50}) was 29.8 ± 1.6 and 22.7 ± 2.3 μ M in the basal and high glucose conditions, respectively (Figure 3.1A, B). Next, we performed an MTT assay to evaluate the cytotoxicity of zafirlukast in the MIN6 cell line. The absorbances at concentrations up to nearly 80 μ M were comparable between the control and zafirlukast-treated groups and cell viability was not altered following exposure to zafirlukast at E_{max} , indicating zafirlukast potentiates insulin secretion without inducing cell cytotoxicity (Figure 3.1C). Thus, zafirlukast induces regulated exocytosis of insulin vesicles, resulting in increased insulin secretion in pancreatic β -cells.

Only zafirlukast among CysLTR1 antagonists induced insulin secretion

To determine whether the effect of zafirlukast on pancreatic β -cells was related to its intrinsic function as a CysLTR1 antagonist, we performed the insulin secretion assay with other antagonists, namely montelukast and pranlukast, which also act on CysLTR1. The chemical structures of these

antagonists are distinct (Figure 3.2A), although they have similar receptor-binding affinities for CysLTR1 [12]. Zafirlukast increased insulin secretion in a dose-dependent manner in low (2.8 mM) and high (16.7 mM) glucose conditions. However, the level of secreted insulin did not change following treatment with pranlukast, and montelukast markedly inhibited glucose-stimulated insulin secretion (GSIS) (Figure 3.2B). To estimate the difference in the internal total insulin contents causes alteration in insulin secretory responses, we measured total insulin content following treatment with montelukast, pranlukast, and zafirlukast. Following stimulation with each of the CysLTR1 antagonists, total insulin content was unaffected in both the low and high glucose conditions (Figure 3.2C). Furthermore, absorbance values were similar between the control and the CysLTR1 antagonist-treated groups at the indicated concentrations, suggesting cell viability was not affected by CysLTR1 antagonist treatment at the different concentrations (Figure 3.2D). Thus, among the CysLTR1 antagonists, only zafirlukast promoted insulin secretion and acted on pancreatic β -cells by triggering activation of the insulin secretory pathway rather than exerting its intrinsic function as a CysLTR1 antagonist.

Zafirlukast, but not montelukast or pranlukast, increased $[Ca^{2+}]_i$ in MIN6 cells

The elevation in $[Ca^{2+}]_i$ level is critical for insulin exocytosis, which subsequently induces insulin secretion [13]. Zafirlukast induced a greater increase in insulin secretion in low (2.8 mM) compared with high (16.7 mM) glucose conditions, suggesting that zafirlukast is directly involved in the regulation of insulin secretion in the absence of glucose stimulation. Next, to assess whether zafirlukast can alter responsiveness, we performed calcium imaging with the fluorescent calcium indicator Fura-2AM. In this study, zafirlukast was tested under low glucose conditions, which eliminates the stimulating effects of glucose on insulin secretion. We analyzed transient calcium response triggered by treatment with zafirlukast, pranlukast, and montelukast during Fura-2AM loading. A significant increase in the Ca^{2+} response was observed following treatment with zafirlukast at the E_{max} of 50 μ M, resulting in an increase in the ratio of 340 nm to 380 nm fluorescence. However, no change in the Ca^{2+} response following treatment with montelukast and pranlukast was observed (Figure 3.3A–C). These results show that zafirlukast specifically evoked a rapid and high increase in $[Ca^{2+}]_i$, resulting in enhanced exocytosis of insulin vesicles in pancreatic β -cells.

Zafirlukast elevates $[Ca^{2+}]_i$ levels and increases insulin secretion in isolated primary islets

Next, to confirm the effect of zafirlukast on insulin secretion *ex vivo*, we performed an insulin secretion assay using primary islets isolated from 8-week-old C57BL/6 mice. In isolated pancreatic

islets, zafirlukast substantially increased insulin release in a dose-dependent manner from 9.6 ± 0.9 to 25.5 ± 1.2 ng·mL⁻¹ in the 2.8 mM glucose condition and from 16.5 ± 1.4 to 25.8 ± 0.8 ng·mL⁻¹ in the 16.7 mM glucose condition (Figure 3.4A). To estimate the influence of zafirlukast on [Ca²⁺]_i in pancreatic islets, we recorded the calcium response in primary pancreatic islets followed by treatment with zafirlukast at the E_{max} of 50 μM. The addition of zafirlukast increased the 340 nm to 380 nm ratio within a few seconds, indicating zafirlukast significantly amplified [Ca²⁺]_i levels. The increase in [Ca²⁺]_i triggered by zafirlukast increased initially and then plateaued (Figure 3.4B,C). In conclusion, zafirlukast significantly potentiates insulin secretion in pancreatic β-cells by increasing [Ca²⁺]_i levels.

Zafirlukast-dependent increase in [Ca²⁺]_i induces activation of key molecular events

The stimulation of pancreatic β-cells by zafirlukast induces an increase in [Ca²⁺]_i. The increase in [Ca²⁺]_i propagates primary signals through the CaMKII, AKT, and ERK signaling pathways, which positively regulate insulin secretion [14-16]. To characterize the activation and regulation of key proteins by zafirlukast, we examined the phosphorylation of CaMKII, AKT, and ERK in a dose- and time-dependent manner in MIN6 cells following zafirlukast treatment. Lysates at the indicated concentrations and times were subjected to western blot analysis to assess phosphorylation. Interestingly, the phosphorylation of CaMKII, AKT, and ERK was significantly increased in a dose-dependent manner and its phosphorylation increased at approximately 15 min and was sustained until 1 h (Figure 3.5A, B). Interestingly, treatment with pranlukast slightly induced the ERK pathway, but not the CaMKII and AKT pathways, resulting in no change in insulin secretion. However, treatment with montelukast markedly suppressed the expression of phosphorylated AKT (p-AKT) and p-ERK (Figure 3.8). These results are consistent with the results of the insulin secretion assay, indicating that zafirlukast stimulates insulin secretion, while pranlukast does not alter insulin secretion, and montelukast inhibits GSIS. Taken together, our results indicate that zafirlukast induces phosphorylation of key signaling proteins in pancreatic β-cells.

Zafirlukast induced extracellular calcium influx through L-type Ca²⁺ channels

We next determined whether the increase in [Ca²⁺]_i was derived from an extracellular or intracellular source of Ca²⁺. In the absence of extracellular Ca²⁺ (Ca²⁺-free KRB medium), the zafirlukast-stimulated [Ca²⁺]_i levels were reduced compared with those observed in Ca²⁺-containing KRB, although the [Ca²⁺]_i level was considerably increased via intracellular Ca²⁺ mobilization by thapsigargin (Figure 3.9). Thus, the results strongly suggest that the effect of zafirlukast on insulin

secretion is primarily due to the entry of extracellular Ca^{2+} rather than intracellular Ca^{2+} from the endoplasmic reticulum. Ca^{2+} influx through L-VDCCs is critical for insulin secretion [17]. Thus, we analyzed the involvement of these channels in zafirlukast-dependent effects using nifedipine, a DHP L-VDCC antagonist, which suppresses GSIS, but has no effect on the basal levels of Ca^{2+} . Fluorescent calcium imaging showed that application of nifedipine significantly inhibited the zafirlukast-dependent increase in $[\text{Ca}^{2+}]_i$ (Figure 3.6A). Consistent with this, nifedipine potently reduced zafirlukast-dependent insulin secretion in both low (2.8 mM) and high (16.7 mM) glucose conditions (Figure 3.6B). Moreover, zafirlukast stimulated the phosphorylation of CaMKII, AKT, and ERK in pancreatic β -cells, but this effect was prevented in the presence of nifedipine (Figure 3.6C). These findings suggest that zafirlukast induces Ca^{2+} influx through L-VDCCs, leading to enhanced insulin secretion in pancreatic β -cells.

Zafirlukast lowers blood glucose levels by increasing insulin secretion *in vivo*

To assess whether zafirlukast induced hypoglycemic effects in mice, 8-week-old C57BL/6J mice were administered 0.1 mg/g zafirlukast via intraperitoneal injection and blood glucose levels were monitored. Upon injection of zafirlukast, blood glucose levels dropped in the normal glycemic mice ($33.5 \pm 0.6\%$ reduction, $P = 0.01$ compared with vehicle; Figure 3.7A). The glucose-lowering effect of zafirlukast was accompanied by an increase in the level of plasma insulin at 90 min (0.07 ± 0.01 and $0.14 \pm 0.02 \text{ ng}\cdot\text{mL}^{-1}$ for vehicle and zafirlukast, respectively) and 120 min (0.06 ± 0.003 and $0.16 \pm 0.02 \text{ ng}\cdot\text{mL}^{-1}$ for vehicle and zafirlukast, respectively), suggesting that the effects of zafirlukast were a result of the increased insulin secretion from pancreatic β -cells (Figure 3.7B). We next measured blood glucose levels in C57BL/6J mice following glucose administration to rigorously assess the glucose-lowering effect of zafirlukast (0.1 mg/g) *in vivo*. The glucose-lowering effect of zafirlukast was also observed in mice at 90 and 120 min during an intraperitoneal glucose tolerance test ($29.3 \pm 7.9\%$ reduction, $P = 0.01$ for 90 min and $46.7 \pm 6.6\%$ reduction, $P = 0.001$ for 120 min; Figure 3.7C). These results show that zafirlukast functions as an insulin secretagogue and exhibits glucose-lowering effects *in vivo*.

3.4. Conclusion and Discussion

It has long been known that zafirlukast have important role in the inflammatory processes of asthma [1]. However, its function and underlying mechanism in pancreatic β -cells remains to be elucidated. In this study, we examined the effects of zafirlukast on hypoglycemia in terms of insulin

secretion in pancreatic β -cells. We demonstrated that zafirlukast potentiates insulin secretion in MIN6 cells and primary pancreatic islets in both basal (2.8 mM) and high glucose (16.7 mM) conditions. The increase in insulin secretion is primarily elicited by extracellular Ca^{2+} influx through L-VDCCs, resulting in increased phosphorylation of CaMKII, AKT, and ERK. Taken together, these findings suggest that zafirlukast can increase $[\text{Ca}^{2+}]_i$ levels and activate key signaling molecules, resulting in increased insulin secretion.

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to alleviate chronic inflammation by blocking the cyclooxygenase pathway by inhibiting cyclooxygenase, which contributes to the formation of pro-inflammatory mediators such as prostaglandins and thromboxane [18, 19]. It has been reported that at high doses NSAIDs such as salicylates lower blood glucose levels [20, 21] and ibuprofen use in diabetic patients has been shown to induce hypoglycemia [22]. Furthermore, meclofenamic acid stimulates insulin secretion from pancreatic β -cells by inhibiting K_{ATP} channels, which is followed by an increase in $[\text{Ca}^{2+}]_i$. Flufenamic acid and acetylsalicylic acid have also been shown to depolarize INS-1 cells by inhibiting K_{ATP} channels [23]. In addition, zafirlukast provides effective relief of inflammation by inhibiting the 5-lipoxygenase pathway by blocking CysLTR1 [2, 24]. We wondered whether the zafirlukast-dependent increase in insulin secretion was related to its intrinsic anti-inflammatory function. We examined the effects on insulin release of pranlukast and montelukast, which, like zafirlukast, function as CysLTR1 antagonists, but with different chemical structures (Figure 3.2A). Interestingly, potentiation of insulin secretion was zafirlukast-dependent, and not pranlukast- or montelukast-dependent (Figure 3.2B). Montelukast suppressed monocyte chemotactic protein 1 (MCP-1)-dependent $[\text{Ca}^{2+}]_i$ increase and phospho-p38 mitogen-activate protein kinase expression in THP-1 cells, abolishing monocyte chemotaxis induced by MCP-1 [25]. Our results show that montelukast inhibited GSIS from pancreatic β -cells, which could be caused by inhibition of glucose-stimulated $[\text{Ca}^{2+}]_i$. Furthermore, CysLTR1, which zafirlukast binds to as an antagonist, is rarely expressed in pancreatic β -cells [26], suggesting that zafirlukast-induced insulin release was not associated with its intrinsic function as a CysLTR1 antagonist. Altogether, these results suggest that there might be potential off-target effects of zafirlukast in pancreatic β -cells.

Various signaling pathways might maintain or increase insulin secretion [27]. In pancreatic β -cells, the rise in $[\text{Ca}^{2+}]_i$ results in activation of CaMKII, which mediates ERK phosphorylation to exert an insulin secretory response [14, 28, 29]. This induces cytoskeletal rearrangement [30, 31] and phosphorylation of components of the secretory machinery, such as synapsin I [32], microtubule-associated protein 2 [33], and vesicle-associated membrane protein (VAMP)/synaptobrevin [34]. Furthermore, AKT pathways optimize β -cell function, including cell survival [16] and insulin secretion

by inducing activation of SNARE proteins such as STX1, SNAP25, and VAMP2 [15, 35] and modulation of L-VDCCs [36]. Interestingly, zafirlukast was able to stimulate insulin secretion by inducing Ca^{2+} entry and phosphorylation of CaMKII, AKT, and ERK. These results were not observed in MIN6 cells treated with pranlukast and montelukast. Rather, the AKT and ERK pathways were substantially inhibited following treatment with montelukast, which suppressed GSIS (Figure 3.8).

Our results showed that zafirlukast failed to induce an increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} sources, indicating that zafirlukast primarily promoted the increase in $[\text{Ca}^{2+}]_i$ via extracellular Ca^{2+} rather than intracellular Ca^{2+} . In pancreatic β -cells, Ca^{2+} influx via L-VDCCs is essential for insulin secretion [17]. Indeed, treatment with nifedipine, a DHP L-VDCC antagonist, reduced the effect of zafirlukast on both $[\text{Ca}^{2+}]_i$ and insulin secretion. Furthermore, nifedipine exposure eliminated the zafirlukast-dependent phosphorylation of CaMKII, AKT, and ERK. We observed a small nifedipine-resistant portion of the zafirlukast-induced elevation in $[\text{Ca}^{2+}]_i$ and insulin secretion, suggesting either incomplete blockage of L-VDCCs or that another type of Ca^{2+} channel, such as N- or P/Q-type Ca^{2+} channels [37], might be targeted by zafirlukast. However, the blockade of L-VDCCs via a specific DHP-sensitive inhibitor significantly inhibited the zafirlukast-dependent effects on pancreatic β -cells, indicating that increased insulin secretion by zafirlukast was primarily mediated via L-VDCCs. Zafirlukast also effectively regulated insulin secretion and glucose levels *in vivo*, evidenced by the increased insulin levels and decreased blood glucose following injection of zafirlukast in mice. Zafirlukast has been prescribed for asthma patients to be taken on an empty stomach [7], which represents a physiological *in vivo* state similar to low (2.8 mM) glucose levels *in vitro*. Our evidence showed that zafirlukast has a greater effect on insulin secretion in low levels of glucose. Therefore, taking zafirlukast on an empty stomach can cause severe adverse effects such as hypoglycemia and our results suggestion that greater attention may be necessary when prescribing zafirlukast, especially in low glucose conditions. On the other hand, zafirlukast also affects insulin secretion in high glucose (16.7 mM) conditions, indicating the possible use of zafirlukast in the control of blood glucose levels. The potential use of zafirlukast in the treatment of type 2 diabetes deserves further investigation.

Overall, we have identified a novel function of zafirlukast as a regulator of insulin secretion in pancreatic β -cells. We have shown that zafirlukast can stimulate insulin secretion by promoting Ca^{2+} entry via L-VDCCs, resulting in increased phosphorylation of CaMKII, AKT, and ERK. In addition to its function in the prevention of asthma symptoms, zafirlukast may be useful in the regulation of type 2 diabetes.

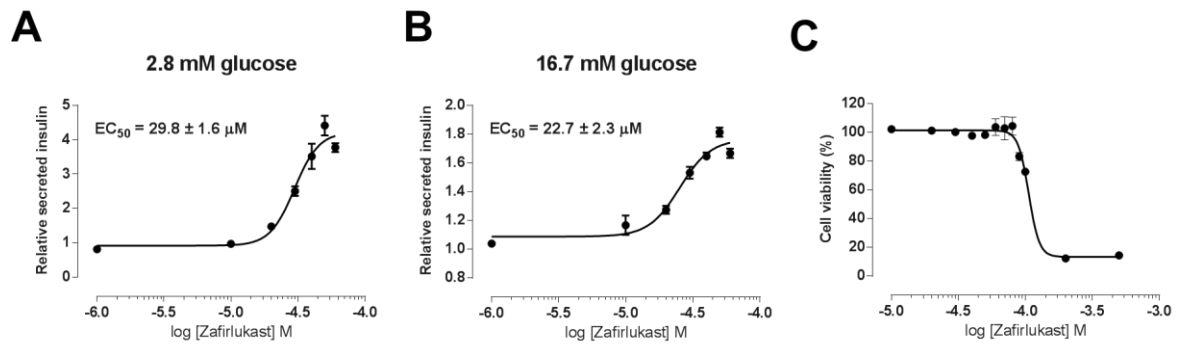


Figure 3.1. Zafirlukast-dependent potentiation of insulin secretion in MIN6 cells.

(A) Dose-response curve showing the effect of zafirlukast on insulin secretion in low (2.8 mM) and (B) high (16.7 mM) glucose conditions (n = 4). (C) Dose-response curve showing the effect of zafirlukast on cell viability (n = 3). Error bars represent standard error of the mean (SEM).

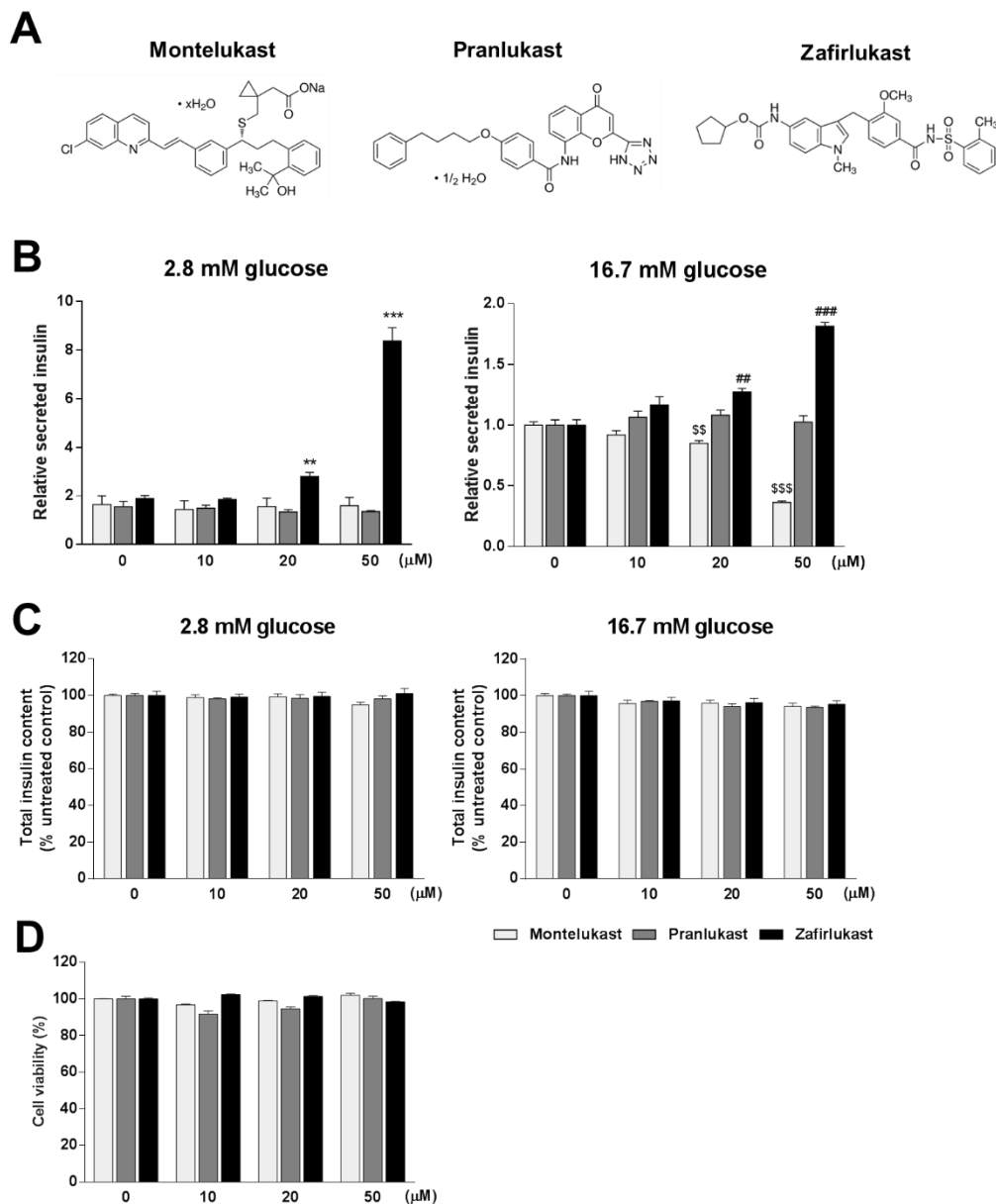


Figure 3.2. Distinct effect of zafirlukast on insulin secretion among cysteinyl leukotriene receptor 1 (CysLTR1) antagonists.

(A) Chemical structures of the CysLTR1 antagonists montelukast, pranlukast, and zafirlukast. (B) Level of secreted insulin following treatment with CysLTR1 antagonists in MIN6 cells in low (2.8 mM) and high (16.7 mM) glucose conditions ($n = 4$). (C) Total insulin content and (D) cell viability determined with the MTT assay following exposure to CysLTR1 antagonists ($n = 3$). Error bars represent SEM. $**P < 0.01$ and $***P < 0.001$, compared with the control in low glucose (zafirlukast). $##P < 0.01$ and $###P < 0.001$, compared with the control in high glucose (zafirlukast). $$$P < 0.01$ and $$$$P < 0.001$, compared with the control in high glucose (montelukast).

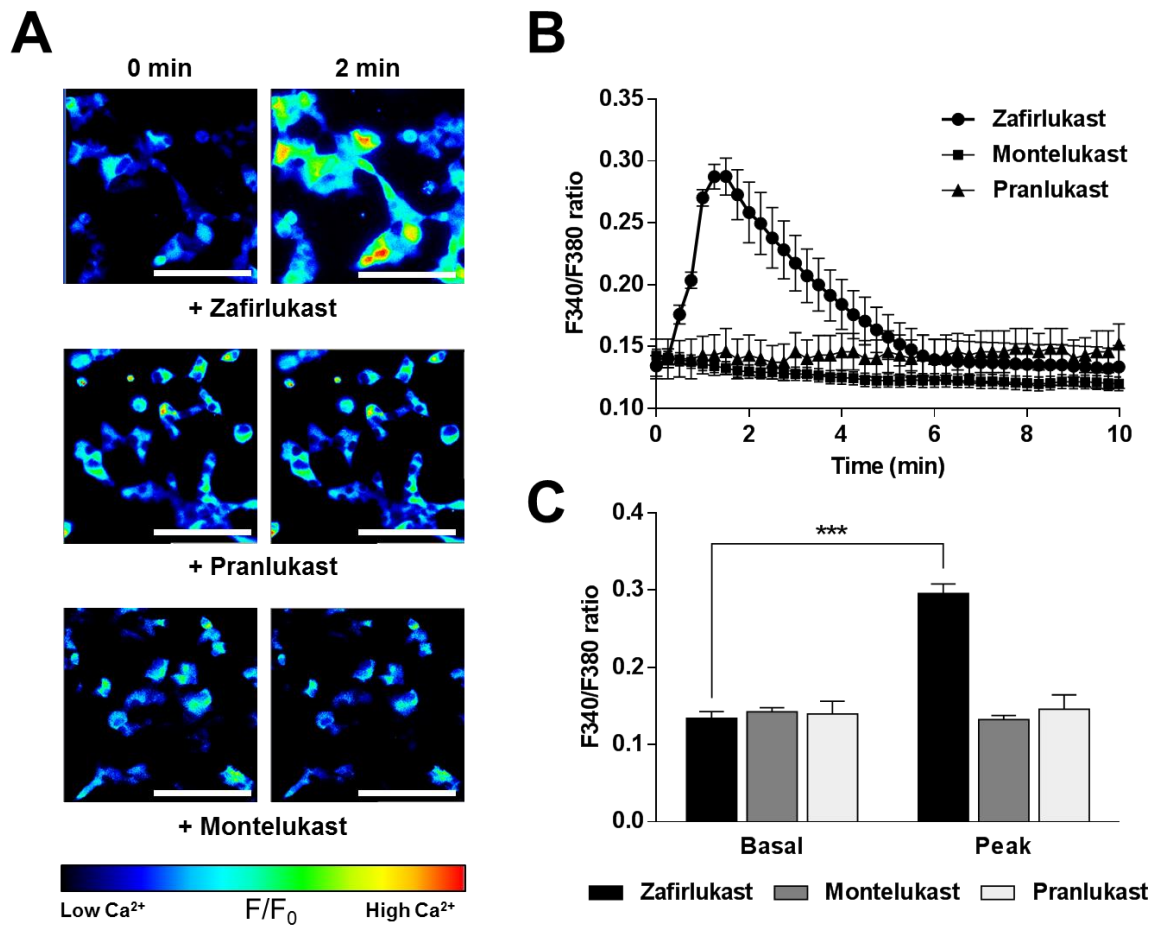


Figure 3.3. Zafirlukast-induced increase in intracellular calcium ($[Ca^{2+}]_i$) in MIN6 cells.

(A) Pseudocolored images showing the relative concentration of calcium visualized by the fura-2-acetoxymethyl ester (Fura-2AM) fluorescence ratio (F340/F380 nm) following stimulation with each CysLTR1 antagonist in MIN6 cells. Blue and red correspond to low and high $[Ca^{2+}]_i$, respectively. (B) Change in the F340/F380 nm ratio at different time points and (C) basal and peak F340/F380 nm ratio following treatment with each CysLTR1 antagonist ($n = 7-12$ per group). Scale bar: 100 μ m. Error bars represent SEM. *** $P < 0.001$.

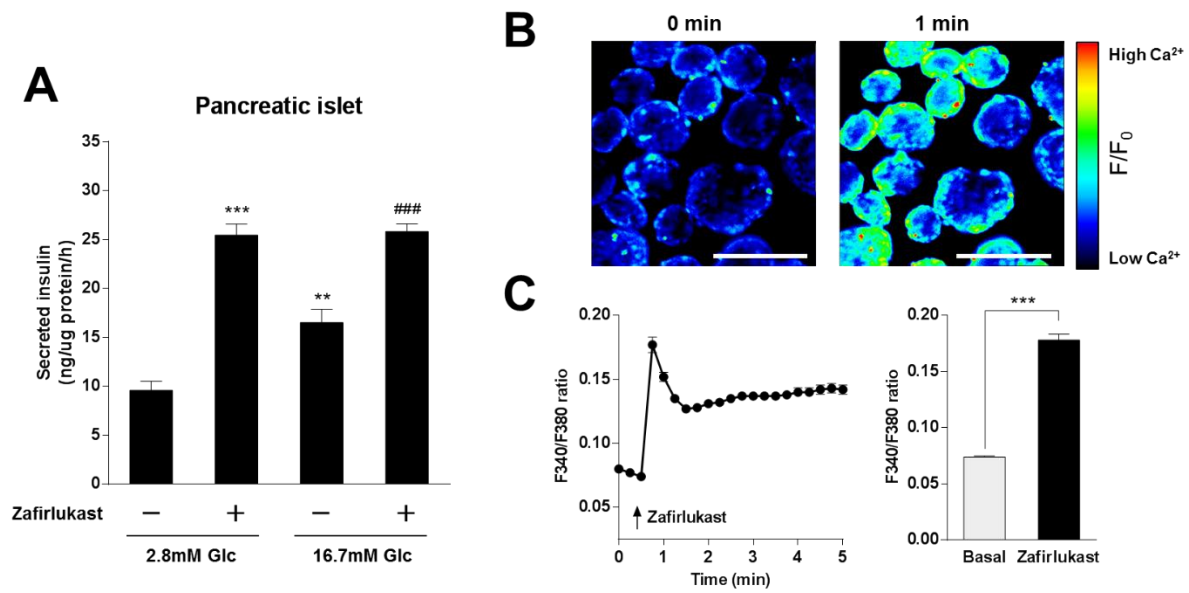


Figure 3.4. Increase in insulin secretion and $[Ca^{2+}]_i$ following stimulation with zafirlukast in primary pancreatic islets.

(A) Secreted insulin level following stimulation with zafirlukast in low and high glucose conditions in primary pancreatic islets. (B) Pseudocolored image of the F340/F380 nm ratio following zafirlukast treatment in cultured primary islets (C) Change in the F340/F380 nm ratio at different time points and (C) basal and peak F340/F380 nm ratio following exposure to zafirlukast (n = 10 per group).. Scale bar: 200 μ m. Error bars represent SEM. ***P < 0.001.

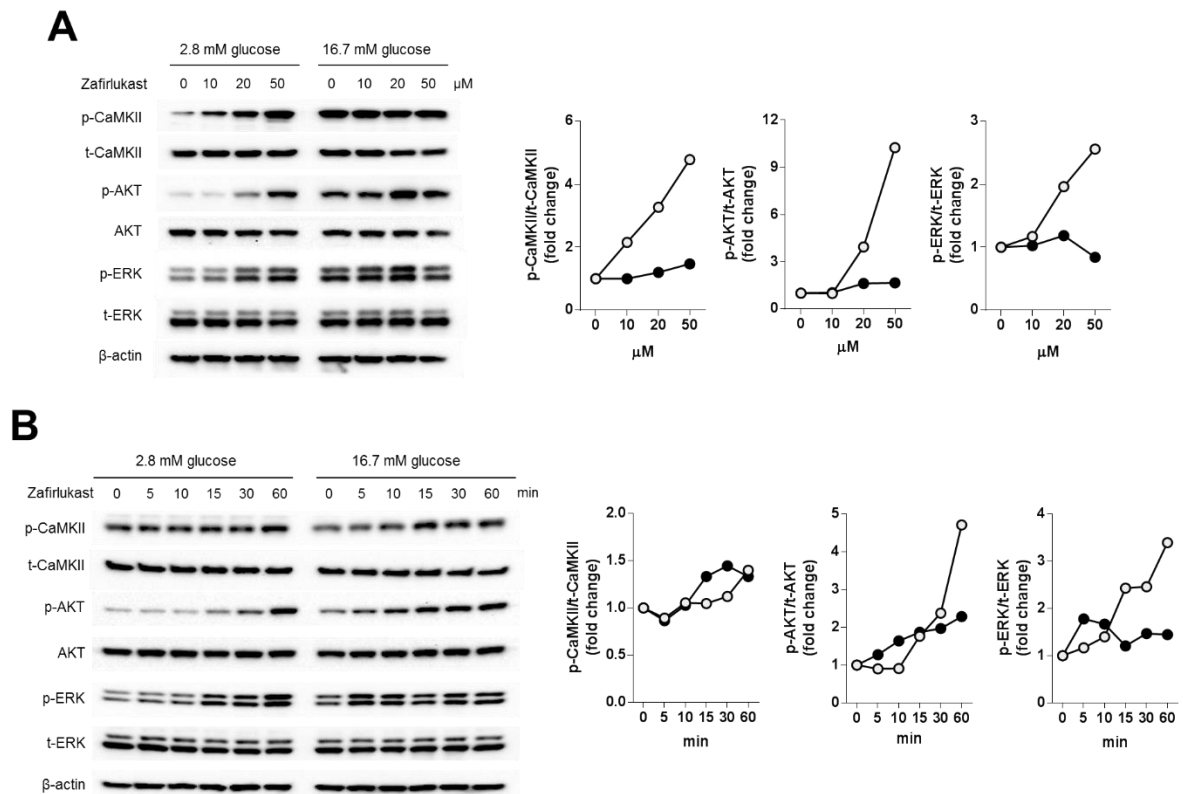


Figure 3.5. Zafirlukast-induced phosphorylation of CaMKII, AKT, and ERK.

(A) Western blot analysis showing phosphorylation of CaMKII, AKT, and ERK proteins following treatment of MIN6 cells with 50 μM zafirlukast for 1 h in a dose-dependent and (B) time-dependent manner. MIN6 cells were incubated with zafirlukast at the indicated concentrations for 1 h and protein lysates were used for western blot analysis. To assess time-dependent changes, proteins were harvested at the indicated times following treatment with 50 μM zafirlukast in basal and high glucose conditions.

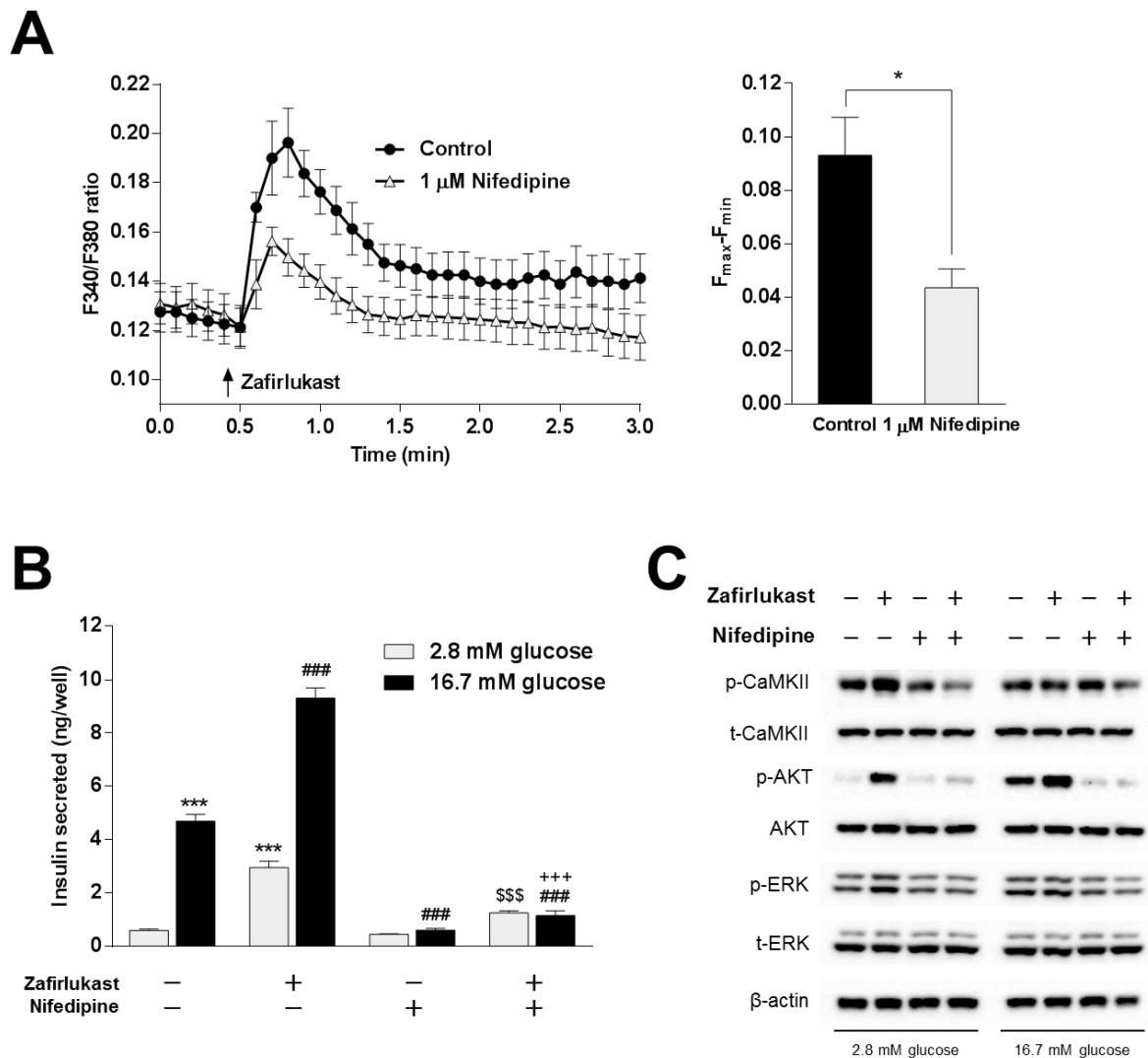


Figure 3.6. Effect of zafirlukast on insulin secretion elicited by extracellular Ca^{2+} influx through L-type voltage dependent calcium channels (L-VDCCs).

(A) Change in the F340/F380 nm ratio (left panel) and maximum change in the F340/F380 nm response (right panel) following zafirlukast treatment in the absence or presence of nifedipine. MIN6 cells were incubated with nifedipine before the addition of zafirlukast and the change in the F340/F380 nm ratio was monitored following treatment with zafirlukast ($n = 8$ per group). (B) Secreted insulin level ($n = 3$) and (C) western blot analysis in MIN6 cells in the presence of basal or high glucose with or without $1 \mu\text{M}$ nifedipine as indicated. Error bars represent SEM. *** $P < 0.001$, compared with the control in low glucose. #### $P < 0.001$, compared with the control in high glucose. \$\$\$ $P < 0.001$, compared with the control in low glucose with zafirlukast. +++ $P < 0.001$, compared with the control in high glucose with zafirlukast.

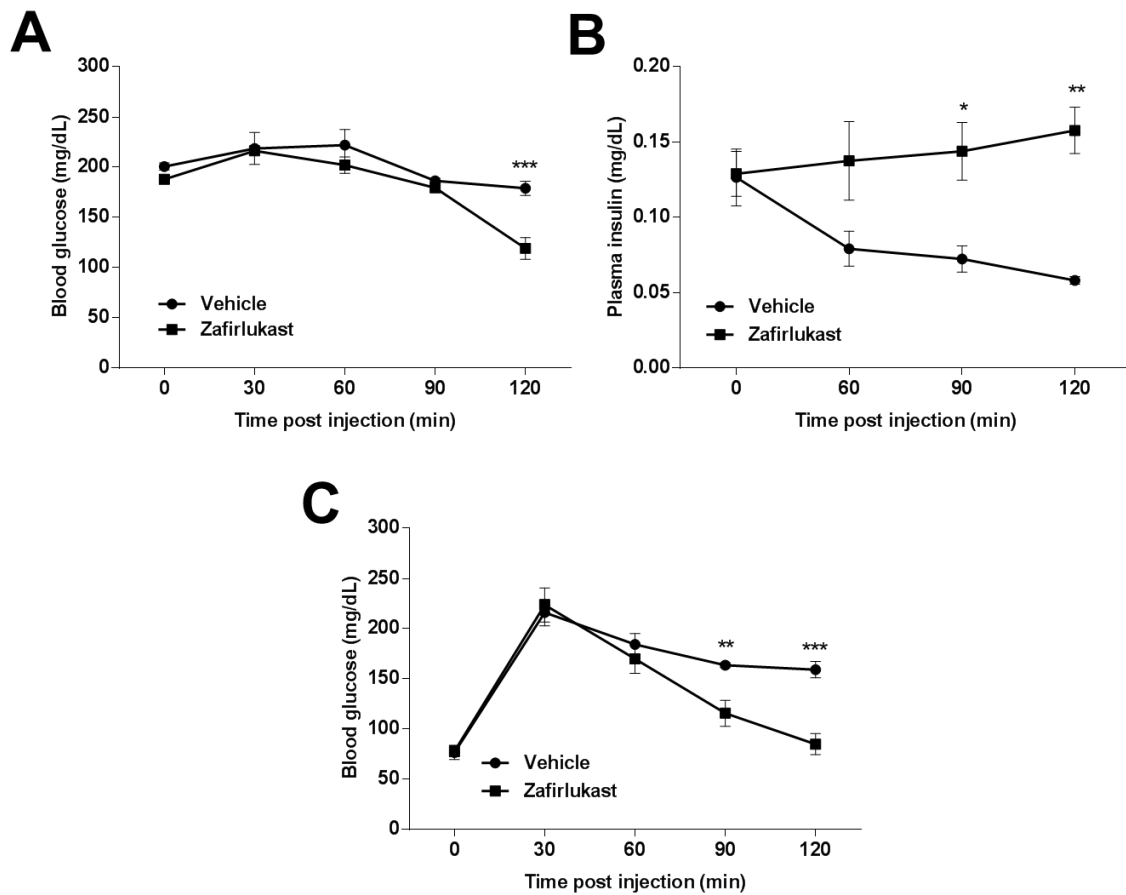


Figure 3.7. Glucose-lowering effect of zafirlukast as a result of increased insulin secretion *in vivo*.

(A) Blood glucose and (B) serum insulin levels in 8-week-old C57BL/6J mice treated with vehicle or 0.1 mg/g zafirlukast ($n = 3$ per group). (C) Blood glucose levels during an intraperitoneal glucose tolerance test in 8-week-old C57BL/6J mice dosed with vehicle or 0.1 mg/g zafirlukast ($n = 5$ per group) Error bars represent SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

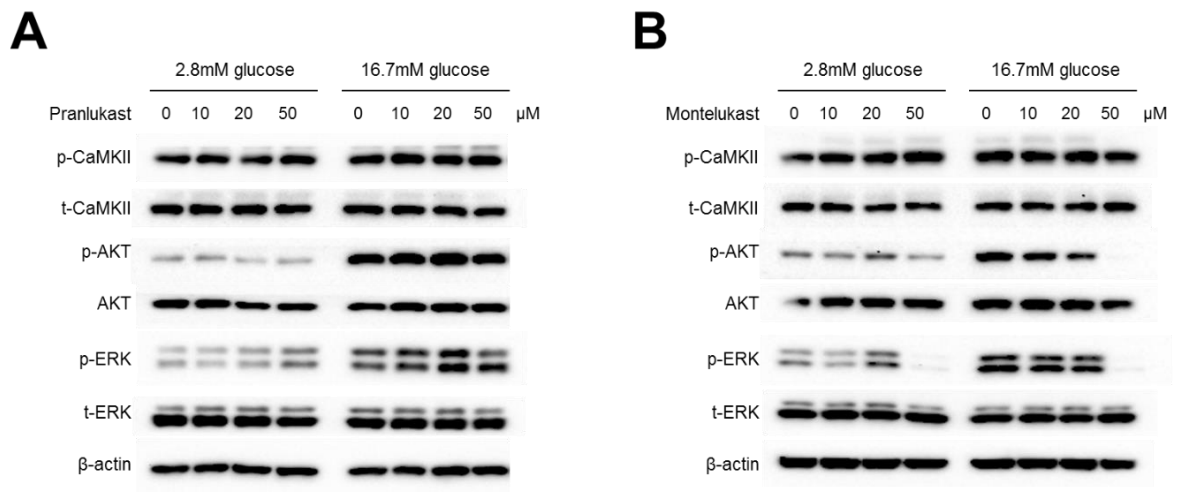


Figure 3.8. Pranlukast- and montelukast-induced phosphorylation of CaMKII, AKT, and ERK.

(A) Western blot analysis showing phosphorylation of CaMKII, AKT, and ERK in a dose-dependent manner following treatment with pranlukast and (B) montelukast for 1 h in MIN6 cells. MIN6 cells were incubated in basal and high glucose medium with pranlukast and montelukast at the indicated concentration for 1 h and protein lysates were analysed by western blot.

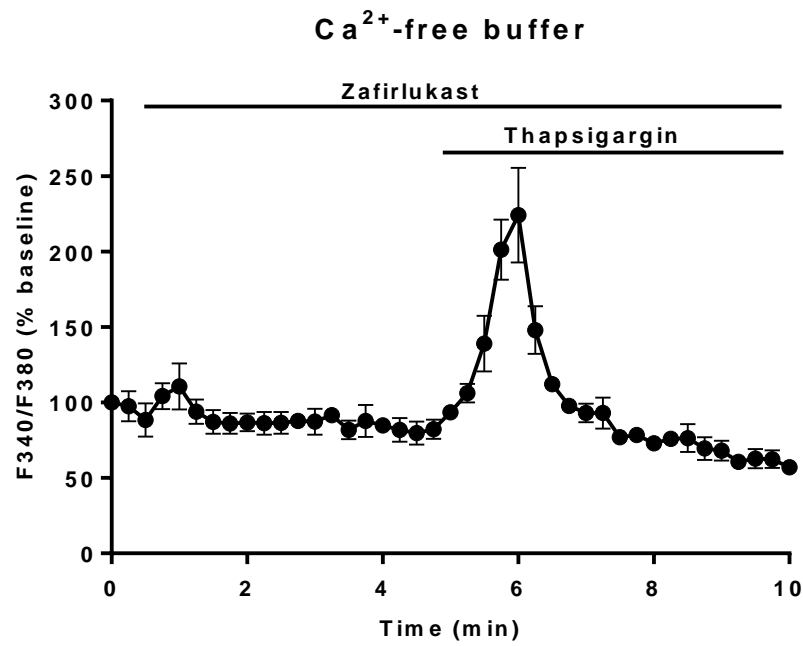


Figure 3.9. Effect of zafirlukast on [Ca²⁺]_i in Ca²⁺-free KRB medium.

(A) Changes in the F340/F380 nm ratio at different time points in Ca²⁺-free KRB medium (n = 6). The Fura-2AM-loaded MIN6 cells were incubated in Ca²⁺-free KRB and stimulated with 50 μM zafirlukast and 1 μM thapsigargin at the indicated times.

3.5. References

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Chapter IV

Acknowledgements

유니스트에서 공부를 시작한지 어느덧 9년이라는 시간이 흘렀습니다. 박사 과정을 무사히 마무리하기까지 많은 분들의 도움이 있었습니다. 이 글을 통해 감사의 인사를 드리 고자 합니다.

제일 먼저, 항상 저의 연구에 여러 가지 가능성과 많은 경험의 기회를 주신 서판길 교수님 감사합니다. 교수님께서 몸소 가르쳐 주신 사람 인연의 소중함, 연구 윤리와 연구자의 도덕적 면모 또한 명심하겠습니다. 바쁘신 와중에도 진심 어린 조언 해주시고 논문을 끝까지 마무리할 수 있도록 많은 도움 주신 최장현 교수님, 채영찬 교수님 감사합니다. 학부 연구 참여 기간 동안 많은 실험 기술을 가르쳐 주신 박찬영 교수님께도 감사의 인사를 드립니다.

박사 학위 논문을 마무리하면서 되돌아보면 힘든 일도, 포기하고 싶었던 적도 많았습니다. 그때마다 실험실 선배와 동료분들로부터 많은 힘을 받았습니다. 지도 교수님만큼 의지했던 저의 사수 현준선배와 엄마처럼 든든했던 유진선배 감사합니다. 어려울 때마다 불러 조언해주시고 도움 주신 용렬선배 감사합니다. 항상 긍정적인 힘을 주신 서영교 박사님, 강두석 박사님, 이철 박사님, 박광일 박사님, 김광연 박사님, 한 공간에서 함께 연구할 수 있어 영광이었습니다. 항상 옳은 생각이 무엇인지 알려주었던 경진선배, 도움 청할 때마다 흔쾌히 손을 내주었던 밝은 혜윤이도 정말 고맙습니다. 행정적으로 실험적으로 지원해주신 김영미 선생님, 이경은 선생님, 김도희 선생님, 정우도 감사합니다. 다른 곳에서 꿈을 펼치고 있는 균희, 서림, 셋별, 슬기, 제표오빠, 원명이 때문에도 정말 기쁩습니다. 흔쾌히 채장 세포 분리를 알려주신 김가영 박사님과 조언을 해주신 김재운 박사님께도 감사드립니다. 이제 막 입학하여 많은 시행착오를 겪고 있는 예지, 희원, 누리, 도홍이도 고맙고 훌륭한 성과 내기를 바랍니다.

누구보다 제가 박사학위를 무사히 마무리할 수 있기를 바랬을 저의 부모님, 아빠 엄마 정말 감사합니다. 언제나 진심으로 응원해주신 언니들 형부들께도 감사의 인사를 전합니다. 항상 저에게 기쁨과 웃음을 주는 우리 조카 예준, 지호, 준우, 윤호도 고마워. 저의 박사학위를 진심으로 기뻐해주신 오빠 어머니 아버지께도 감사드립니다. 가족들의 아낌없는 격려와 사랑 덕분에 학위를 잘 마칠 수 있었습니다.

마지막으로, 한결 같은 사랑으로 저를 챙겨주었던 동선 오빠에게 박사 학위 취득의 영광을 돌리고 싶습니다. 진심으로 고맙고, 저도 평생 사랑합니다.

