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ULSAN NATIONAL INSTITUTE OF SCIENCE AND TECHNOLOGY

Doctoral Thesis

Studies on regulation of insulin secretion in pancreatic β-cells

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Studies on regulation of insulin secretion in pancreatic β-cells

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Studies on regulation of insulin secretion in pancreatic β-cells

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Abstract

The type 2 diabetes mellitus (T2DM) was one of the most significant public health challenges in 21th 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 Cβ (PLCβ) 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 PLCβs and high-throughput tools for discovering regulator of insulin secretion. In the second chapter, I described the PLCβ1-mediated insulin secretion. Phospholipase Cβ (PLCβ) exerts biological influences through G protein-coupled receptors (GPCRs). GPCRs are involved in regulating glucose-stimulated insulin secretion (GSIS). Previous studies have suggested that PLCβs might play an important role in pancreatic β-cells. However, due to a lack of the specific inhibitors of PLCβ isozymes and appropriate genetic models, the *in vivo* function of specific PLCβ 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 PLCβ1 was crucial for β-cell function by generation of each PLCβ conditional knockout mice. Mice lacking PLCβ1 in β-cells exhibited a marked defect in GSIS, leading to glucose intolerance. In *ex vivo* studies, the secreted insulin level and Ca^{2+} response in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets was lower than those in the *Plcb1*$^{f/f}$ islets under the high glucose condition. PLCβ1 led to potentiate insulin secretion via stimulation of particular G_q protein-coupled receptors. *Plcb1*$^{f/f}$; *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

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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 PLCβ1 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.

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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
Emax	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
IP_3	Inositol triphosphate
IP_3R	inositol trisphosphate receptors
IP_4	Inositol 1,3,4,5-tetrakisphosphate

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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
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
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
$[Ca^{2+}]_i$	Intracellular calcium level

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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

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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 Cβ (PLCβ) that preferentially couples to the $G\alpha_q$ 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 PLCβs as important regulators in the pathway for insulin release. This review provides a comprehensive understanding of the potential regulatory modes of PLCβs 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-VDCC), inducing an influx of extracellular Ca^{2+}. The elevated intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) 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 $G\alpha_s$ and $G\alpha_i$ proteins couple to adenylyl cyclase (AC), and activation of $G\alpha_s$ proteins stimulates AC and insulin secretion, but activation of $G\alpha_i$ 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 $G\alpha_q$ proteins promote lipid-derived signaling through PLCβs, which hydrolyze membrane-bound phosphatidylinositides and induce increased $[Ca^{2+}]_i$ mobilization. Also, pancreatic β-cells contains a

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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 PLCβ-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-PLCβ signaling are described below.

1.3. The PLCβ-mediated regulation of GSIS

Distinct structures and expression patterns of PLCβs in pancreatic β-cells

To date, 13 mammalian PLC isozymes have been characterized and classified into six different subtypes: PLCβ1-4, PLCγ1,2, PLCδ1,3,4, PLCε, PLCζ, and PLCη1,2 [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 (PIP_2) and βγ-subunits of G proteins. The EF hand is a known Ca^{2+}-binding region, which promotes $G\alpha_q$-mediated GTP hydrolysis. The X–Y linker contains the catalytic activity. The C2 domain regulates PLC activity involving Ca^{2+}-dependent membrane targeting. Additionally, there are other unique domains specific to each subtype. Notably, PLCβs 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 PLCβ isozyme has different PDZ-binding motifs, enabling each PLCβ to interact with specific G_qPCRs via particular PDZ proteins [36, 37].

The expression patterns and regulation of PLCβs, PLCβ1-4, are somewhat different. PLCβ1 and PLCβ4 are enriched in neural tissue, PLCβ2 is limited to hematopoietic cells, and PLCβ3 is more widely expressed [38]. In pancreatic β-cells, the expression of PLCβ 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 PLCβ1, PLCβ2, and PLCβ3 are detectable, but the expression levels of these isozymes are slightly reduced in rat islets [39]. The PLCβ1 is especially localized in the nucleus as well as the cytoplasm in the mouse insulinoma cell line, MIN6 [40]. Regarding rat primary tissue, all PLCβ isozymes have been detected in the islets of Langerhans by western blot analyses. Using immunohistochemistry (IHC) of the rat pancreas, PLCβ1 and PLCβ4 have

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been found in endocrine tissues. PLCβ2 and PLCβ3 are mainly expressed in the periphery of the islets [41]. The use of monoclonal antibodies revealed high levels of PLCβ4 and moderate levels of PLCβ3 in the pancreatic islets in the IHC of the mouse pancreas. PLCβ1 was mainly found in exocrine tissue [42]. However, there is other evidence that both rat and mouse islets express PLCβ1. [43]. Recently, we found that PLCβ1, PLCβ3, and PLCβ4 were expressed in the MIN6 cell line and mouse primary islets (unpublished data). As the physiological function of each PLCβ isozyme in pancreatic β-cells remains largely unknown, it is important to clearly define the regulatory mode of each PLCβ during insulin secretion.

Activation of PLCβs 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 PLCβs. As a result of liquid chromatography-mass spectrometry/mass spectrometry-based proteomics of human islets after 24 h of high glucose exposure, PLCβ4 was characterized as a differentially abundant protein, with PLCβ4 downregulated under conditions of high glucose levels [44]. The exposure to high glucose levels for several days induced expression of PLCβ1 and endoplasmic reticulum (ER) stress markers, such as GRP78, sXBP1, ATF4, and Grp78. Losartan, a selective angiotensin II type 1 receptor blocker, inhibits the PLCβ-IP_3-Ca^{2+} pathway and prevents glucose-induced expression of PLCβ1 and ER stress markers, leading to alleviated glucose toxicity conditions [45]. Several proteins, including PLCβs, 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 PLCβ 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, PLCβ1-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 PLCβ1 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

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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 PLCβs.

PLCβ-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 $G\alpha_q$ and, in turn, activates membrane-associated PLCβs. The PLCβs hydrolyze PIP_2 into inositol triphosphate (IP_3) and diacylglycerol (DAG), which serve as important second messengers in signal transduction [50]. IP_3 is targeted to inositol trisphosphate receptors (IP_3Rs) on the ER, inducing Ca^{2+} mobilization from the ER and rapid elevation of cytosolic Ca^{2+} 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 PIP_2 and increased DAG directly activate TRPC3 [56]. The release of the Gβγ complex also plays a role in insulin secretion. When the βγ subunit-binding domains, such as the Gα subunit of the retinal G-protein transducing or the PH domain of G-protein-coupled receptor kinase 2, were expressed, the Gβγ-mediated signaling was masked, and insulin secretion was inhibited [57]. Also, the Gβγ-dependent pathway is involved in the kisspeptin-10-dependent increase in insulin secretion [58]. However, the role of the Gβγ 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 IP_3-mediated release of Ca^{2+} from internal stores, PLCβ can facilitate a further increase in $[Ca^{2+}]_i$ by activation of store-operated channels (SOC) [60]. The PLCβ-IP_3-induced depletion of Ca^{2+} 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 Ca^{2+} entry. In addition to the GPCR-regulatory mode, the PLCβ1 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 PLCβ signaling.

Muscarinic receptor (M_3)

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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 PLCβ activity. Carbachol and oxotremorin M increase PLCβ-induced IP_3 production and transient $[Ca^{2+}]_i$ and insulin secretion, and their effects are abolished by aminosteroid U-73122, a PLC inhibitor [64]. Muscarinic receptor stimulation leads to rapid and sustained PLCβ activity, and the periodic activation of PLC is tightly regulated by dynamic $[Ca^{2+}]_i$ oscillations, whereas sustained PLCβ activity is enhanced by increased cytosolic Ca^{2+} 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. IP_3-mediated Ca^{2+} mobilization by PLCβs during cholinergic activation causes Ca^{2+}/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]. PLCβ signaling has an essential role in FFA-induced insulin secretion [70]. Additionally, treatment with an inhibitor for $G\alpha_q$, YM-254890, and inhibitor for PLC, U73122 and neomycin, results in reduced insulin secretion and FFA1-mediated Ca^{2+} efflux from the ER [71, 72]. The increase in $[Ca^{2+}]_i$ induced by stimulation of FFA1 is attenuated by nitrendipine, a LTCC blocker, which indicates that not only $G\alpha_q$-PLCβ-mediated intracellular Ca^{2+} efflux from the ER but also extracellular Ca^{2+} 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–PLCβ 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 $G\alpha_q$-PLCβ [75]. However, whether activation of the ERK1/2 pathway correlates with induction of insulin secretion remains controversial [76].

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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 vehicles 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 $G\alpha_q$ protein, which activates the PLCβ–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_qPCRs, are functionally coupled to PLCβ-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 PLCβ-IP_3-mediated efflux of Ca^{2+} from the ER [89]. PLCβ signaling has been shown to be involved in the Ang II-mediated regulation of GSIS. Future studies should therefore characterize the role of PLCβ 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

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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 IP_3 receptor, have been used to show that IL-6 enhances GSIS in PLCβ-IP_3-dependent pathways. Among the isozymes of PLC, PLCβ1 knockdown in MIN6 cells abolishes the stimulatory effect of IL-6 on GSIS, implying that PLCβ1 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>>OLFR21) [94]. As a ligand for OLFR15, octanoic acid (OA) mediates GSIS potentiation [94]. In the presence of an inhibitor for PLC and IP_3, neomycin and Xestospongin C, OA-enhanced GSIS is attenuated. Additionally, among primary PLCs (PLCβ and PLCγ), inhibition of PLCβ1 results in attenuated enhancement of GSIS in response to OA [95], suggesting that the G_q–PLCβ1–IP_3 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

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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.

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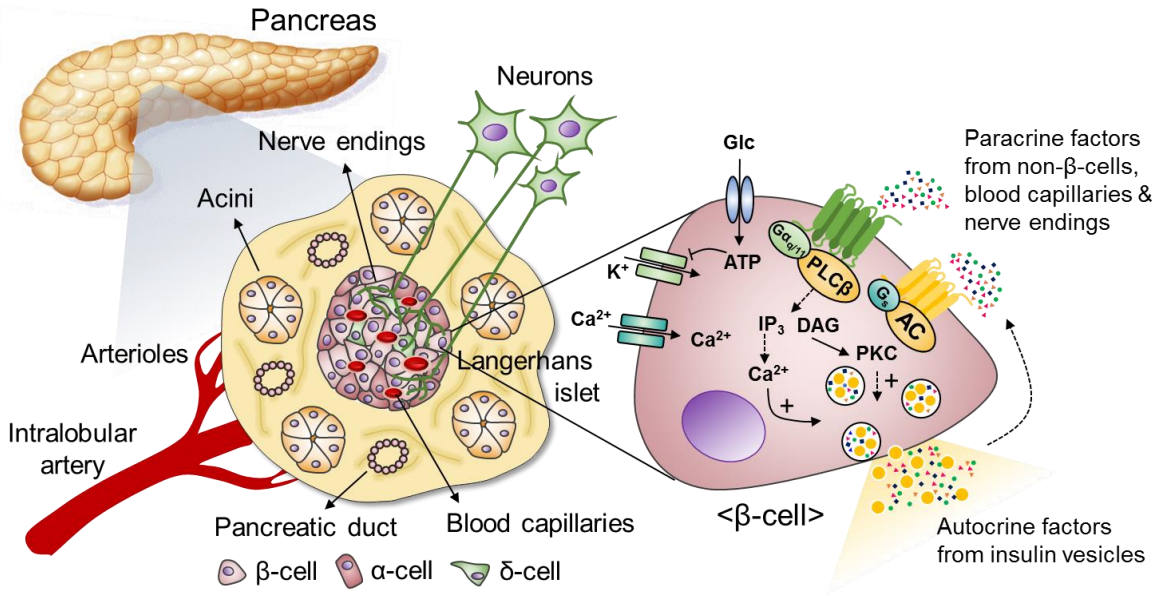

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Figure 1.1. Vascularization and innervation of Langerhans islets

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 Cβ (PLCβ) 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].

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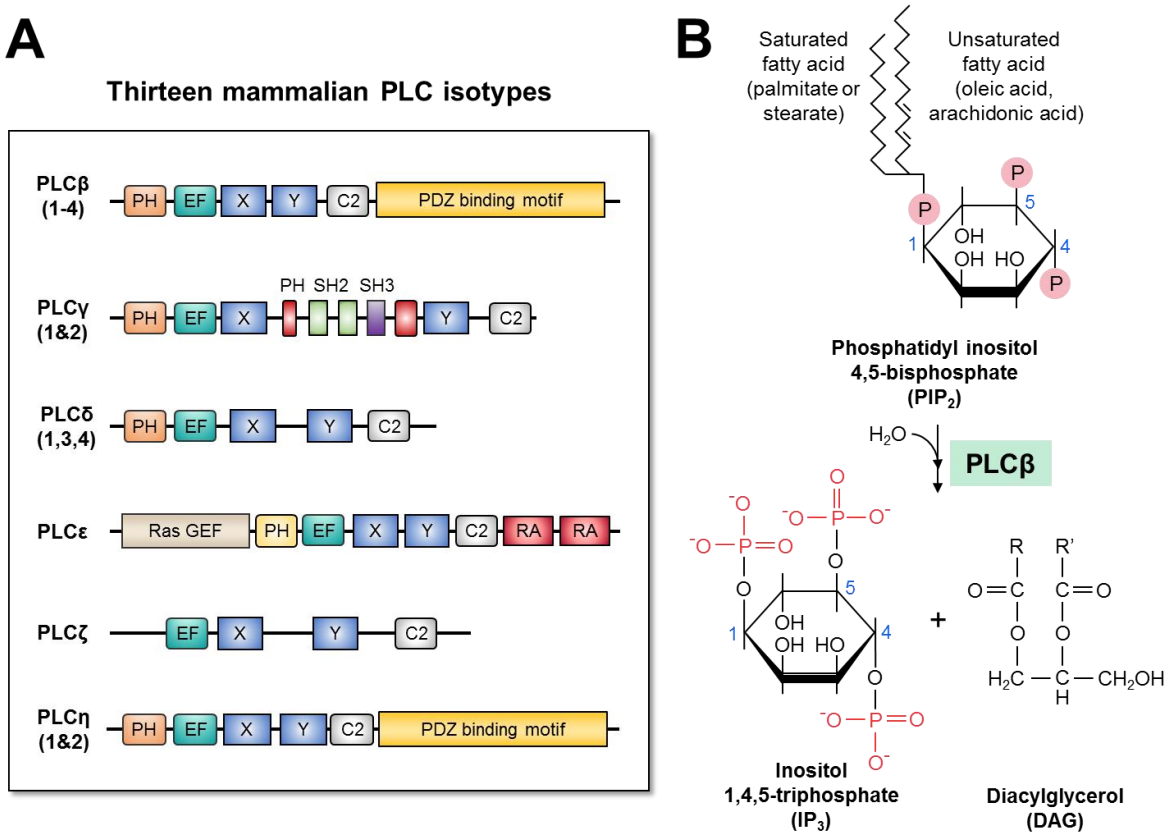

Figure 1.2. Structure of mammalian PLC isotypes and detailed action of PLCβs

(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 hare a highly conserved domain. The PH domain enables PLC to anchors plasma membrane, C2 domain could regulate PLC activity, XY domain has catalytic activity and EF hand is Ca^{2+}-binding region which triggers GTP hydrolysis of $G\alpha_q$. The PLCβ isozymes contain different PDZ binding motif which binds to particular PDZ scaffolding proteins. (B) The PLCβs selectively catalyzes the hydrolysis of phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) on glycerol portion of the phosphodiester bond. It results in release of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). PH; pleckstrin homology, SH2; SRC homology 2, GEF; guanine nucleotide exchange factor, PDZ; PSD-95/Discs-large/ZO-1.

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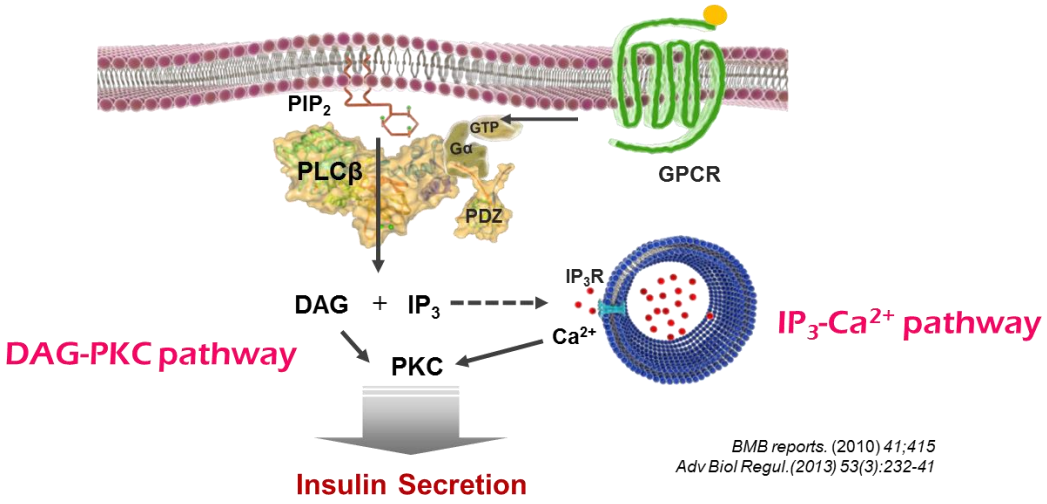
- The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2)
- Generation of second messenger (DAG and IP_3)
- Four PLCβ isozymes (PLCβ1~4)

  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 PIP_2 into IP_3 and DAG, which are important second messengers in the downstream signal pathway, regulating Ca^{2+} 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 IP_3-Ca^{2+} pathway and DAG-PKC pathway. PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PDZ, PSD-95/Discs-large/ZO-1.

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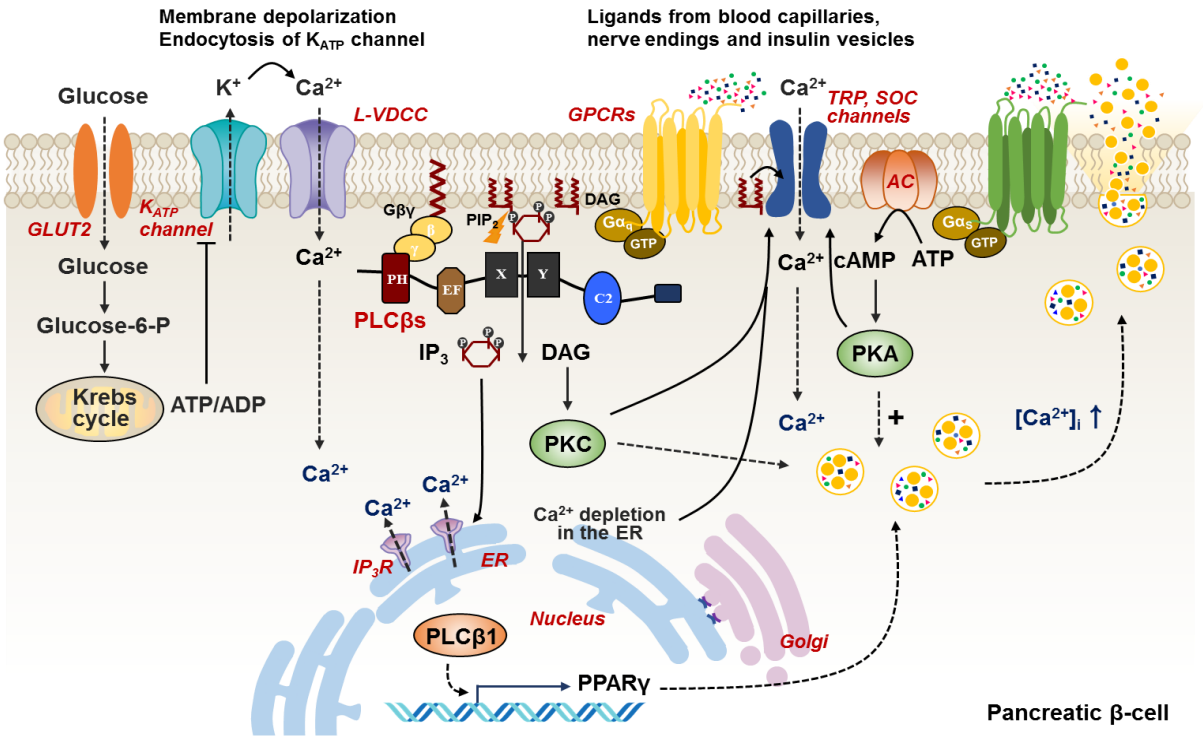

Figure 1.4. Overview of phospholipase Cβ-mediated insulin secretion pathways

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 KATP channels and endocytosis of KATP 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 PLCβ and AC is coupled to Gαq and Gαs proteins of GPCRs, respectively. PLCβ hydrolyzes membrane-bound PIP_2 into IP_3 and DAG, and AC generates cAMP, which activates PKA. The IP_3 binds to IP_3R 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 RRPs adjacent to the PM. The nuclear PLCβ1 facilitates induction of PPAR-γ expression and an increase in insulin secretion.

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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; PLCβ, phospholipase Cβ; AC, adenylyl cyclase; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; ER, endoplasmic reticulum; IP_3R, IP_3 receptor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PPAR-γ, peroxisome proliferator-activated receptor-γ.

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

Receptor name (alias)	***Natural ligand***	***G protein***	***References***
Muscarinic acetylcholine M_3 receptor (M_3)	*Acetylcholine*	*G_q, (G_s)*	*Proc Natl Acad Sci U S A.(2010) 107:21181-6 Diabetes.(2001) 50(11):2505-13*
Cholecystokinin A receptor (CCK_1)	*Cholecystokinin-8*	*G_q, (G_s)*	*Br J Pharmacol. (2015) 172:5050-67*
Free fatty acid receptor 1 (GPR40, FFA1)	*Mid- and long FFAs*	*G_q*	*Sci Rep. (2016) 6:25912*
KiSS1-derived peptide receptor (GPR54)	*Kisspeptin-10*	*G_q*	*Diabetologia (2009) 52:855–862*
Vasopressin receptor 1B (V_{1B})	*Vasopressin*	*G_q*	*J Biol Chem.(1990) 265, 15724-15730*
Serotonin receptor (HTR2B)	*Serotonin*	*G_q*	*Diabetologia. (2016) 59:744-54*
Angiotensin II receptor type 1 (AT_1)	*Angiotensin II*	*G_q*	*Diabetologia. (2006) 49:321-31*
Interleukin-6 receptor (IL-6R, CD126)	*Interleukin-6*	*G_q*	*Diabetes. (2011) 60:537-47*
Olfactory receptors 15 (OLFR15)	*Octanoic acid*	*G_q*	*Sci Rep. (2018) 8:1499*

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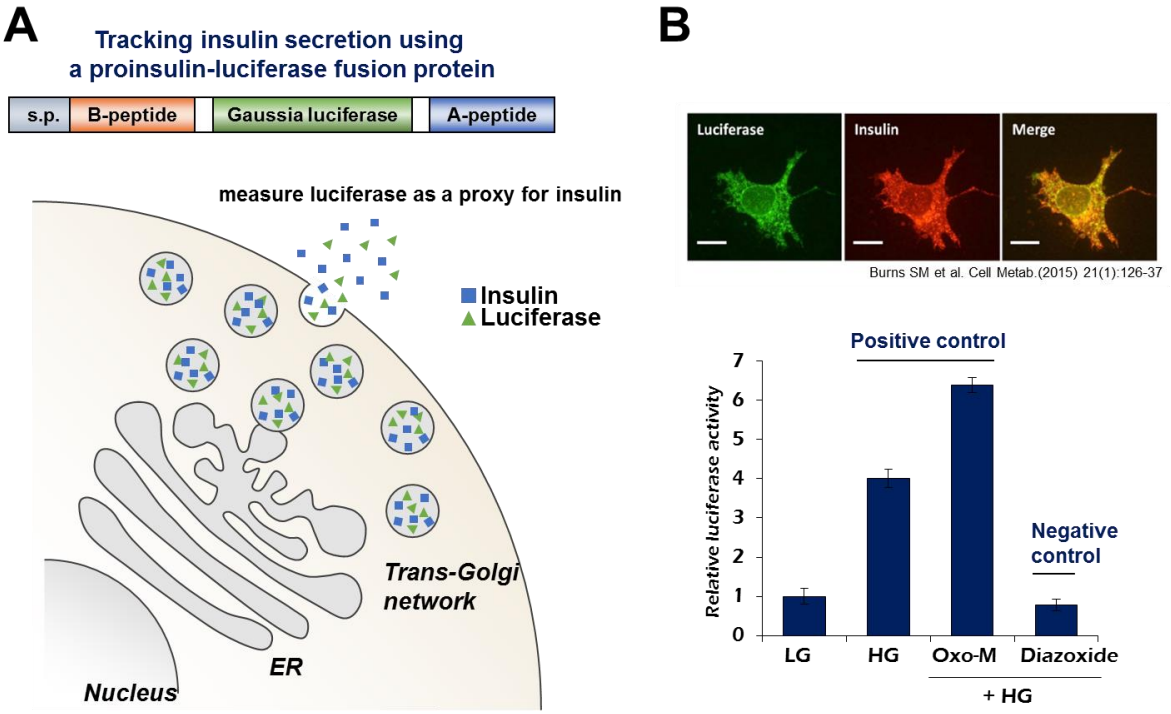

Figure 1.5. Schematic diagram of a proinsulin-luciferase fusion protein and validation of luciferase activity as a proxy for secreted insulin

(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 Cβ1 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 Cβ (PLCβ) is one of the six enzyme subtypes (β, γ, δ, ε, ζ, and η) that play a principal role in GPCR-mediated signaling. PLCβ hydrolyzes membrane-bound phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG, which are important secondary messengers in the downstream

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signal pathway, regulating Ca^{2+} mobilization and protein kinase C (PKC) activation, respectively. The PLCβ family consists of four isozymes, PLCβ1 to β4, 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 PLCβs are considered crucial effectors in GPCR-mediated signaling transduction for potentiating insulin secretion. β-cell-specific ablation of G protein α-subunits $G\alpha_q$ and $G\alpha_{11}$, which activate PLCβs, results in glucose intolerance and impaired insulin secretion. This indicates that PLCβs also contribute to the regulation of insulin secretion [16, 17]. However, previous studies have not focused on the roles of the specific PLCβ isozymes, and have only demonstrated the putative role of PLCβs 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 PLCβ regulates insulin secretion via which GPCR.

To address the physiological role of PLCβs, we generated PLCβ isozyme-deficient mice in pancreatic β-cells. β-cell-specific PLCβ1 conditional knockout (cKO) mice (*Plcb1*$^{f/f}$; *Pdx1-CreERt2*) showed impaired glucose tolerance due to deficiency in insulin secretion, with more severe phenotypes seen in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice fed high-fat diets (HFDs). Furthermore, we discovered that particular GPCR-induced insulin release was PLCβ1-dependent. Taken together, the data presented here show that PLCβ1 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

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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).

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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 CO_2 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 KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 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; DiscoveRx), 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 CO_2 cell incubator.

Determination of intracellular Ca^{2+}

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

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37°C. Glucose (16.7 mM) and ligands (50 μM 5-HT and 20 μM 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 μL of acid–ethanol extract with 100 μL 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 μm sections were sliced and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) using standard procedures or immunostained with PLCβ1 (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 ΔΔCt method using 36B4 as an internal control. The primer sets used are listed in Table 2.1.

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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

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*$p < 0.05$ considered as statistically significant.

2.3. Results

PLCβ1, 3, and 4 isozymes are moderately expressed in pancreatic β-cells

PLCβs 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 PLCβ isozymes, PLCβ1 to β4, differ in expression pattern and regulation. However, studies of PLCβ isozyme expression patterns have been controversial [22-24], with additional investigations necessary to determine the expression levels of individual PLCβ isozymes. First, to confirm the PLCβ isozyme expressed in pancreatic β-cells, we assessed PLCβ 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). PLCβ1 is enriched in the cerebral cortex and hippocampus [25], whereas PLCβ4 is highly expressed in the cerebellum [26]. Expression of PLCβ2 is largely restricted to hematopoietic cells [27], while PLCβ3 is ubiquitously expressed [28]. Indeed, PLCβ1 and PLCβ4 were highly expressed in the brain, while PLCβ2 was limited to the thymus enriched origin of hematopoietic cells. In the case of mouse pancreatic β-cells in the present study, PLCβ1, β3, and β4 expressions were apparent; however, PLCβ2 was detected at only trace levels in MIN6 cells and pancreatic islets. Taken together, these results led us to further investigate PLCβ isozymes as a potential regulator of β-cell function.

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

To gain insight into the physiological role of each PLCβ 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 PLCβ isozyme cKO mice in adults by mating floxed/floxed mice for each PLCβ 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 PLCβ isozyme was confirmed in protein extracts from islets prepared from control and PLCβ isozyme cKO mice

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

Loss of PLCβ1 in adult pancreatic β-cells resulted in impaired glucose tolerance

Next, we assessed glucose homeostasis in the PLCβ 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*$^{f/f}$; *Pdx1-CreERt2* mice exhibited a lower ability to clear glucose from the blood than the *Plcb1*$^{f/f}$ 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*$^{f/f}$; *Pdx1-CreERt2* mice showed similar insulin sensitivity to that of the *Plcb1*$^{f/f}$ 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*$^{f/f}$; *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*$^{f/f}$; *Pdx1-CreERt2* mice after overnight fasting as well as 15 and 30 min after intraperitoneal glucose challenge. Plasma insulin levels of *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice were lower than that of *Plcb1*$^{f/f}$ 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*$^{f/f}$; *Pdx1-CreERt2* islets showed a corresponding decrease in GSIS, we performed GSIS assays using islets isolated from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice. At basal glucose levels (2.8 mM), insulin levels secreted from *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets were similar to *Plcb1*$^{f/f}$ islets. After high glucose stimulation (16.7 mM) for 1 h, *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets secreted lower insulin than that of the *Plcb1*$^{f/f}$ 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*$^{f/f}$ and *Plcb1*$^{f/f}$; *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

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from *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice than in islets from the *Plcb1*$^{f/f}$ mice (Figure 2.4D). This abnormal Ca^{2+} response in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice islets may have caused the impaired GSIS. These results suggest that PLCβ1 affects pancreatic β-cell function, with a potential role in mediating insulin secretion.

Insulin production and islet morphological characteristics were normal in *Plcb1*$^{f/f}$; *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 PLCβ1 by performing immunohistochemistry analysis. Consistent with the previously published data [22, 33], PLCβ1 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 PLCβ1 staining (Figure 2.10). Double staining for insulin and PLCβ1 on the pancreas sections indicated that PLCβ1 expression was selectively eliminated in the β-cell compartment in the pancreas of tamoxifen-treated mouse (Figure 5A). Also, mRNA levels of PLCβ1 were diminished in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets relative to *Plcb1*$^{f/f}$ 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*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice was measured by ELISA and the insulin mRNA levels were determined by qRT-PCR from islets isolated from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice (Figure 2.5B, C). The total insulin content was similar in the pancreases of the *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice and there were no differences in insulin mRNA levels (*Ins1* and *Ins2*) between *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets in which the *Plcb1* gene was stably ablated. In H&E-stained sections, *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets appeared similar in shape, size, and distribution in the pancreas compared to *Plcb1*$^{f/f}$ islets (Figure 2.5D). Furthermore, pancreatic β-cells from the *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice had similar areas (Figure 2.5E). Histological analysis revealed no pathological clues in *Plcb1*$^{f/f}$; *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_qPCRs) are involved in regulating potentiation of GSIS, with PLCβs acting as principal effector molecules downstream of GPCRs [16,

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34]. Our initial findings indicated that PLCβ1 was engaged in the regulation of GSIS. To examine whether PLCβ1 was also associated with G_qPCR-dependent potentiation of GSIS, we performed an insulin secretion assay using islets isolated from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice. Isolated islets were incubated with ligands known to augment insulin release via G_qPCRs, 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*$^{f/f}$ mice; however, these potentiation effects were diminished in the *Plcb1*$^{f/f}$; *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_qPCR-dependent insulin secretion.

PLCβ1 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*$^{f/f}$; *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*$^{f/f}$; *Pdx1-CreERt2* islets compared to *Plcb1*$^{f/f}$ 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*$^{f/f}$; *Pdx1-CreERt2* and *Plcb1*$^{f/f}$ islets (Figure 2.6C). Next, we intraperitoneally injected 5-HT into *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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*$^{f/f}$; *Pdx1-CreERt2* mice (Figure 2.6D). Additionally, we performed intraperitoneal injections of bethanechol, a nonsubtype-selective muscarinic agonist, in *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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 PLCβ1 is required for GSIS in a selective G_qPCR-dependent manner, particularly as a 5-HT receptor, in pancreatic β-cells.

High-fat diet caused severe defects in glucose homeostasis in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice

Microarray analysis revealed that PLCβ1 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 PLCβ1 might contribute to the pathophysiology of type 2 diabetes [38]. To test these effects, *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice were challenged with a HFD that mimicked the status of the human

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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*$^{f/f}$ and *Plcb1*$^{f/f}$; *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*$^{f/f}$; *Pdx1-CreERt2* mice during HFD intake. *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice receiving the HFD exhibited marked defects in glucose clearance in the blood relative to *Plcb1*$^{f/f}$ 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*$^{f/f}$; *Pdx1-CreERt2* mice (Figure 2.7E). Correspondingly, the GSIS assay in cultured islets from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice fed a HFD showed that the stimulatory effect of high glucose was lower in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets than in those of *Plcb1*$^{f/f}$ islets (Figure 2.7F). Thus, PLCβ1 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 PLCβ1 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 PLCβ1 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 PLCβ1 and PLCδ1 in mice, indicating a critical role of PLCβ1 in β-cell function [24]. Furthermore, the silencing of nuclear PLCβ1 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 PLCβ 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 PLCβ1 enabled β-cells to manage their highly specialized function, particularly insulin secretion. We demonstrated that the conditional ablation of PLCβ1 in pancreatic β-

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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 PLCβ1 expression was decreased in hypothalamus, *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice showed no alteration in food intake or body weight (Figure 2.12A, B). The impaired insulin secretion in *Plcb1*$^{f/f}$; *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*$^{f/f}$ mice. Our data showed that PLCβ1 exerted its effects exclusively via GSIS augmentation, making it important to determine the mechanism through which glucose induces PLCβ1-mediated insulin secretion. Glucose can stimulate the production of inositol phosphates in pancreatic islets, and PLCβ 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 PLCβ-mediated production of inositol phosphates and insulin secretion [17, 42].

Here, we confirmed that PLCβ1 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 PLCβ-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/PLCβ1 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*$^{f/f}$; *Pdx1-CreERt2* mice, there was no remarkable change in β-cell proliferation and β-cell turnover (Figure 2.13A, B). However, PLCβ1 signaling that regulates GSIS through serotonergic pathways might also be active during pregnancy, and PLCβ1 could be the genetic cause of gestational diabetes, which has great potential for developing into type 2 diabetes mellitus [45].

GPCRs can bind specific PLCβs 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/PLCβ1 signaling.

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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*$^{f/f}$; *Pdx1-CreERt2* and *Plcb1*$^{f/f}$ mice. One possible explanation for this distinction is functional compensation. Although there was no compensatory upregulation of PLCβ isozymes (Figure 2.14A, B), due to a shared primary structure and similar enzymatic activity, other still expressed PLCβ isozymes might be engaged with these G_qPCRs. 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 PLCβ1-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), PLCβ 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 PLCβ 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/μg protein/h, as compared to those fed normal chow diet (NCD), about 0.1 ng/μg 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*$^{f/f}$; *Pdx1-CreERt2* mice, HFD-feeding induced defects in glucose homeostasis. Similar to the role of PLCβ1 in regulating insulin secretion in normal *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice, the HFD-fed *Plcb1*$^{f/f}$; *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 PLCβ1 back in MIN6 cell lines (Figure 2.15A, B). These result strongly support that effect on reduced insulin secretion was due to the PLCβ1 deletion and PLCβ1 can be considered a candidate to improve therapeutic intervention related to diabetes mellitus [55].

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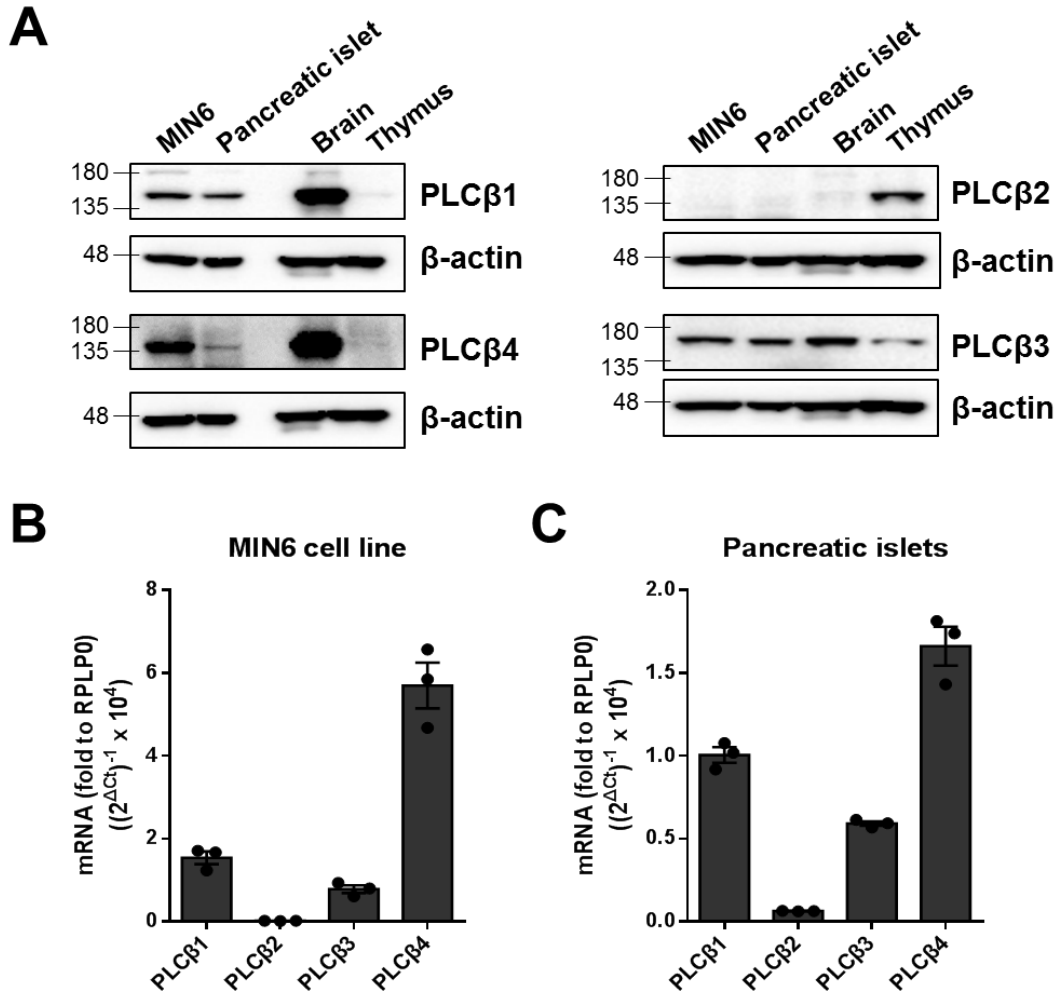

Figure 2.1. Expression profile of PLCβ isozymes in pancreatic β-cells

(A) Western blot analysis showing PLCβ isozyme 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 isozyme 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.

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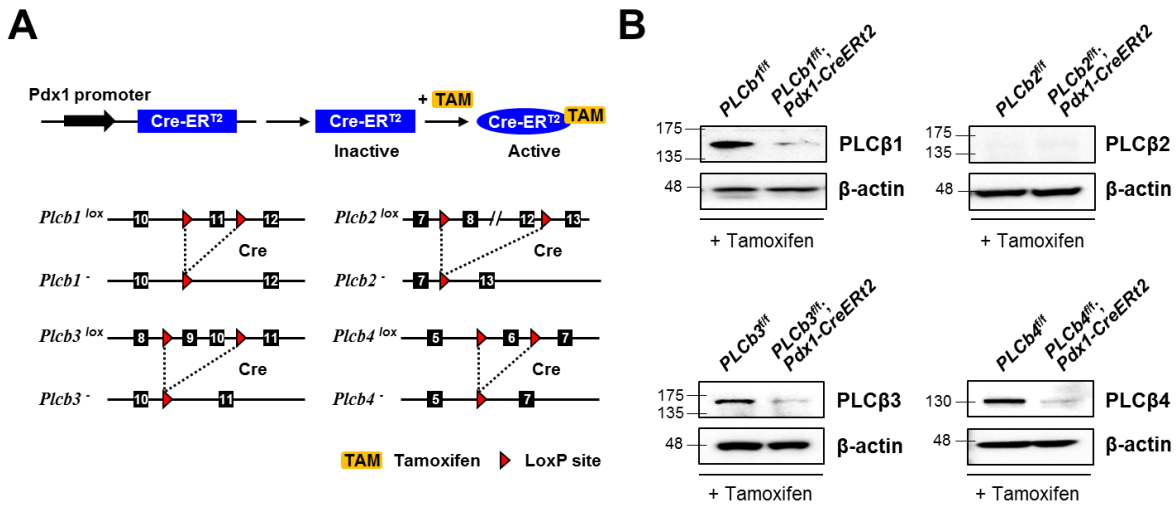

Figure 2.2. Inducible ablation of PLCβ isozymes in adult pancreatic β-cells

(A) Graphical diagram of adult *Plcb1, 2, 3,* or *4*$^{f/f}$ mice carrying the *Pdx1-creERt2* transgene injected with tamoxifen to induce β-cell-specific deletion of PLCβ1, 2, 3, or 4 isozymes. (B) Western blot analysis showing the reduction in PLCβ isozyme protein contents from extracts of isolated islets from each *Plcb*$^{f/f}$ and *Plcb*$^{f/f}$; *Pdx1-creERt2* mice following tamoxifen treatment.

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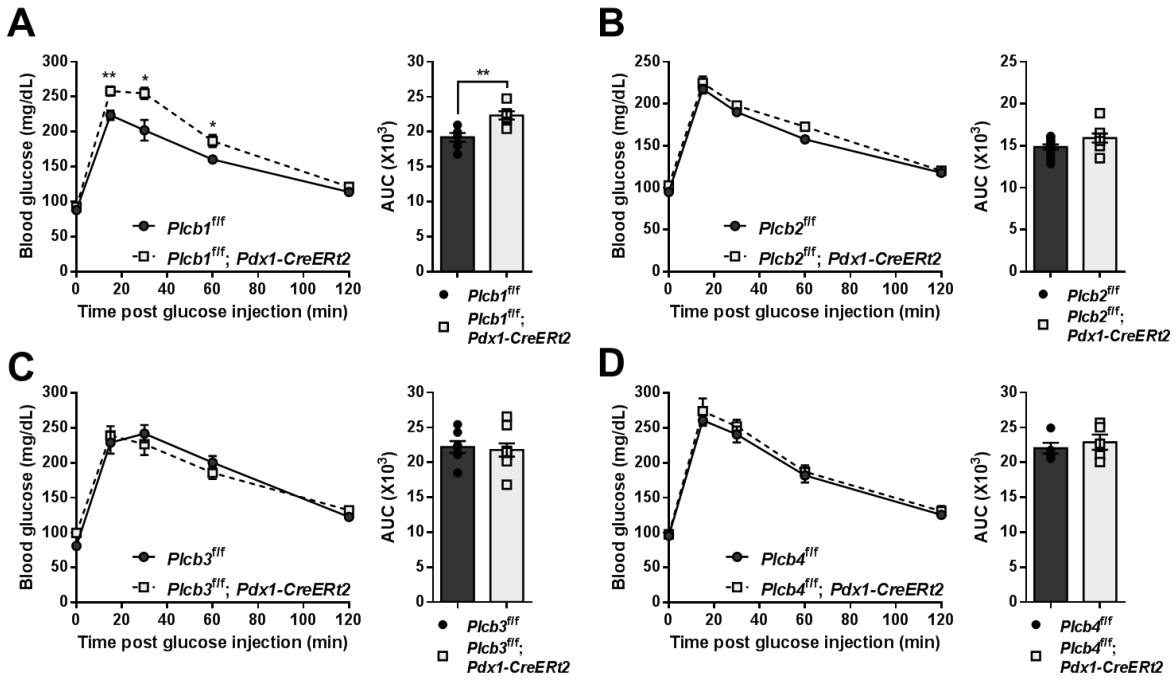

Figure 2.3. Impaired glucose tolerance in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice

(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 ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$.

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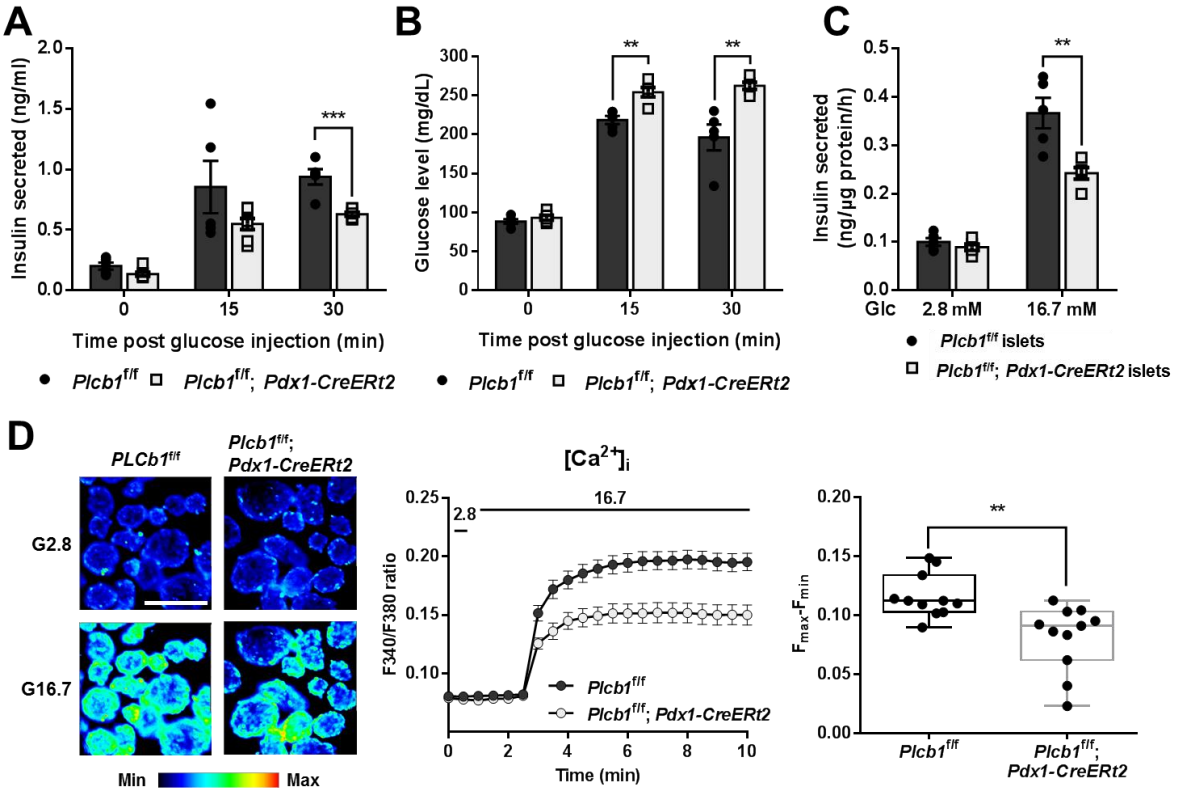

Figure 2.4. Defect in GSIS in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice

(A) Plasma insulin and (B) glucose levels after overnight fasting (16 h), 15 min, and 30 min post glucose injection in *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice (*Plcb1*$^{f/f}$, n = 5 vs *Plcb1*$^{f/f}$; *Pdx1-CreERt2*, n=7). (C) Secreted insulin levels from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice. Pseudo-colored image of the fura-2AM fluorescence ratio (F340/F380) following high glucose

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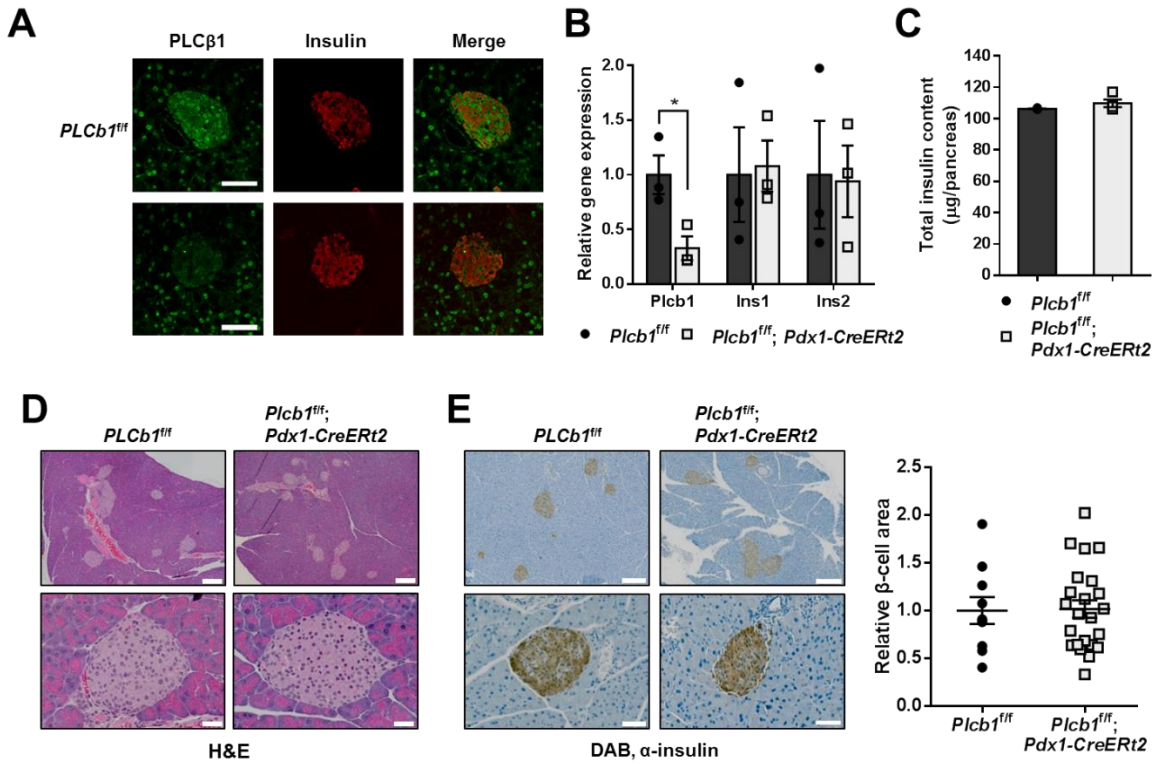

Figure 2.5. Normal insulin production and islet morphology

(A) Pancreatic sections stained with insulin and PLCβ1 antibodies. (B) mRNA expression of insulin genes from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice stained with H&E. (E) Insulin staining of pancreatic sections and quantification of β-cell area in *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice (four to five islets were analyzed from the *Plcb1*$^{f/f}$, n = 4 vs. *Plcb1*$^{f/f}$; *Pdx1-CreERt2*, n = 5). Scale bars, low magnification: 200 μm, high magnification: 50 μm. Error bars represent ± SEM. *$p < 0.05$.

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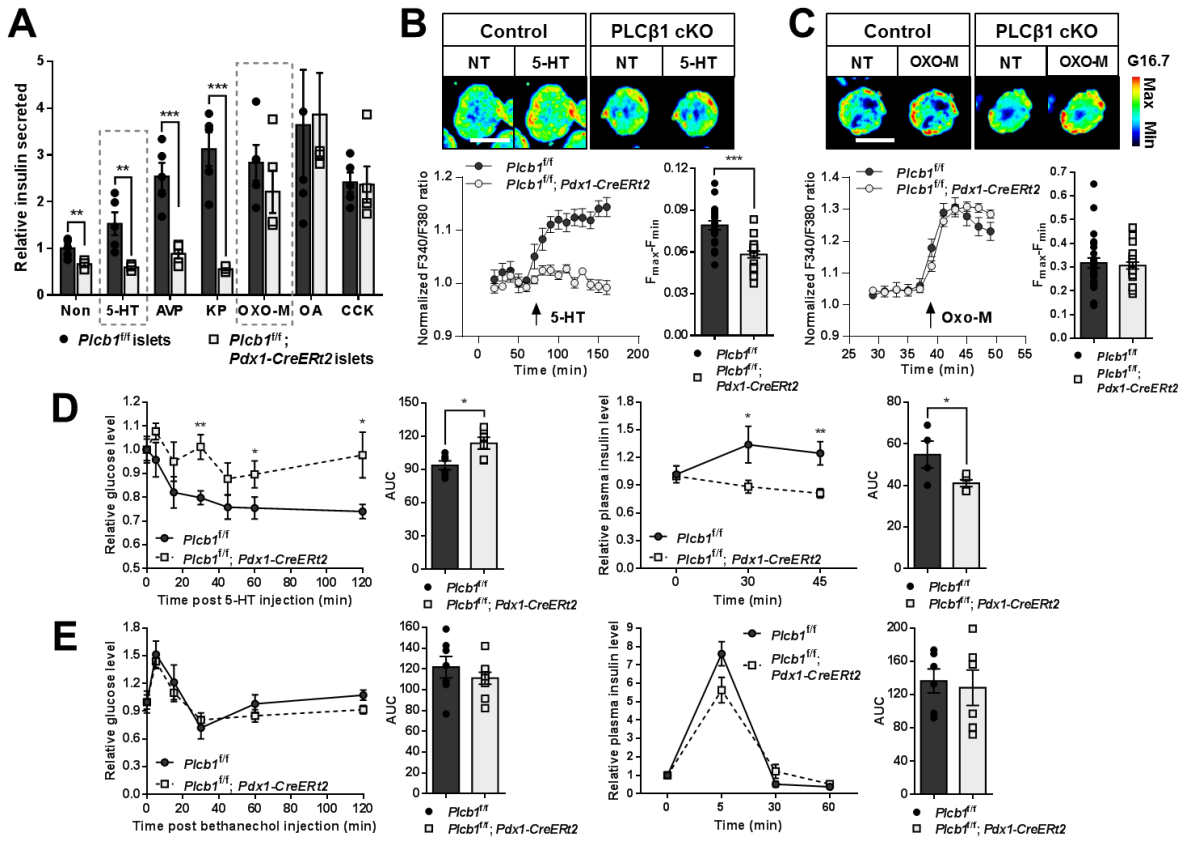

Figure 2.6. Decreased selective G_qPCR-induced insulin release in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets

(A) Selective augmentation of insulin secretion in pancreatic islets. Isolated pancreatic islets prepared from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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 μM), 5-HT (50 μM), KP (1 μM), CCK (500 nM), OXO-M (20 μM), and OA (10 μM). 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 μM, n = 20 per group) and (C) to OXO-M treatment (20 μM, n = 25 per group). Blood glucose and insulin levels of *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice and *Plcb1*$^{f/f}$ littermates after receiving (D) a single dose of 5-HT (0.3 mg per mouse, *Plcb1*$^{f/f}$, n = 5 vs. *Plcb1*$^{f/f}$; *Pdx1-CreERt2*, n = 6) and (E) a single dose of bethanechol (2 μg/g, intraperitoneal injection, *Plcb1*$^{f/f}$, n = 6 vs. *Plcb1*$^{f/f}$; *Pdx1-CreERt2*, n = 7). Plasma glucose and insulin levels were measured at the indicated time points. Scale bar: 400 μm. Error bars represent ± SEM. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

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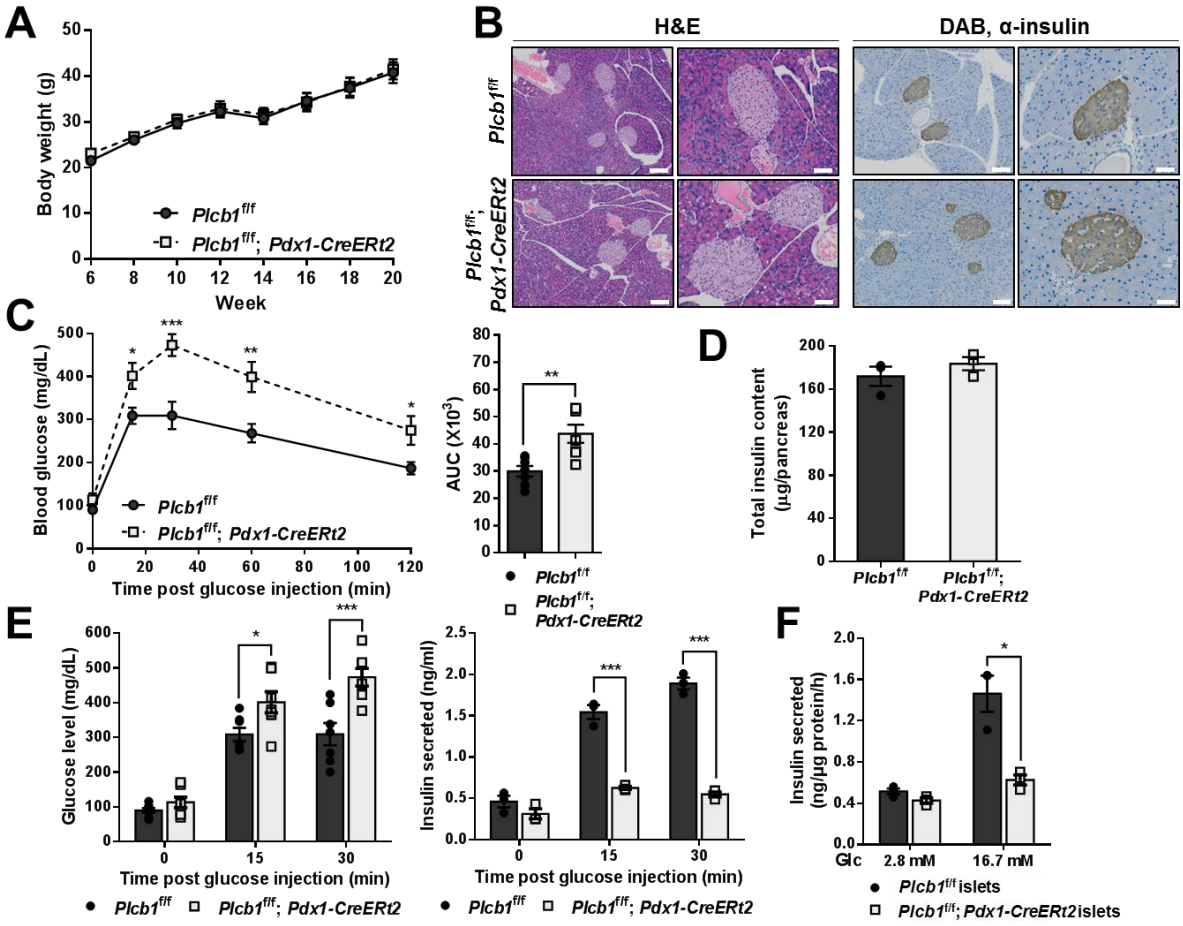

Figure 2.7. Impaired insulin secretion in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice fed a HFD

(A) Body weight during HFD feeding (*Plcb1*$^{f/f}$, n = 5 vs. *Plcb1*$^{f/f}$; *Pdx1-CreERt2*, n = 4). (B) Pancreatic sections from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice (n = 5–7 per group) post glucose injection. (F) Secreted insulin levels from *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *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.

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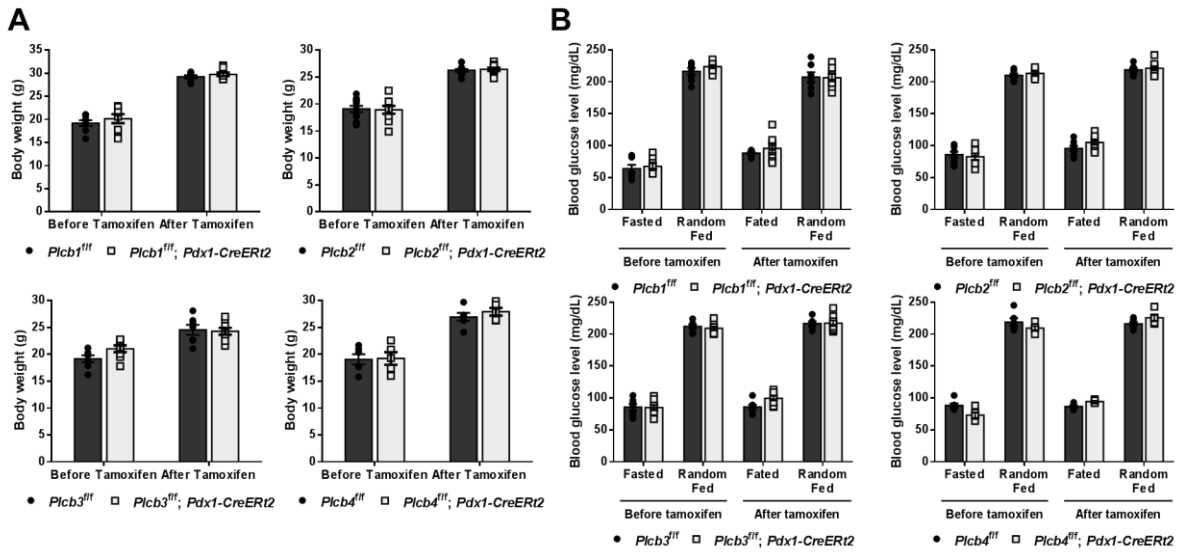

Figure 2.8. Similar body weight and fasting and re-feeding glucose levels between control and PLCβ cKO mice following tamoxifen treatment

(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.

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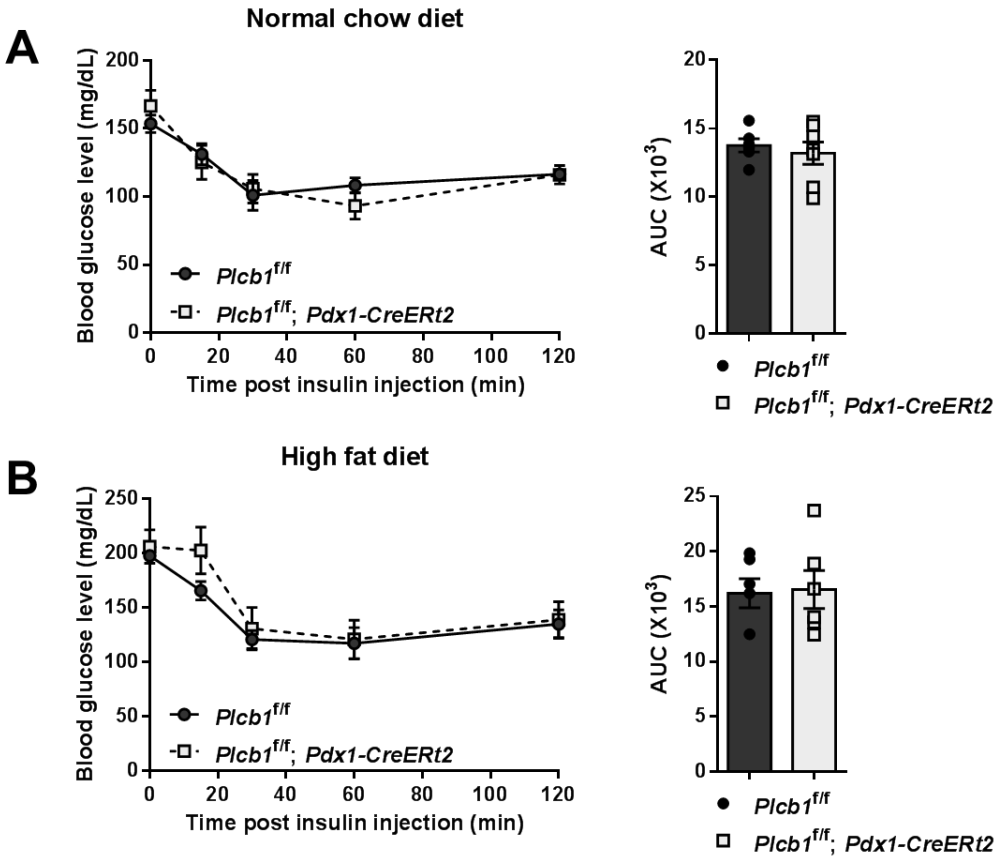

Figure 2.9. Normal insulin sensitivity in *Plcb1*$^{f/f}$*; Pdx1-CreERt2*mice fed a normal chow diet and HFD

(A) Insulin tolerance test (0.3 U/kg) for control and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice fed a normal chow diet (*Plcb1*$^{f/f}$, n = 6 vs. *Plcb1*$^{f/f}$; *Pdx1-CreERt2*, n = 7) and (B) *Plcb1*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice fed a HFD (n = 6 per group).

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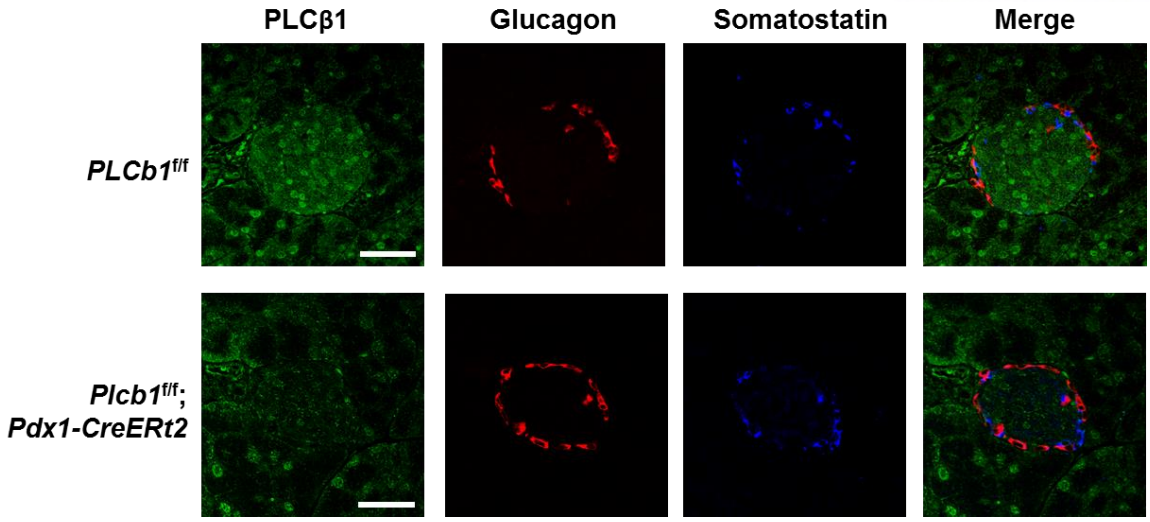

Figure 2.10. PLCβ1 protein expression in α-cell and δ-cells in pancreatic islets from control and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets

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

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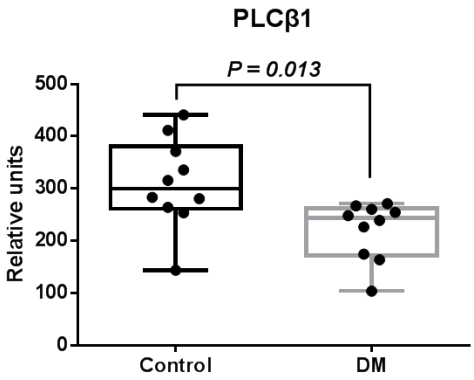

	Control	Type 2 diabetic donors
Gender (male/female)	4/6	7/3
Age (years)	60±5	67±7
BMI (kg/m2)	30.5±6.5	30.9±6.2 (n=9)
Cause of death	8 CVD, 1 TR, 1 SA	8 CVD, 1 TR
Known diabetes duration (years)	-	5.3±2.3 (n=7)

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).

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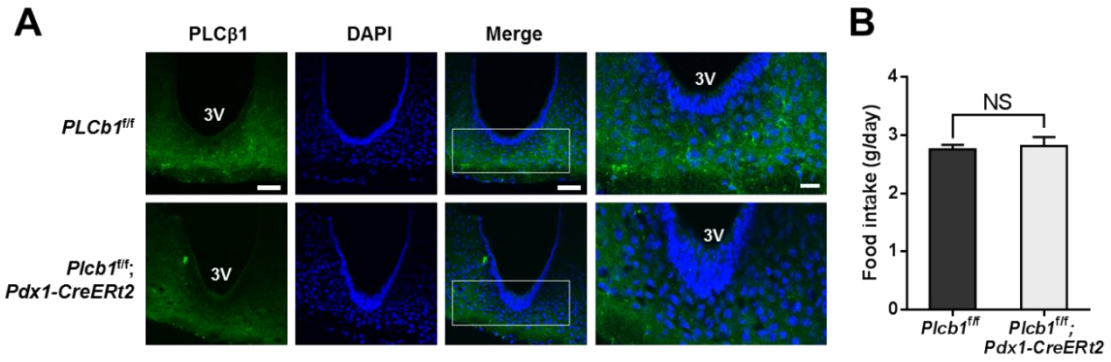

Figure 2.12. Reduced PLCβ1 expression in hypothalamus results in no alteration in food intake

(A) Brain sections stained with PLCβ1 antibodies in hypothalamus. (B) Absolute food intake (n = 6 per group). 3V; Third ventricle. Scale bars, low magnification: 50 μm, high magnification: 100 μm. Error bars represent ± SEM.

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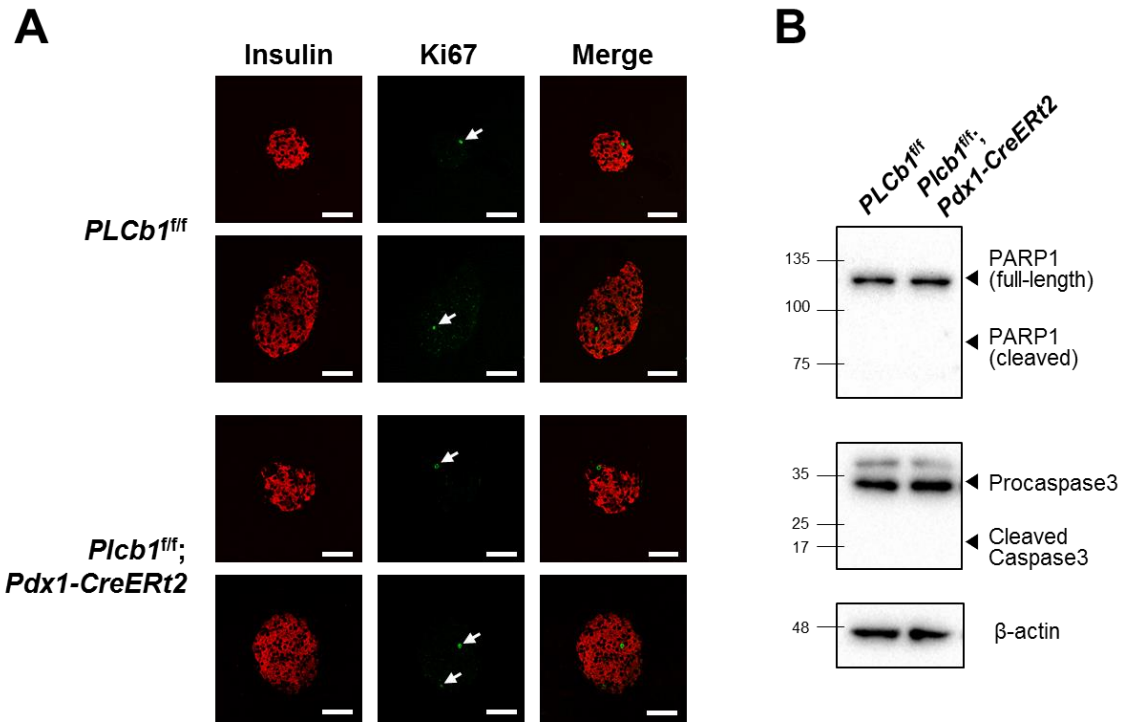

Figure 2.13. No change in β-cell proliferation and β-cell turnover in *Plcb1*$^{f/f}$; *Pdx1-CreERt2* islets

(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*$^{f/f}$ and *Plcb1*$^{f/f}$; *Pdx1-CreERt2* mice. Scale bars, 50 μm.

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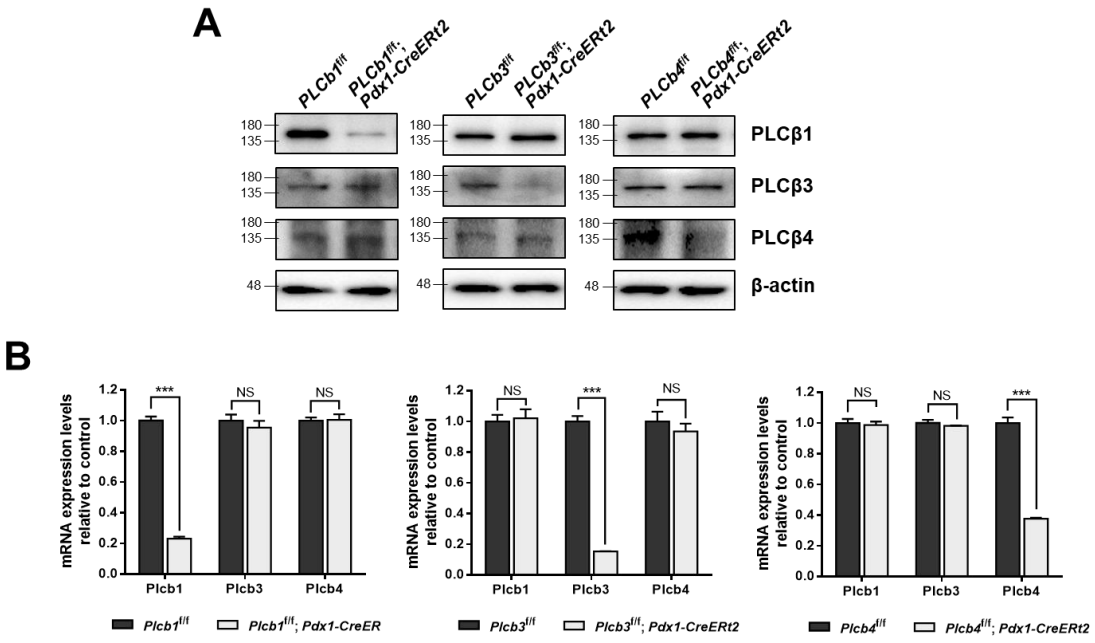

Figure 2.14. PLCβ isozyme expression in each PLCβ isozyme conditional knockout islets

(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 ± SEM. ***$p < 0.001$.

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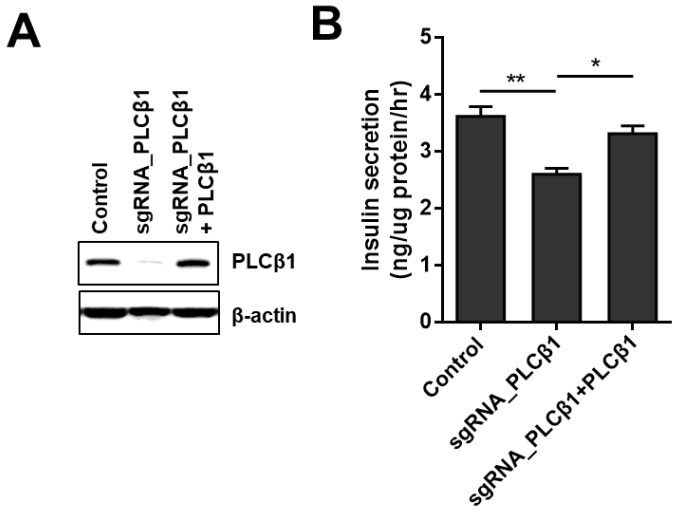

Figure 2.15. Reconstitution of PLCβ1 expression to rescue cells from abnormal insulin secretion

(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 ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$.

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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 Ca^{2+} channels. An increase in the intracellular calcium ($[Ca^{2+}]_i$) level induces exocytosis of insulin vesicles by a Ca^{2+}-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 $[Ca^{2+}]_i$ levels in pancreatic β-cells. Among montelukast, pranlukast, and zafirlukast, only zafirlukast treatment induced increases in insulin secretion and $[Ca^{2+}]_i$ in MIN6 cells, triggering phosphorylation of Ca^{2+}/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 $[Ca^{2+}]_i$ 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 Ca^{2+} channel (L-VDCC) antagonist, suggesting that L-VDCCs are involved in the

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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

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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 Ca^{2+}

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 μM) in KRB for 1 h. The coverslips were then washed three times and inserted into a microscope holder in contact with 500 μL of buffer at 37°C. The chemicals (50 μM zafirlukast, montelukast, or pranlukast; 1 μM nifedipine; 1 μM 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 Bicoll solution (Biochrom) and handpicked under a dissection microscope. Islets were cultured in RPMI-1640 medium in a CO_2 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

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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 μM 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 μM 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 μM 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

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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 μM, 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

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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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ levels. The increase in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ levels.

Zafirlukast-dependent increase in $[Ca^{2+}]_i$ induces activation of key molecular events

The stimulation of pancreatic β-cells by zafirlukast induces an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ 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 Ca^{2+} channels

We next determined whether the increase in $[Ca^{2+}]_i$ was derived from an extracellular or intracellular source of Ca^{2+}. In the absence of extracellular Ca^{2+} (Ca^{2+}-free KRB medium), the zafirlukast-stimulated $[Ca^{2+}]_i$ levels were reduced compared with those observed in Ca^{2+}-containing KRB, although the $[Ca^{2+}]_i$ level was considerably increased via intracellular Ca^{2+} mobilization by thapsigargin (Figure 3.9). Thus, the results strongly suggest that the effect of zafirlukast on insulin

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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 $[Ca^{2+}]_i$ (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 ± 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 ± 0.02 $ng \cdot mL^{-1}$ for vehicle and zafirlukast, respectively) and 120 min (0.06 ± 0.003 and 0.16 ± 0.02 $ng \cdot mL^{-1}$ 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 ± 7.9% reduction, P = 0.01 for 90 min and 46.7 ± 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

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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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ 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

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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 $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} sources, indicating that zafirlukast primarily promoted the increase in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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.

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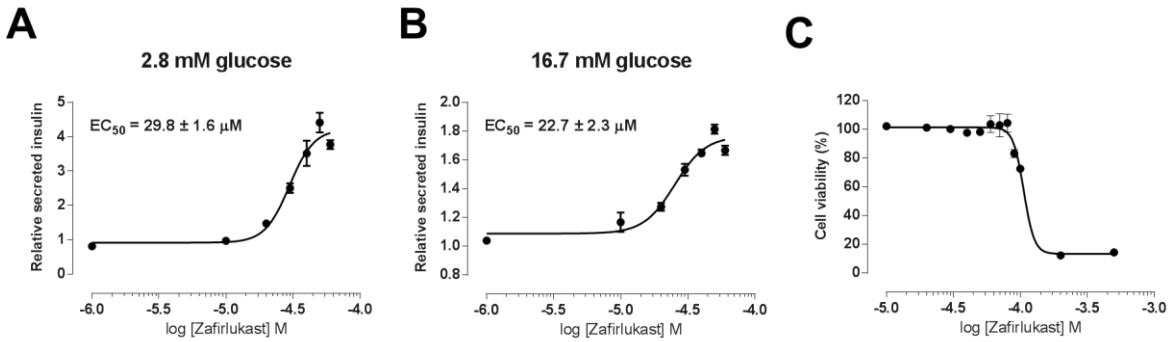

Figure 3.1. Zafirlukast-dependent potentiation of insulin secretion in MIN6 cells.

(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).

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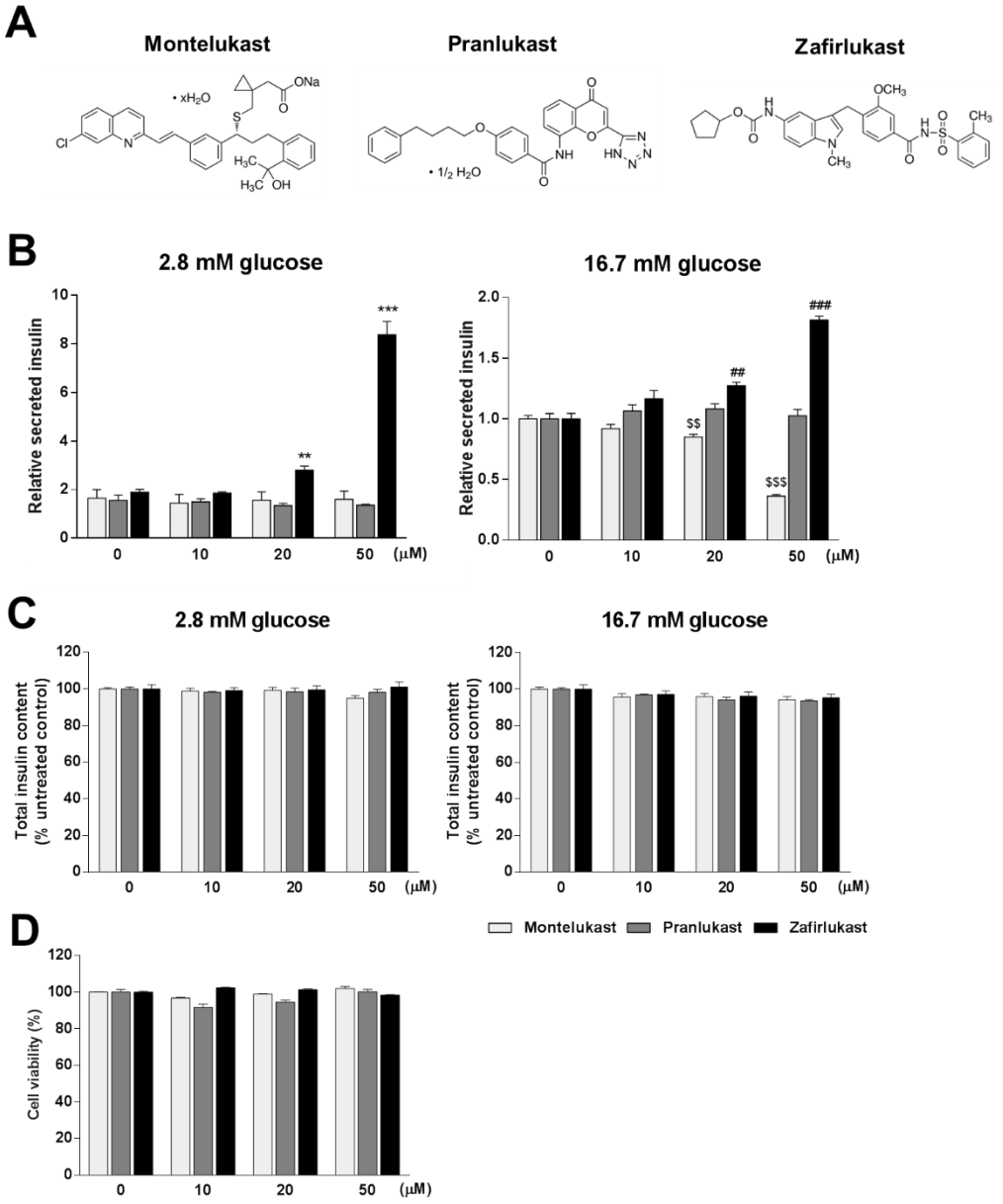

Figure 3.2. Distinct effect of zafirlukast on insulin secretion among cysteinyl leukotriene receptor 1 (CysLTR1) antagonists.

(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).

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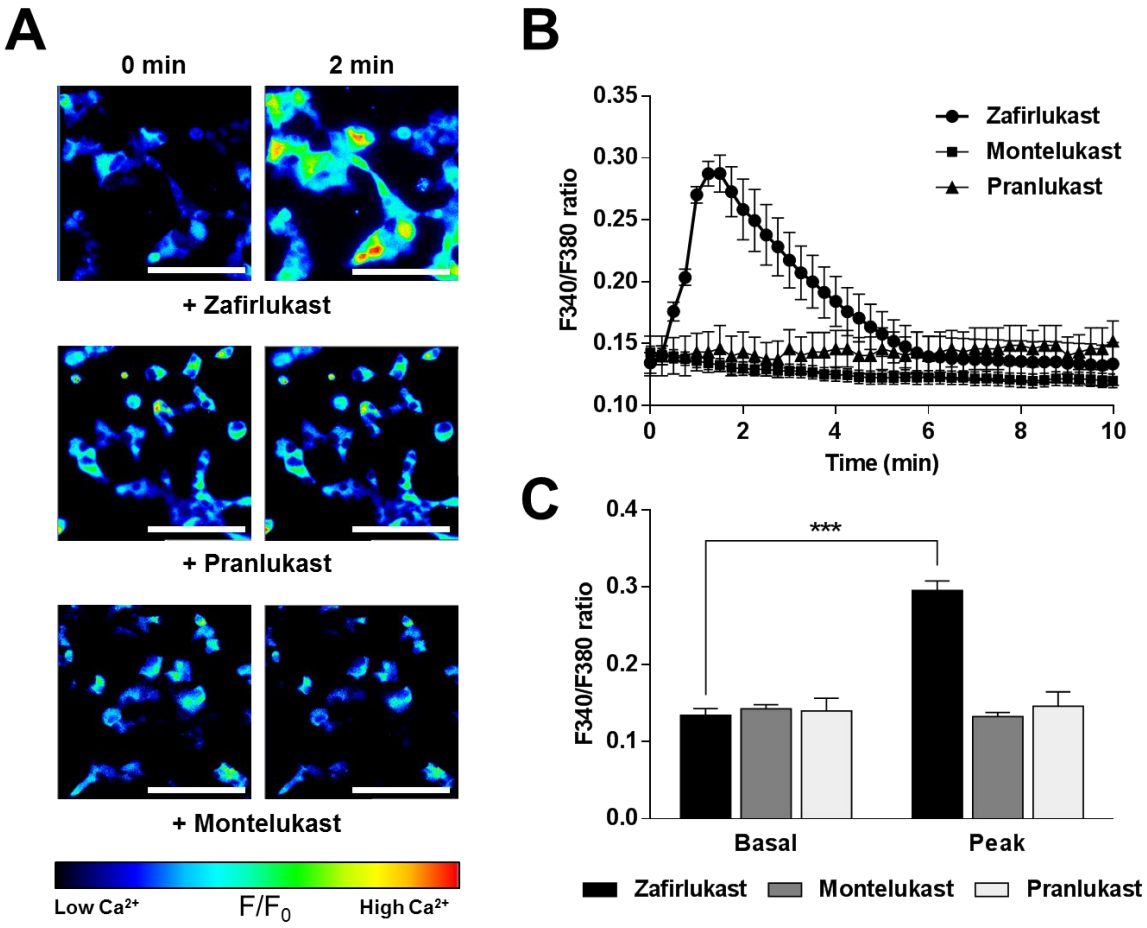

Figure 3.3. Zafirlukast-induced increase in intracellular calcium ($[Ca^{2+}]_i$) in MIN6 cells.

(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 μm. Error bars represent SEM. ***P < 0.001.

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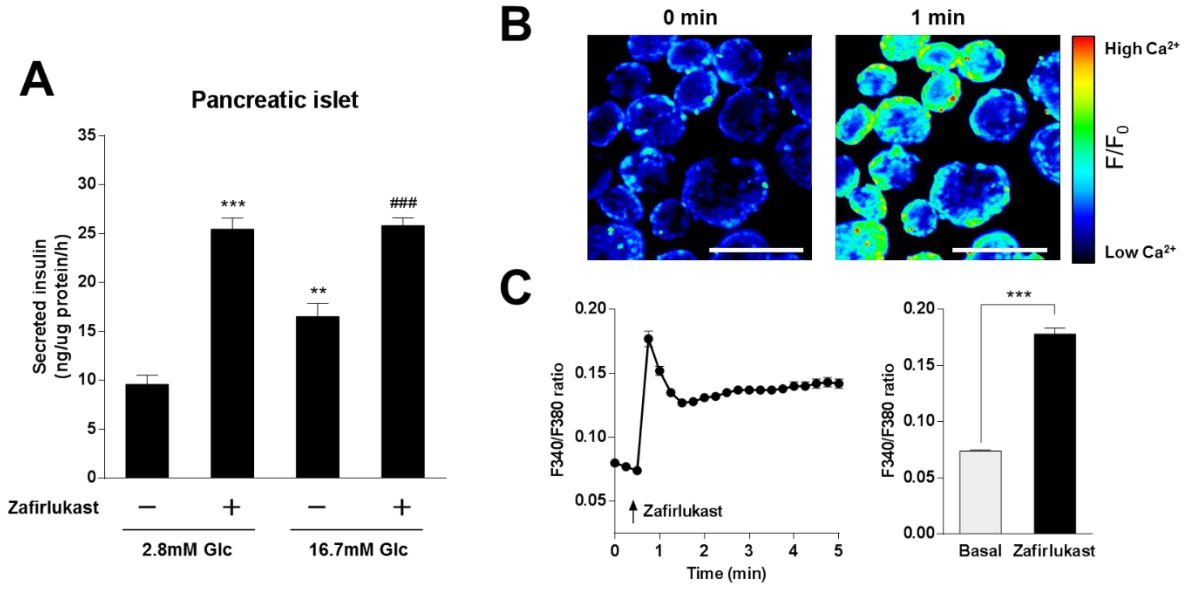

Figure 3.4. Increase in insulin secretion and $[Ca^{2+}]_i$ following stimulation with zafirlukast in primary pancreatic islets.

(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 μm. Error bars represent SEM. ***P < 0.001.

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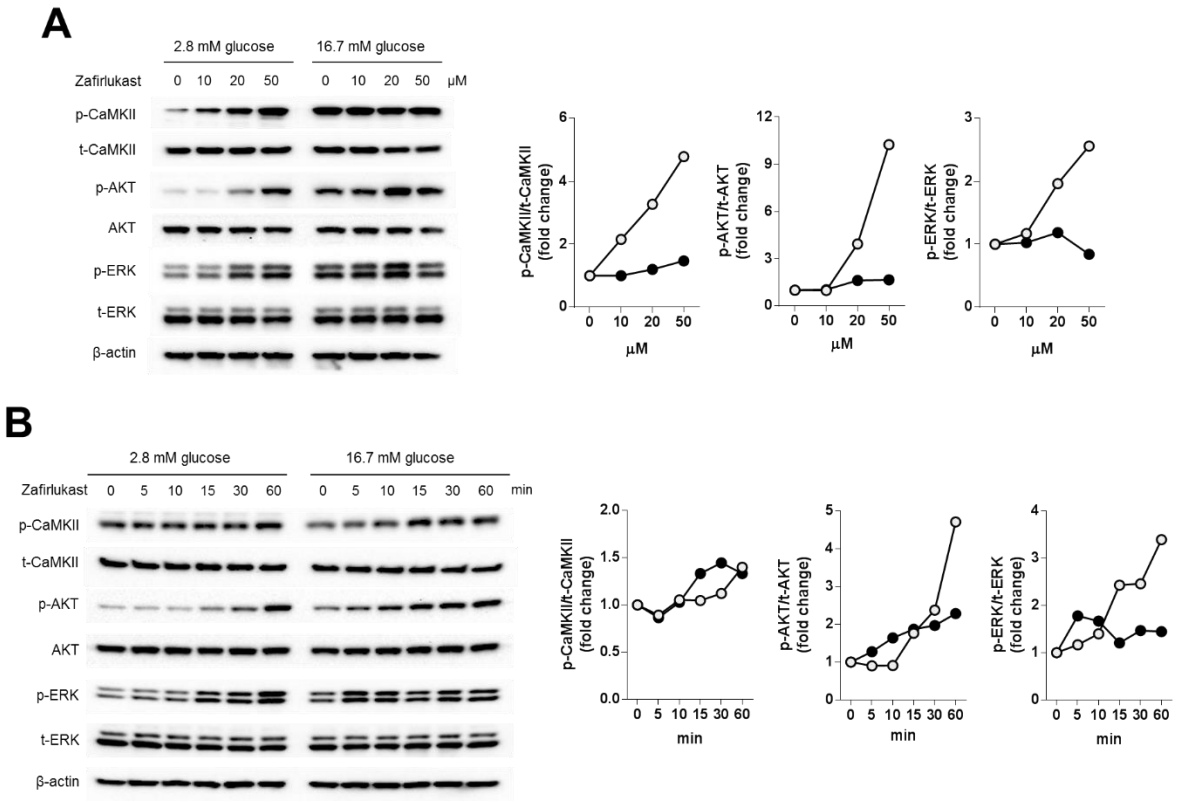

Figure 3.5. Zafirlukast-induced phosphorylation of CaMKII, AKT, and ERK.

(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.

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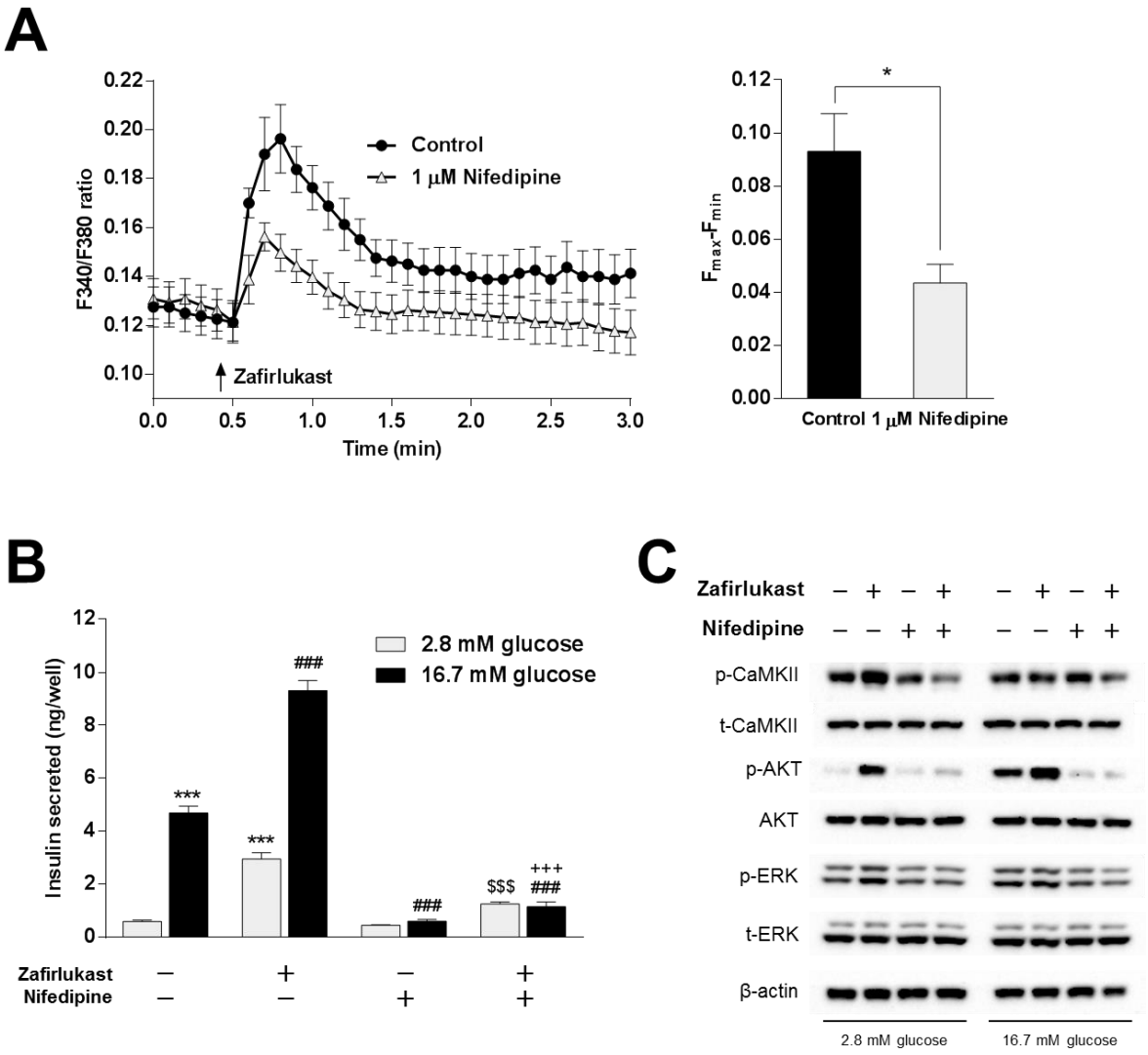

Figure 3.6. Effect of zafirlukast on insulin secretion elicited by extracellular Ca^{2+} influx through L-type voltage dependent calcium channels (L-VDCCs).

(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 μM 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.

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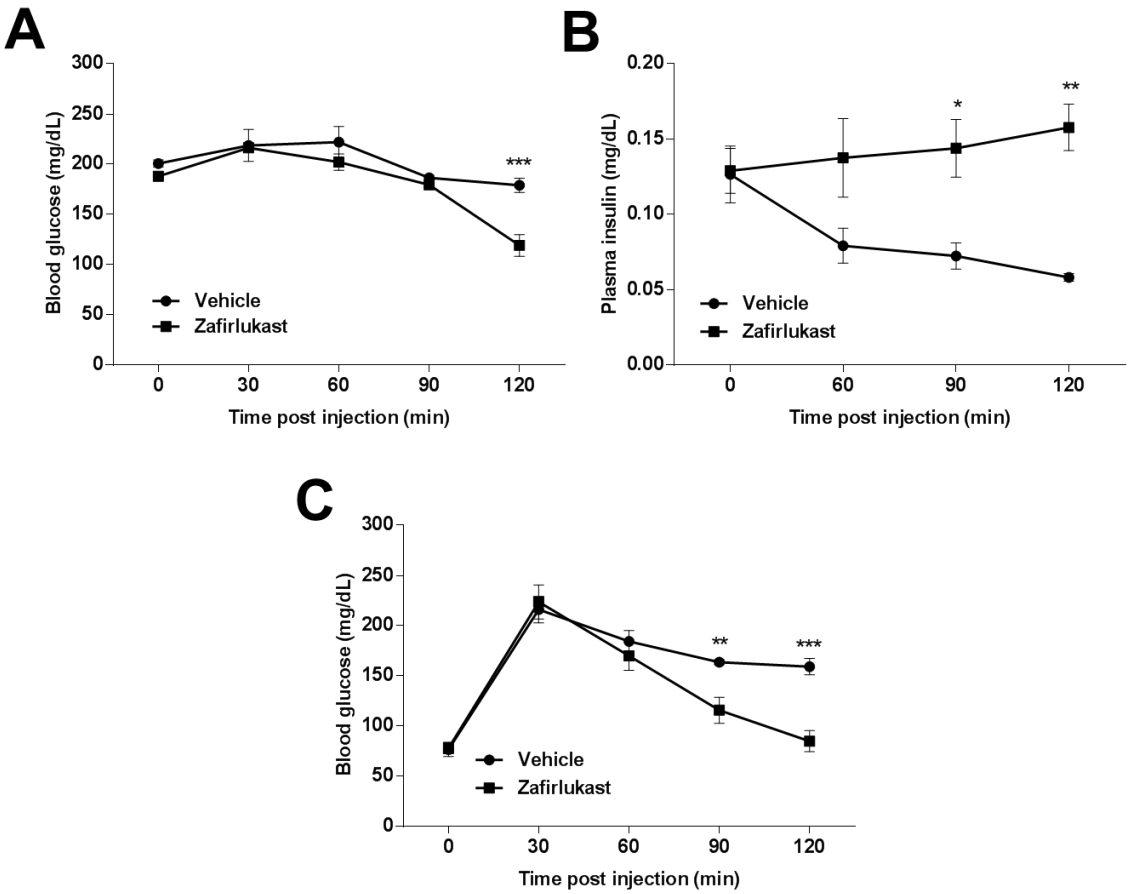

Figure 3.7. Glucose-lowering effect of zafirlukast as a result of increased insulin secretion *in vivo*.

(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.

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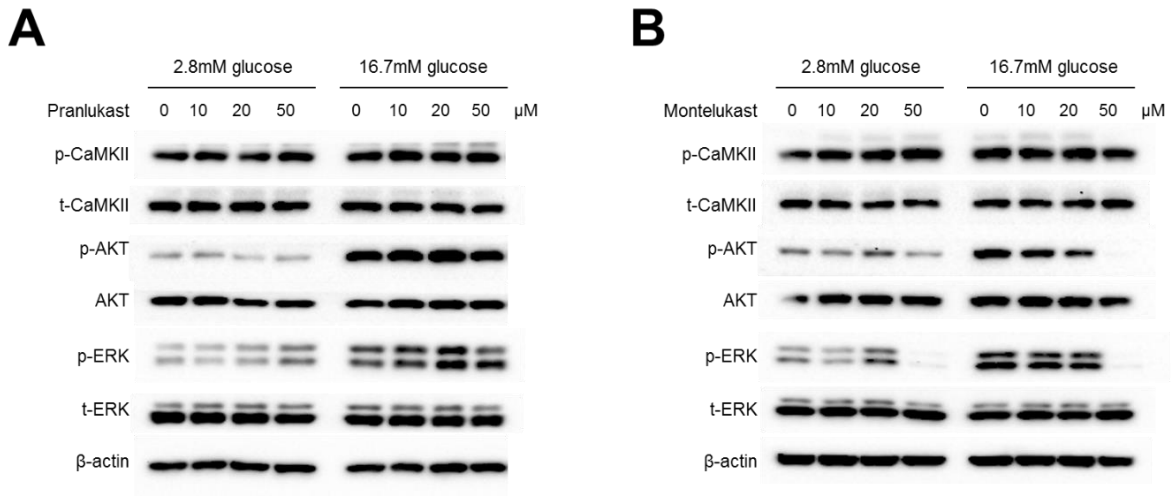

Figure 3.8. Pranlukast- and montelukast-induced phosphorylation of CaMKII, AKT, and ERK.

(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.

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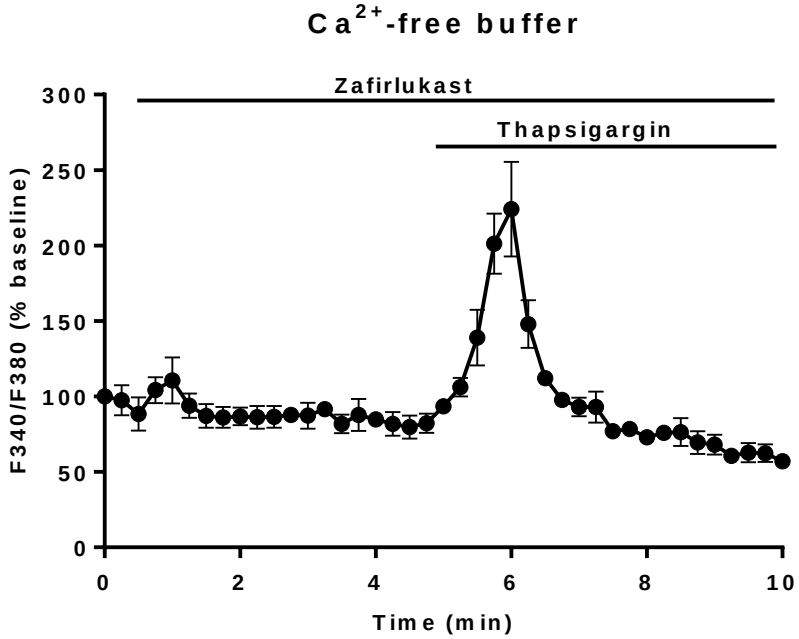

Figure 3.9. Effect of zafirlukast on $[Ca^{2+}]_i$ in Ca^{2+}-free Kreb's buffer (KRB) medium.

(A) Changes in the F340/F80 nm ratio at different time points in Ca^{2+}-free KRB medium (n = 6). The Fura-2AM-loaded MIN6 cells were incubated in Ca^{2+}-free KRB and stimulated with 50 μM zafirlukast and 1 μM thapsigargin at the indicated times.

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3.5. References

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Chapter IV

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