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ROLE OF INOSITOL PYROPHOSPHATES IN PANCREATIC BETA CELL FUNCTION

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Role of inositol pyrophosphates in pancreatic beta cell function

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By

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To my family

ABSTRACT

Inositol pyrophosphates are high energy diphosphate containing molecules that are ubiquitous in eukaryotic cells. They have been implicated in diverse cellular processes ranging from DNA repair, telomere length regulation, ribosome synthesis, cell cycle regulation and apoptosis to osmoregulation, phosphate homeostasis, insulin sensitivity, vesicle trafficking, cytoskeletal rearrangement and exocytosis. The inositol pyrophosphate diphosphoinositol pentakisphosphate (IP_7) is present in high levels in pancreatic β-cells. These cells secrete insulin to regulate blood glucose homeostasis. Previous work has shown that IP_7 is important for maintaining the immediate exocytotic capacity of β-cells and thus the potential to secrete insulin. However, the physiological regulation and role of IP_7 in these cells, especially in response to glucose, remained unexplored. The aims of this Ph.D. work were to investigate the dependence of IP_7 on cellular bioenergetic status, the consequent action of IP_7 in glucose-induced insulin secretion and IP_7's broader role in cellular regulation.

We initially discovered the dependence of IP_7 on the cellular ATP/ADP levels in insulin secreting HIT-T15 cells. Off-target reduction in ATP/ADP, upon use of a selection of signal transduction inhibitors, decreased IP_7 levels. The compounds tested included inhibitors of phosphatidylinositol 3-kinase, PI3K, (wortmannin, LY294002), phosphatidylinositol 4-kinase, PI4K, (Phenylarsine Oxide, PAO), phospholipase C, PLC, (U73122) and the insulin receptor (HNMPA). We demonstrated for the first time a direct positive correlation between intracellular changes in endogenous ATP/ADP and IP_7, pinpointing the regulation of IP_7 by the cellular bioenergetic status. This is in agreement with the enzymatic properties of the inositol hexakisphosphate kinases (IP6Ks) that synthesize IP_7. Their high K_m for ATP makes IP6Ks sensitive to ATP changes. We have also revealed that some inhibitors (PAO, U73122 and LY294002) directly inhibit IP6Ks.

We then investigated how physiological changes in ATP/ADP regulate IP_7 production in β-cells. Glucose stimulation induced a transient increase in IP_7 levels in insulin secreting cell lines and primary islets. Other secretagogues known to increase ATP/ADP, e.g. leucine, also increased IP_7 levels. Silencing IP6K1, but not IP6K2, decreased glucose-mediated IP_7 production and first phase insulin secretion. Therefore, IP6K1 acts as a key metabolic sensor. In diabetic ob/ob mouse islets the deranged ATP/ADP levels were mirrored by perturbed IP_7 production and insulin secretion. Altogether these studies show that metabolic changes in the β-cells are reflected in IP_7 levels, which consequently affect exocytosis under both physiological and pathophysiological conditions.

IP_7 inhibition of Akt/PKB had been described in insulin-sensitive tissues, such as liver, muscle and white fat. β-cells are also regulated by insulin. Therefore, we examined the role of IP_7 in modulating the activity of Akt/PKB. To our surprise, the increase in IP_7 generated by IP6K1 in glucose-stimulated β-cells was associated with higher Akt/PKB phosphorylation on the T308 and S473 sites. This indicates that IP_7 activates Akt/PKB. The results cannot be explained by a direct effect of IP_7 on Akt/PKB, because of the inhibitory nature of this interaction. Instead, we propose that Akt/PKB is indirectly activated by IP_7 through the IP_7-induced increase of insulin secretion and the consequent potentiation of the insulin feedback signaling on β-cell insulin receptors.

In conclusion, the dependence of IP_7 on cellular bioenergetics status suggests that IP6K1, i.e. the kinase that produces IP_7 under glucose stimulation, is a new metabolic sensor in β-cells and is a likely hostage to the disrupted metabolism of type-2 diabetes. The work on Akt/PKB also exposes the complexity of inositol pyrophosphate signaling in different biological settings. Collectively our new findings have considerably broadened the understanding of IP_7 regulation and function in β-cells and islets under both physiological and diabetic conditions.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles, which in the text will be referred by their Roman numerals

I. **Protein kinase- and lipase inhibitors of inositide metabolism deplete IP_7 indirectly in pancreatic β-cells: Off-target effects on cellular bioenergetics and direct effects on IP6K activity**
Subu Surendran Rajasekaran[*], Christopher Illies[*], Stephen B. Shears, Huanchen Wang, Thais S. Ayala, Joilson O. Martins, Elisabetta Daré, Per-Olof Berggren[*], Christopher J. Barker[*].
Cellular Signalling 2018 Jan;42:127-133.

II. **Inositol hexakisphosphate kinase 1 is a metabolic sensor in pancreatic β-cells**
Subu Surendran Rajasekaran, Jaeyoon Kim, Gian-Carlo Gaboardi, Jesper Gromada, Stephen B. Shears, Karen Tiago dos Santos, Eduardo Lima Nolasco, Sabrina de Souza Ferreira, Christopher Illies, Martin Köhler, Chunfang Gu, Sung Ho Ryu, Joilson O. Martins, Elisabetta Daré, Christopher J. Barker[*], Per-Olof Berggren[*].
Cellular Signalling 2018 Jun;46:120-128.

III. **Inositol pyrophosphates and Akt: is the pancreatic β-cell the exception to the rule?**
Jaeyoon Kim, Subu Surendran Rajasekaran, Elisabetta Daré, Per-Olof Berggren[*], Christopher J. Barker[*].
Manuscript

[*]These authors contributed equally

Publication not included in the thesis:

- **One-step purification of functional human and rat pancreatic alpha cells**
  Martin Köhler[*], Elisabetta Daré[*], Muhammed Yusuf Ali, Subu Surendran Rajasekaran, Tilo Moede, Barbara Leibiger, Ingo B. Leibiger, Annika Tibell, Lisa Juntti-Berggren, Per-Olof Berggren.
  Integrative Biology 2012 Feb;4(2):209-219.

[*]These authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
Akt/PKB	Akt/protein kinase B
ATP	Adenosine triphosphate
$[Ca^{2+}]_i$	Cytoplasmic free Ca^{2+} concentration
DIPP	Diphosphoinositol polyphosphate phosphohydrolase
FBS	Fetal bovine serum
HNMPA-$(AM)_3$	Hydroxy-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor 1
IP_3	Inositol 1,4,5-trisphosphate
IP_6	Inositol hexakisphosphate
IP6K	Inositol hexakisphosphate kinase
IP_7	Diphosphoinositol pentakisphosphate
IP_8	Bisdiphosphoinositol tetrakisphosphate
IRS	Insulin receptor substrate
K_{ATP} channel	ATP-sensitive potassium channel
KO	Knockout
mTORC	Mammalian target of rapamycin complex
PAO	Phenylarsine oxide
PFA	Paraformaldehyde
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PI4K	Phosphatidylinositol 4-kinase
PI4P	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPIP5K	Diphosphoinositol pentakisphosphate kinase
SNARE	Soluble NSF (N-ethylmaleimide-sensitive factor)-attachment protein receptor
T1D	Type-1 diabetes
T2D	Type-2 diabetes
TBB	4, 5, 6, 7-tetrabromobenzotriazole
TCA	Trichloroacetic acid
TNP	N^2-(m-Trifluorobenzyl), N^6-(p-nitrobenzyl)purine

1 INTRODUCTION

Inositol pyrophosphates are molecules belonging to the family of water soluble inositol derivatives, namely the inositol phosphates. They are evolutionarily conserved from yeast to mammals and are involved in virtually every aspect of cell biology, from telomere length regulation and cell growth to vesicle trafficking and exocytosis [1-3]. Inositol pyrophosphates possess 'high energy' diphosphates and contain nature's most crowded three-dimensional array of phosphate molecules [4], up to 8 phosphates on an inositol ring. This thesis focuses on the specific contribution of inositol pyrophosphates in regulating the function of β-cells, a key component of the endocrine system maintaining glucose homeostasis. The following sections will review the physiological role of β-cells and the consequences of their malfunction. This will be followed by a detailed consideration of the inositol pyrophosphates and how they might influence β-cell specific cellular processes.

1.1 GLUCOSE HOMEOSTASIS AND ITS DERANGEMENT IN DIABETES

Maintaining homeostasis of the blood sugar level is important for the normal functioning of all cells in the body. This is highly regulated by hormones and other peptides that are secreted by a complex network involving pancreas, brain, liver, intestine and adipose tissue. The islets of Langerhans, which constitute the endocrine pancreas, play a central role by secreting key anabolic (insulin) and catabolic (glucagon) hormones. Secreted insulin lowers blood glucose levels via increasing systemic glucose uptake by muscle, fat and liver, whereas the counteracting action of glucagon increases blood sugar levels by triggering glucose mobilization from the liver. Impaired secretion of these hormones is central for the development of metabolic disorders such as diabetes mellitus, simply known as diabetes [5]. Diabetes is characterized by elevated blood glucose levels. These high glucose levels reflect the body's inability to produce sufficient insulin or to utilize insulin effectively [6, 7]. Human lifestyle and behavioral changes over the past century have led to a striking increase in the occurrence of this disease and its serious complications [8]. This growing epidemic affects over 400 million people worldwide. According to the International Diabetes Federation, 10% of the world population will be diabetic by 2040 [7]. Apart from the devastating health problems that patients undergo, managing the impact of diabetes is a big toll on a country's economy.

Diabetes is broadly classified into two types: type-1 diabetes (T1D) and type-2 diabetes (T2D). T1D, previously known as juvenile diabetes or insulin dependent diabetes, is caused by an autoimmune destruction of pancreatic β-cells resulting in very low or no insulin

production. About 5% of the diabetes patients account for T1D [9]. The most common form in humans, comprising over 90% cases, is T2D [8, 10]. It is caused by heterogeneous factors, both environmental and genetic, that impair pancreatic β-cell function and/or peripheral tissue insulin sensitivity, resulting in the body's ineffective production and/or utilization of insulin, respectively [6, 7, 10, 11]. Furthermore, T2D is also associated with defective glucagon and somatostatin secretion [11, 12]. All these important hormones are secreted by the pancreatic islets. This highlights the importance of these micro-organs in the disease development.

1.2 THE PANCREATIC ISLETS

The pancreas itself is a vital glandular organ of the alimentary tract located posterior to the bottom part of the stomach in the upper left abdomen. It has both exocrine and endocrine function to facilitate nutrient digestion and blood glucose homeostasis, respectively. The majority (98%) of the pancreas is composed of exocrine cells that secrete enzymes to aid digestion. The remaining 1-2% of the pancreas consists of clusters of endocrine cells, which are the pancreatic islets. There are at least five different cell types in the islet: glucagon secreting α-cells, insulin secreting β-cells, somatostatin secreting δ-cells, pancreatic polypeptide secreting PP-cells and ghrelin secreting ε-cells [5]. Much of the understanding of diabetes and islet cell function has come from rodent models. However, there are differences in the cytoarchitecture and cellular composition of islets between rodents and humans. The main difference in islet structure is that in rodent islets β-cells form a core surrounded by the other islet cells, in contrast in the human islet the different cell types are interdispersed [13]. The percentage of these different cells per islet in the human and mouse are as follows: β-cells (50% in human and 75% in mice), α-cells (35-40% in humans and 15-20% in mice), δ-cells (10-15% in human and ~ 5% in the mouse) [14]. Although all these cells participate in the regulation of glucose homeostasis, insulin secreting β-cells play the central role.

1.3 REGULATION OF INSULIN SECRETION IN PANCREATIC BETA CELLS

After a meal, the β-cell responds to increases in blood glucose levels by secreting appropriate amounts of insulin to maintain blood glucose within normal limits. There is a quick response, which is aided by a network of capillaries enabling the islet to receive 10 times more blood compared to the surrounding exocrine part of the pancreas. This unique architecture enables the β-cells to rapidly sense the change in blood glucose levels [15]. In addition to the prime stimulus glucose, insulin release is also mediated by other factors such as nutrients (monosaccharides, amino acids, fatty acids and vitamins), incretins, neurotransmitters, paracrine and autocrine signals [11, 15].

1.3.1 Stimulus-secretion coupling

Several decades of research on β-cell physiology have shed light on the stimulus-secretion coupling mechanisms of insulin secretion. Increases in blood glucose concentration are sensed by a complex metabolic pathway. This starts with the uptake of glucose into the β-cell via dedicated high capacity plasma membrane glucose transporters. GLUT 2 and GLUT 1 are the primary glucose transporters in rodents and humans, respectively. Since glucose readily enters the β-cell via facilitated diffusion, glucose transport is not the rate limiting step of glucose metabolism. The next step is the formation of glucose-6-phosphate by glucokinase. This enzyme has a high K_m for glucose and determines the flux of glycolysis. Thus glucokinase is considered to be the most important glucose sensor in β-cells [14-16]. Phosphorylated glucose is metabolized through glycolysis to form ATP, NADH and pyruvate. Pyruvate is further oxidized in the mitochondria by tricarboxylic acid cycle and oxidative phosphorylation to produce more ATP molecules.

Altogether, glucose metabolism through glycolysis and mitochondrial respiration results in the increase in cellular ATP/ADP levels [16]. This increase leads to the closure of ATP-sensitive potassium channels (K_{ATP} channels). These channels thus act as important metabolic sensors in stimulus secretion coupling. The relative contribution of the ATP obtained through glycolysis versus oxidative phosphorylation in regulating K_{ATP} channel remains under debate [16-19]. The closure of K_{ATP} channels results in the depolarization of the plasma membrane and increases Ca^{2+} influx through the opening of voltage gated L-type Ca^{2+} channels (Ca_v1 [20, 21]). The increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is the primary trigger for insulin secretion [22] (Figure 1).

There are also other glucose generated signals which act independently of the K_{ATP} channels. Although this kind of signaling does not increase $[Ca^{2+}]_i$ *per se* it augments insulin secretion when $[Ca^{2+}]_i$ is kept high through other modes, such as through high potassium stimulation. Thus both K_{ATP}- dependent and independent exocytosis occurs in the presence of increased $[Ca^{2+}]_i$ [23]. Nonetheless, there are instances where glucose can augment insulin secretion when the increase of $[Ca^{2+}]_i$ is prevented. These mechanisms work only when there is maximal activation of protein kinase A (PKA) and protein kinase C (PKC). However, when extracellular Ca^{2+} is completely absent the amount of glucose-induced insulin release is only a minor fraction compared to insulin secretion in the presence of physiological Ca^{2+}. Thus, although glucose-mediated insulin release may involve $[Ca^{2+}]_i$ independent steps, the physiological regulation by glucose is mostly accomplished through $[Ca^{2+}]_i$ dependent

pathways. This places $[Ca^{2+}]_i$ as an inevitable requirement for proper insulin secretion [22, 23].

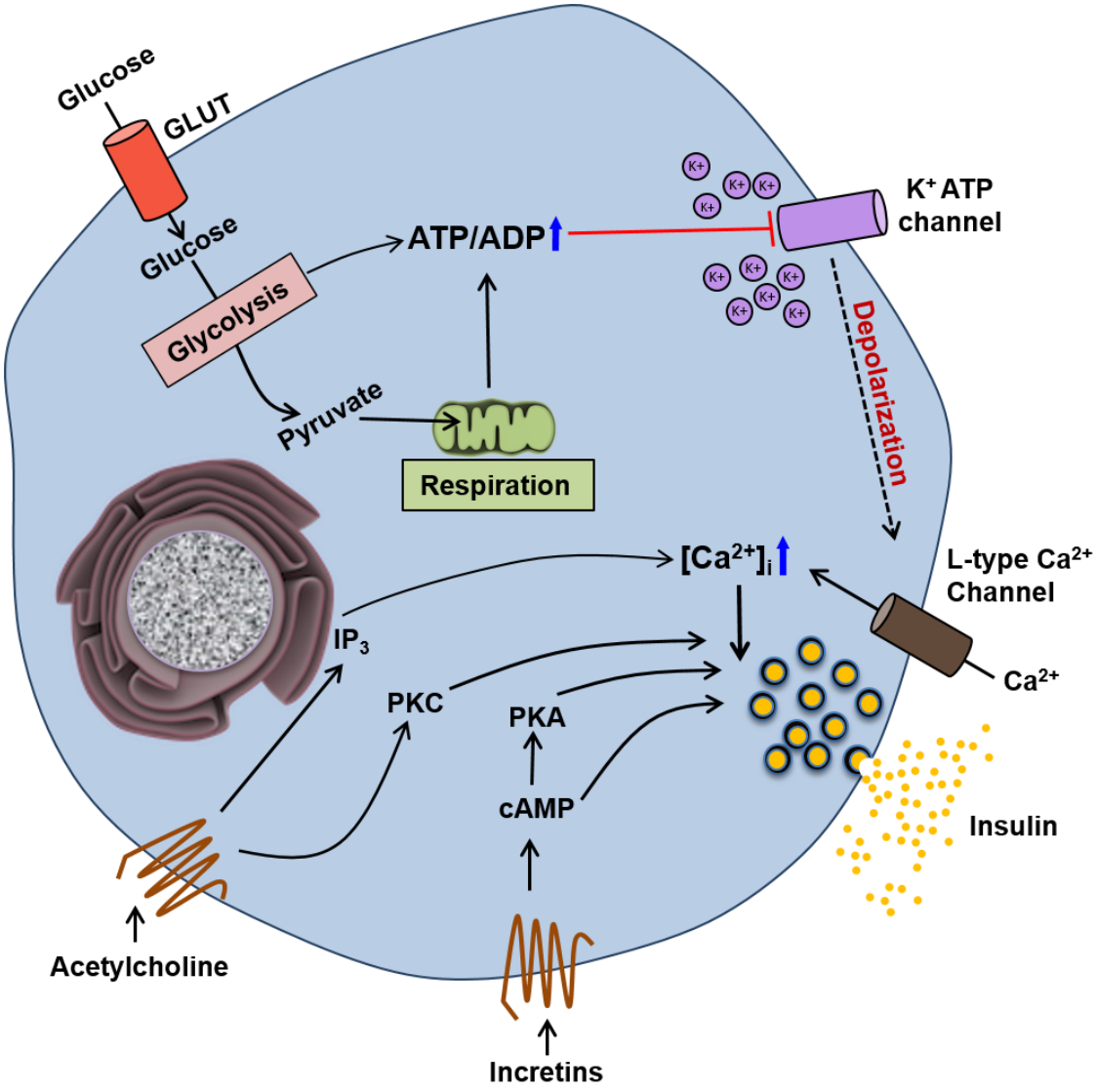

Figure 1. Schematic representation of the main mechanisms that mediate insulin secretion in pancreatic β-cells.

In addition to glucose there are also other G-protein-dependent pathways that increase $[Ca^{2+}]_i$ to amplify insulin secretion [15, 24]. One such pathway, activated by acetylcholine, drives an increase in inositol 1,4,5-trisphosphate (IP_3) levels that mobilize Ca^{2+} from intracellular endoplasmic reticulum stores [22, 25-27]. Interestingly, the IP_3 metabolite inositol hexakisphosphate (IP_6) also increases $[Ca^{2+}]_i$ by activating the voltage dependent L-type Ca^{2+}

channels [28]. This illustrates that inositol phosphates can influence two major pathways mediating increases in $[Ca^{2+}]_i$.

Another very important G-protein-dependent amplifying pathway is driven by incretins such as glucagon-like peptide-1 and gastric inhibitory polypeptide. They are secreted from the enteroendocrine cells in the intestinal wall upon nutrient ingestion and mediate increases in cAMP, which in conjunction with glucose-stimulation augment insulin release [15, 26, 29]. This type of signal-derived increase in insulin secretion occurs only when glucose is present.

1.3.1.1 Biphasic insulin exocytosis

The glucose-mediated increase in insulin secretion is biphasic. The first phase is rapid, transient and occurs during the first 5-10 min of stimulation followed by a sustained second phase that can even last for hours if the blood glucose remains elevated. The sustained release is achieved by the large excess number of insulin containing granules that are present in the β-cells [23, 30]. The biphasic pattern of insulin release is proposed to be a consequence of the granules existing in physically or functionally distinct pools [16, 23, 31].

The first phase of insulin secretion is thought to be mediated by the readily releasable pool (RRP) of granules consisting of 1-2% of the total granules, whereas the second sustained phase draws upon the reserve pool which consists of the remaining granular pools in the cell. The original studies carried out using electrophysiological techniques and electron microscopy identified the RRP as granules that are primed, pre-docked at the plasma membrane and then rapidly fuse to release insulin upon a stimulus [15, 31]. More recent models using total internal reflection fluorescence microscopy have indicated that under glucose stimulation the first phase secretion may be formed dynamically from what was previously called the reserve pool [32]. This new model is still contentious, however.

1.3.1.2 Vesicle fusion

The final process of the granule membrane fusion is facilitated by several proteins. Among them a group of proteins forming the soluble NSF (N-ethylmaleimide-sensitive factor)-attachment protein receptor (SNARE) complex play an essential role in the fusion process. The SNARE complex is formed by the association of synaptosomal-associated protein 25 (SNAP-25) and accessory protein syntaxin which are in the plasma membrane with synaptobrevin/VAMP-2 that is present in the secretory vesicle membrane. Moreover, the SNARE proteins interact with the voltage dependent Ca^{2+} channels, thus allowing increases in $[Ca^{2+}]_i$ in close proximity to the machinery driving the final vesicle fusion events [15, 16, 31, 33].

1.3.1.3 Defects in the stimulus secretion coupling in diabetes

Under T2D, the insulin secretion is deranged and compromised. During the early disease progression, the basal serum insulin levels are increased [34, 35]. Upon glucose stimulation, the β-cell is unable to further respond with proper first phase secretion. Loss of first phase insulin secretion is one of the early hallmarks of T2D [36-38]. As the disease progresses the second phase insulin secretion is also compromised, resulting in full blown diabetes [10, 34]. In addition to the increased basal insulin levels, the basal metabolism is also altered in diabetic islets. It is characterized by high basal ATP levels and a compromised glucose-stimulated ATP/ADP generation [39-41]. Furthermore, increasing evidence from genetic studies indicates that defects in the secretory machinery are important in disease development [42, 43]. This highlights that defects in insulin release are central to diabetes.

1.4 INSULIN FEEDBACK SIGNALING

Insulin released from the β-cell feeds back in an autocrine fashion to initiate a secondary wave of signaling. This signal positively regulates a number of β-cell functions such as transcription, translation, ion flux, insulin exocytosis, cell survival and proliferation [44]. The first step in the insulin feedback loop is the binding of the released insulin to insulin receptors. In addition, insulin also binds to insulin-like growth factor 1 (IGF-1) receptors and can exert its effect through them [45]. It was first reported in the 1990s that glucose-induced insulin release triggers the β-cell insulin receptor and activates its downstream effectors, such as insulin receptor substrate (IRS) and phosphatidylinositol 3-kinase (PI3K) [46, 47]. However, these studies did not show if the impact of this autocrine insulin feedback led to a positive or negative influence on insulin secretion. In the late 1990s it was shown experimentally that autocrine feedback of insulin enhances the on-going insulin secretion, most likely through an insulin mediated increase in $[Ca^{2+}]_i$ level [45, 48, 49]. The role of insulin feedback in secretion was also supported by animal models with β-cell specific knockout (KO) of the insulin [50] and IGF-1 receptors [51, 52], global KO of IRS1 [52] and islet cell specific KO of IRS2 [53]. These mice exhibited decreased insulin secretion which resulted in impaired glucose tolerance. However, there are other studies showing that insulin feedback had either a negative impact or no effect on insulin secretion [45]. The difference in the effect of insulin on insulin exocytosis could be due to the differences in experimental set ups, the time or concentration at which the cells were stimulated with exogenous insulin and the presence of other factors in the extracellular environment, particularly glucose [44, 45].

The most widely studied insulin mediated signaling pathway is based on PI3K activation, both in β-cells and other peripheral cells. This kinase phosphorylates the 3 hydroxyl position

of phosphatidylinositol 4-phosphate (PI4P) or phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the formation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), respectively. These lipids then activate the downstream Akt/protein kinase B (PKB) signaling [54]. The following section will briefly touch upon the actions of Akt/PKB.

1.4.1 Akt/PKB signaling

PI3K/Akt/PKB signaling is ubiquitous in almost all cells in an organism [54, 55]. It is the major growth, survival and anti-apoptotic signal in the body [56, 57]. Increases in PIP_3 production, induced by growth factors such as insulin, recruit pleckstrin homology (PH) containing Akt/PKB to the plasma membrane. Akt/PKB is then activated by phosphorylation at two positions, threonine 308 (T308) and serine 473 (S473). Phosphorylation of both the sites is necessary for maximal activation. The threonine residue is phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) and the serine residue is primarily phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) [54, 58].

Activated Akt/PKB then phosphorylates many substrates that belong to different functional classes of proteins, to exert a wide repertoire of cellular events. Key protein classes activated include protein and lipid kinases, transcription factors, metabolic enzymes and cell cycle regulators. The most important and widely studied Akt/PKB downstream proteins, that have multiple functions, are glycogen synthase kinase 3 (GSK3), forkhead Box O (FoxO) and mTORC1 [54].

In certain insulin sensitive tissues, such as skeletal muscle and fat, Akt/PKB is one of the major mediators of the systemic glucose uptake, whereas in pancreatic islets it regulates β-cell mass and functions such as insulin secretion. Transgenic mice overexpressing β-cell specific Akt/PKB resulted in increased β-cell proliferation, hypertrophy and decreased apoptosis. Furthermore, this overexpression increased insulin secretion resulting in hyperinsulinemia. In addition, mice with reduced β-cell specific Akt/PKB activity displayed defective insulin exocytosis [59]. This suggests a critical role of PI3K/Akt/PKB signaling in the β-cell.

Akt/PKB signaling has also been shown to be negatively regulated by the inositol pyrophosphate diphosphoinositol pentakisphosphate (IP_7) in insulin sensitive tissues [60, 61]. Inositol pyrophosphates, which belong to the water soluble family of inositol phosphates, are built on the structural backbone of inositol. The following section will now consider inositol and inositol based molecules in more detail.

1.5 INOSITOL PHOSPHATES

Inositol is a carbohydrate molecule that has the same molecular formula, $C_6H_{12}O_6$, as glucose, but a different structure. Although inositol exists in nine possible stereoisomers, the most commonly found form in living cells is *myo*-inositol or *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, which has one axial hydroxyl group (at 2-position, perpendicular to the plane of the ring) and five equatorial hydroxyl groups (in the plane of the ring) (Figure 2). *Myo*-inositol (or colloquially called inositol) is biosynthesized in a two-step process from phosphorylated glucose (glucose-6-phosphate). Cells can also directly take up *myo*-inositol from the plasma through sodium ion- and proton coupled inositol transporters [62-64]. This uptake is the main source of *myo*-inositol in β-cells. *Myo*-inositol is then phosphorylated at different positions on the cyclohexane ring substituting a hydroxyl group with an α-phosphate to form inositol phosphates. The most widely studied inositol phosphates are inositol tris-, tetrakis-, pentakis- and hexakisphosphates (IP_3, IP_4, IP_5 and IP_6). In the majority of cells, IP_6 is the most abundant inositol phosphate. It is present in quantities approximately 10-100 times higher than one of the most widely studied intracellular second messengers, IP_3. The concentration of IP_6 varies between 15-60 µM in mammalian cell types [1, 65, 66]. In pancreatic β-cells it exists at about 50 µM [28]. These inositol phosphates play a very important role in cellular signaling. Mathematically, it is possible to build 63 different inositol phosphates [3, 67].

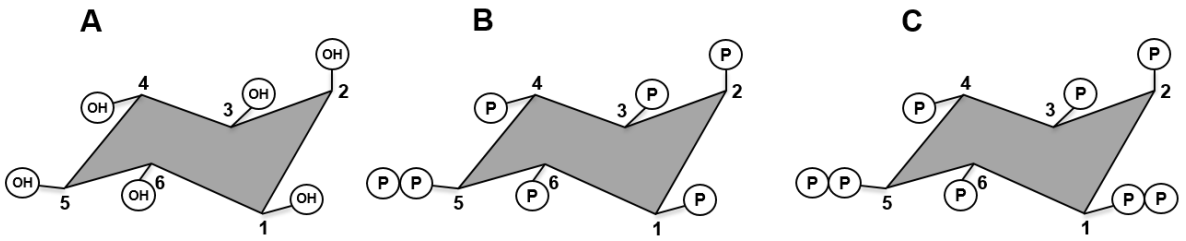

Figure 2. Structure of inositol and inositol pyrophosphates. (A) *myo*-inositol chair conformation (B) diphosphoinositol pentakisphosphate, 5-PP-IP_5/5-IP_7 (C) bisdiphosphoinositol tetrakisphosphate, 1,5-$(PP)_2$-IP_4/IP_8.

1.6 INOSITOL PYROPHOSPHATES

As well as the conventional inositol phosphates, it is possible to form another distinct molecular type by adding a β-phosphate to an already existing α-phosphate on the inositol

ring. The molecules so formed are called inositol pyrophosphates. They can be based on IP_5 or IP_6, but the two best characterized inositol pyrophosphates, diphosphoinositol pentakisphosphate (IP_7) and bisdiphosphoinositol tetrakisphosphate (IP_8), are derived from IP_6 (Figure 2). In simple terms, inositol pyrophosphates are molecules that have 'high energy' diphosphates attached to a given position on the inositol ring [2, 3, 68, 69]. They have a free energy of hydrolysis similar to that of ATP [65]. They are evolutionarily conserved from yeast to mammals [1-3]. This thesis focuses particularly on one of the inositol pyrophosphates, IP_7.

Though researchers had observed more polar products than IP_6 in HPLC traces [66, 67], inositol pyrophosphates were officially discovered in 1990s by laboratories led by Mayr, Stephens and Shears [70-72]. In most eukaryotic cells, inositol phosphate synthesis is initiated by the phospholipase C (PLC) mediated breakdown of PIP_2 into diacylglycerol and IP_3. The latter is then phosphorylated to higher inositol phosphates, including inositol pyrophosphates, by a series of specific enzymes [73-75].

1.6.1 Concentrations and turnover of inositol pyrophosphates in cells

IP_7 concentrations present in yeast and mammalian cells ranges from 0.5 μM to 5 μM [3, 68]. Two isoforms are present, 5-IP_7 and 1-IP_7. In β-cells IP_7 largely consists of the 5-isoform and its levels are maintained at a high concentration, ~ 6 μM [76, 77]. IP_8 has not been studied in β-cells, but in other cell types it was reported to vary from being undetectable to 10-20% of IP_7 levels [68].

Inositol pyrophosphates turn over rapidly. In mammalian cells every hour almost 50% of the IP_6 and 20% of the IP_5 pool are converted into inositol pyrophosphates [71, 78]. IP_7 is approximately 2-10% of IP_6 [68] and turns over substantially each hour. For instance, in hepatocytes, the total IP_7 pool turns around 10 times in 40 minutes [79]. This turnover is dependent on their kinases and phosphatases. It is highly energy demanding to make these inositol pyrophosphates because of the crowded array of phosphates in a small inositol molecule and the strong electronegative charge that they exhibit [2, 4]. In addition, since the steady state turnover of these molecules is high, their cellular levels must be finely controlled and may have important functions in regulating dynamic cellular processes.

1.6.2 Kinases and phosphatases that regulate inositol pyrophosphate levels

As we have seen inositol pyrophosphates are formed by pyrophosphorylating two different parent molecules, IP_5 (the 1,3,4,5,6-IP_5 isoform) and IP_6. An IP_5 derived inositol pyrophosphate is 5-diphosphoinositol (1,3,4,6)-tetrakisphosphate (5-PP-IP_4). IP_6 derived

inositol pyrophosphates are 1-diphosphoinositol (2,3,4,5,6)-pentakisphosphate [1-PP-IP_5 or commonly called 1-IP_7], 5-diphosphoinositol (1,2,3,4,6)-pentakisphosphate [5-PP-IP_5 or commonly called 5-IP_7] and (1,5)-bisdiphosphoinositol (2,3,4,6)-tetrakisphosphate [1,5-$(PP)_2$-IP_4 or commonly known as 1,5-IP_8 or IP_8]. In the case of 5-PP-IP_4, 5-IP_7 and 1,5-IP_8 the reaction is catalyzed by inositol hexakisphosphate kinases (IP6Ks), whereas in the case of 1-IP_7 and 1,5-IP_8 the reaction is catalyzed by diphosphoinositol pentakisphosphate kinases (PPIP5Ks) [3, 66, 68, 80]. Figure 3 illustrates the synthesis of IP_6 derived inositol pyrophosphates. Although both IP_5 and IP_6 serve as parent molecules for inositol pyrophosphate synthesis, IP_6 is preferred as a substrate because of its higher intracellular concentration and the higher affinity of IP6Ks for this inositol phosphate [1, 27].

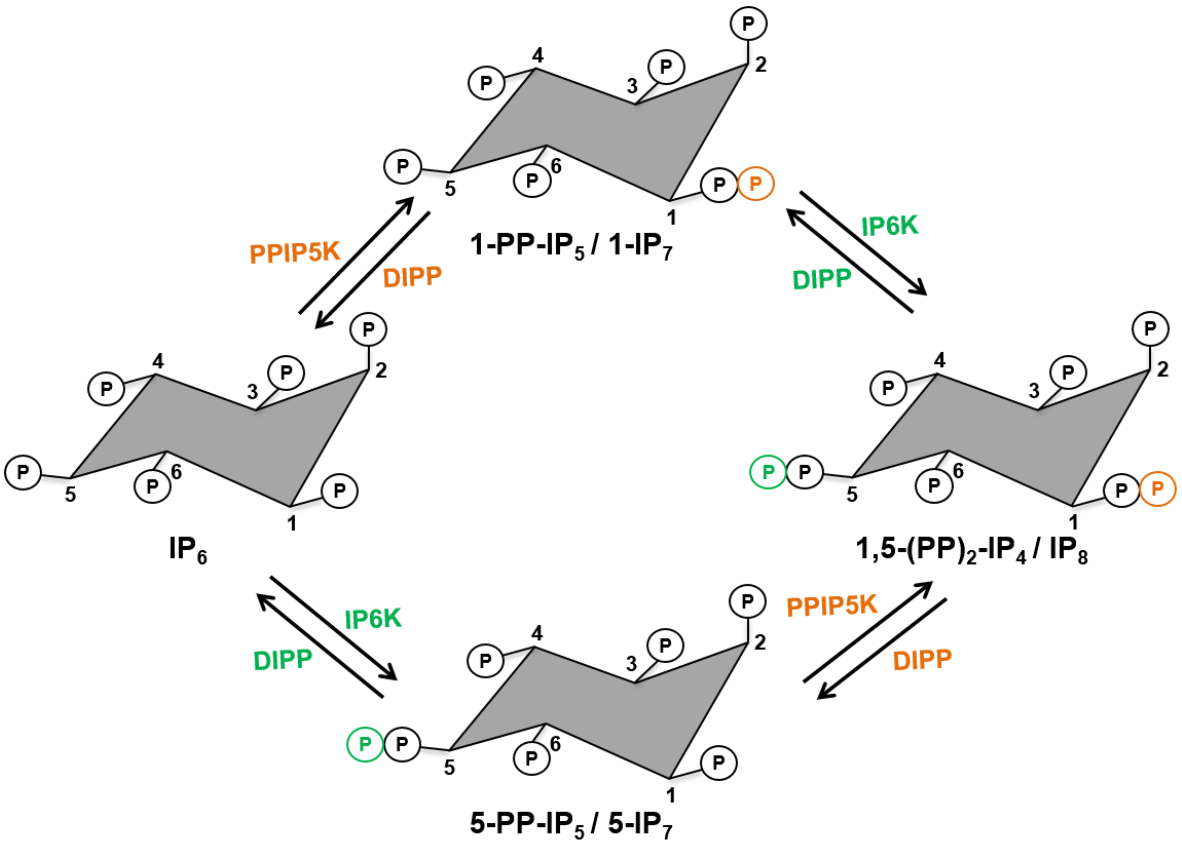

Figure 3. Pathway illustrating the synthesis of IP_6-based inositol pyrophosphates in mammalian cells. IP6K and PPIP5K pyrophosphorylate both IP_6 and IP_7. This is done by adding a β-phosphate (second phosphate) on the inositol ring at either the 5-position (IP6K) or the 1-position (PPIP5K). The β-phosphate is dephosphorylated by diphosphoinositol polyphosphate phosphohydrolase (DIPP).

Mammalian cells express three IP6K isoforms (IP6K1, IP6K2 and IP6K3) and two PPIP5K isoforms (PPIP5K1 and PPIP5K2) [1, 68, 78]. IP6K1 and IP6K2 are present in β-cells, but IP6K3 is absent [76]. The expression of PPIP5Ks in β-cells is not known. IP6Ks and

PPIP5Ks pyrophosphorylate the already existing phosphate on the 5 position and 1 position of IP_6, respectively [4, 27, 80] (Figure 3). Thus 5-IP_7 is formed by IP6Ks. Since it is the dominant form in cells under normal conditions [75], one can assume that IP6Ks act as the physiological IP_7 producing kinases and PPIP5Ks as the IP_8 producing kinases, respectively. This has also been recently confirmed by the laboratories of Shears and Safrany [81]. Inositol pyrophosphates are hydrolyzed by an enzyme called diphosphoinositol polyphosphate phosphohydrolase, DIPP. There are five isoforms of DIPP in the mammalian cell system [75, 78]. DIPPs catalyze the dephosphorylation of the β-phosphate in inositol pyrophosphates [78, 82]. They dephosphorylate IP_8 and IP_7 to IP_7 and IP_6, respectively (Figure 3). The metabolically preferred route for IP_8 synthesis (catalyzed by IP6Ks) is through 5-IP_7 whereas IP_8 dephosphorylation (catalyzed by DIPP) is preferentially through 1-IP_7 (Figure 3) [81]. This thesis focuses on IP_7 and the IP6Ks that produce it in β-cells.

1.6.2.1 Enzymatic properties of IP6Ks

IP6Ks are bi-substrate enzymes using both inositol phosphates and ATP or ADP as substrates [83]. Enzymatically IP6Ks have the potential to act as IP_6 kinases and ATP synthases *in vitro*. For the forward kinase reaction IP6Ks have a K_m value of about 0.43-1.2 µM for IP_6 and 1.1-1.35 mM for ATP [65, 67, 84]. For the reverse ATP synthase reaction, the K_m for IP_7 and ADP are 1.97 µM and 1.57 mM respectively [65]. IP_6 is usually at a saturating concentration for the enzyme, because of its high cellular levels (15-60 µM) [65, 66]. In contrast, free ATP levels are in the range of 1 mM [85], so the main modulating factor in the living cell should be ATP. Furthermore, in living cells the ATP concentration is quite high compared to the ADP concentration. In mammalian cells, under steady state conditions, the physiological ADP levels are ~ 3-10 times lower than ATP levels [41, 86]. This suggests that the physiological ADP concentration is not likely to drive the ATP synthase reaction of IP6Ks.

1.6.2.2 IP6Ks expression and cellular localization

The three mammalian IP6K isoforms range from 46– 49 kDa in molecular weight [2]. Although the amino acids in the catalytic domain are largely conserved in all three isoforms [80], there are some isoform specific sequences that enable unique protein-protein interactions, cellular distribution and post translational modification [78]. While IP6Ks in general are ubiquitously expressed, the isoforms exhibit differential tissue mRNA expression and sub-cellular localization. In mice, IP6K1 is the most abundant isoform in the majority of the tissues, whereas in humans IP6K1 and IP6K2 are almost equally expressed. In both mouse and human IP6K3 expression is quite low in many tissues with few exceptions.

In general in both mouse and human the tissues with the prominent level of expression of these different IP6Ks are as follows: brain and testis (IP6K1); testis, thymus, and brain (IP6K2); muscle (IP6K3) [75, 80, 87]. It has to be noted that although some of the mouse mRNA expression data have been confirmed with protein levels, human data is solely based on mRNA expression levels, which may not necessarily reflect the actual abundance of the protein [80].

IP6Ks display a varied cellular distribution. IP6K1 was reported to be found both in cytoplasm [88, 89] and nucleus [89-91]. IP6K2 contains a nuclear localization sequence [92] and is found predominantly in the nucleus. In certain cell types, such as ovarian carcinoma cells and HEK293 cells, IP6K2 is also localized in cytoplasm [89, 92, 93]. IP6K3 is predominantly localized in the cytoplasm [89]. Since, the localization of the IP6Ks is often based on data obtained from the overexpression of recombinant proteins, these data may not necessarily reflect the location of the endogenous protein.

1.6.2.3 Role of IP6Ks at the organism level

Isoform specific gene deletion on an organism level unveiled the physiological importance of IP6Ks and the molecules generated by them. IP6K1 KO mice exhibited growth retardation, increased immunity, decreased serum insulin levels, increased insulin sensitivity, reduced obesity, resistance to high fat diet, male infertility and compromised hemostasis [80, 88, 94]. IP6K2 KO mice displayed resistance to ionizing radiation but susceptibility to carcinogen-induced squamous cell carcinoma [95]. IP6K3 KO mice exhibited some similarities to IP6K1 gene deletion, such as lower blood glucose levels and reduced insulin levels, but not resistance to high fat diet. Furthermore, they had an increased life span [2, 80, 87].

1.6.3 Functions of inositol pyrophosphates

Inositol pyrophosphates have been found to be important cellular regulators that are involved in many aspects of cell biology. Some examples of the cellular processes that inositol pyrophosphates are involved in are endocytosis, exocytosis, apoptosis, vesicle trafficking, cytoskeletal dynamics, regulation of the binding of PH domain containing proteins to phospholipids, telomere length regulation, chromatin hyper-recombination, neurotransmission, phosphate homeostasis and adaptations to environmental stress. Inositol pyrophosphates exert their function mainly through two molecular mechanisms, allosteric protein binding and protein pyrophosphorylation [3, 67, 68, 78].

1.6.4 Inositol pyrophosphates and β-cells

Pancreatic β-cells express IP6K1 and IP6K2 isoforms that generate the inositol pyrophosphate IP_7. As mentioned earlier inositol pyrophosphates are involved in several cellular functions, but exocytosis is the only process that has been investigated in the β-cell so far [76]. IP_7 was found to be present at high levels in β-cells and was described to be important for preparing these cells for exocytosis. However, this study did not address the physiological role of glucose in determining the IP_7 level.

In the β-cell, ATP/ADP increases are important for secretion. Since IP6Ks have a high K_m for ATP [65, 68, 83, 84, 96], metabolic regulation of IP_7 may play an important role in exocytosis. A crude indication that this was worth investigating further came from a brief study on insulin secreting HIT-T15 cells which showed that IP_7 levels are compromised by treatment with sodium azide, a metabolic poison [97]. This suggests the significance of the substrate ATP in maintaining IP_7 levels and that IP_7 concentration may be an important bioenergetic indicator in the β-cell. It also suggests the importance of investigating the metabolic coupling between glucose, IP_7 level and insulin secretion.

2 AIMS

2.1 GENERAL AIMS

The overall objective of this thesis was to characterize the regulation and role of the inositol pyrophosphate IP_7 in pancreatic β-cells.

2.2 SPECIFIC AIMS

The specific aims were the following:

- To investigate the regulation of IP_7 and to examine this pyrophosphate's dependence on the cellular bioenergetic status.
- To investigate the possible role of IP6Ks as metabolic sensors that integrate cellular ATP/ADP levels with insulin secretion via IP_7 production.
- To determine the role of IP6K1/IP_7 in the regulation of Akt/PKB signaling.

3 METHODOLOGICAL CONSIDERATIONS

3.1 MATERIALS

Cell culture reagents were obtained from ThermoFisher Scientific (Stockholm, Sweden) and other common chemicals were purchased from Sigma (Stockholm, Sweden), Merck KGaA (Darmstad, Germany) and VWR (Leyven, Belgium). Inhibitors such as LY294002, TBB (4, 5, 6, 7-tetrabromobenzotriazole) and wortmannin were obtained from Sigma-Aldrich (Stockholm, Sweden). HNMPA-$(AM)_3$ (Hydroxy-2-naphthalenylmethylphosphonic Acid Trisacetoxymethyl Ester), PAO (Phenylarsine Oxide), U73343 (1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione) and U73122 (1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H–pyrrole-2,5-dione) were purchased from Merck Millipore (Solna, Sweden). HNMPA was purchased from Biomol (Hamburg, Germany). TNP [N^2-(m-Trifluorobenzyl), N^6-(p-nitrobenzyl)purine] was purchased from Sigma-Aldrich or Merck Millipore. Other materials used in this thesis are described in detail in **Paper I**– **III**.

3.2 ANIMALS

All animal experiments were approved by the animal ethics committee of Northern Stockholm, Sweden and carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals. Animals were housed at a constant room temperature (22 °C; 12 h light/dark cycle) with *ad libitum* access to food pellets and tap water.

In this study we used three month-old male ob/ob (leptin deficient) mice and lean littermates [98]. These animals were originally obtained from Umeå, Sweden and then inbred at the Karolinska hospital animal core facility. C57BL/6 mice were obtained from Charles River Laboratories (Germany). NMRI mice were supplied from Bomholtgaard Breeding & Research Center, Bomholtgaard, Ry, Denmark [76].

3.3 ISOLATION OF MOUSE PANCREATIC ISLETS

In **Paper II**, mice were shortly anaesthetized using isofluorane (Baxter, Kista, Sweden) before sacrifice by cervical dislocation. In order to study inositol pyrophosphate metabolism we needed to maximize the islet yield per mouse. Therefore, pancreata were perfused via the pancreatic duct with 1 mg/ml ice-cold collagenase A or P (Roche, Sweden) in isolation buffer (HBSS supplemented with 0.5% BSA, 100 units/ml penicillin G, 100 µg/ml streptomycin and 25 mM HEPES, pH 7.4). The pancreas was digested in a 37 °C water bath with mild shaking for ~ 12 min. Collagenase from the digested tissue was then removed by washing it three

times with isolation buffer. Islets were hand-picked in ice-cold isolation buffer with the help of a microscope. The islets were then cultured temporarily (~ 1 h) in custom made RPMI 1640 supplemented with 10 µM inositol, 11 mM glucose, 2 mM L-glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). These islets were then transferred into culture media containing [^{3}H] *myo*-inositol for labeling (as described in section 3.7). We pooled islets from several mice and used ~ 300 and ~ 1000 islets per condition for measuring IP_7 and IP6K activity, respectively.

3.4 ISOLATION OF HUMAN PANCREATIC ISLETS

Human islets were received from the Nordic Network for Islet Transplantation from deceased donors with total brain infarction after proper consent. Experimental work on human islets was approved by Regional Ethical Review Boards in Uppsala and Stockholm. Human islets were provided through the JDRF award 31-2008-416 (ECIT Islet for Basic Research program).

The islets, isolated at the Division of Clinical Immunology at the University of Uppsala [99], were received in complete CMRL 1066 medium supplemented with 10% human serum. These islets were then handpicked under a microscope into CMRL 1066 medium supplemented with 11 mM nicotinamide, 2 mM L-glutamine, 5 mM sodium pyruvate, 0.25 µg/ml fungizone, 100 units/ml penicillin G, 100 µg/ml streptomycin, 10 mM HEPES, pH 7.4, and 10% FBS as described in **Paper II**.

3.5 CELL CULTURE

The hamster-derived, HIT-T15 cells (**Paper I** and **II**) were cultured in complete RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine at 5% CO_2 and 37 °C. HIT-T15 cells were purchased from ATCC, UK.

The mouse-derived, MIN6m9 cells (**Paper II** and **III)** [100] were cultured in complete DMEM supplemented with 10% FBS, 11 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 75 µM β-mercaptoethanol at 5% CO_2 and 37 °C. MIN6m9 cells were a kind gift from Prof. S. Seino, Kobe University Graduate School of Medicine, Japan.

3.6 RNA SILENCING

MIN6m9 cells were seeded at a density of 26000 cells/cm^2 in complete DMEM. After overnight culture, the cells were transfected with siRNA against IP6K1, IP6K2, or non-targeting controls, siRNA IDs: 188560, 71758, 287702, 292211, 4611 and 4613 (**Paper II**

and **III**). The siRNA mixture was prepared in lipofectamine 2000 and opti-MEM. The ratio of lipofectamine to siRNA was 2.5:1 and the final concentration of each siRNA was 25 nM. A combination of two different siRNAs was used for silencing either IP6K1 or IP6K2. This method of using two different siRNAs against the same gene decreases off-target effects. The next day, cells were replenished with antibiotics free DMEM and cultured for another two days. Silencing efficiency was determined using immunoblotting.

For experiments involving the capacitance measurement in the primary β-cells from NMRI mice (**Paper II)**, the above described siRNAs with Cy3 fluorescent tags at the 5'end of the sense and antisense strands were employed. The cells were selected for capacitance measurement based on the fluorescence signal, indicating efficient transfection.

3.7 [^{3}H] *MYO*-INOSITOL LABELING

HIT-T15 cells were labeled for 120 h in custom made RPMI 1640 supplemented with 10% dialyzed FBS (dialyzed using 1000 M.W. cut off membrane, Spectrum labs, The Netherlands) containing 10 µCi [^{3}H] *myo*-inositol (American radiolabeled chemicals, St. Louis, MO, USA) (**Paper I** and **II**) [28, 76]. During the labeling period, after ~ 72 h, a medium change was carried out.

MIN6m9 cells were labeled for 96 h with 50 µCi [^{3}H] *myo*-inositol in a complete DMEM supplemented with 10% dialyzed FBS (**Paper II**). During the labeling period, after ~ 48 h, a medium change was carried out. For experiments involving the quantification of inositol pyrophosphate levels upon silencing of IP6Ks, the cells were labeled for 72 h in radioactive DMEM.

Mouse and human islets were labelled for 72 h with 20 µCi [^{3}H] *myo*-inositol in a custom-made RPMI 1640 and complete CMRL 1066 (with an addition of 10 µM inositol) supplemented with 10% dialysed FBS (**Paper II**). During the labeling period, after ~ 48 h, a medium change was carried out.

3.8 DYNAMIC INCUBATION ASSAY

MIN6m9 cells (26000 cells/cm^2) were seeded in complete DMEM and cultured for 4 days. For experiments involving the measurement of inositol phosphates, cells were seeded in DMEM containing [^{3}H] *myo*-inositol. On day 4 (~ 96 h of culture), the cells were preincubated for 1 h in modified KREBS buffer containing 119 mM NaCl, 4.6 mM KCl, 2 mM or 4 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 0.5 mg/ml BSA and 20 mM HEPES pH 7.4. The buffer was then exchanged, every min, for

30 min with KREBS containing 0.5 mM glucose and then the cells were stimulated with KREBS buffer containing 10 mM glucose. The perfusates were collected for insulin quantification [101]. The cells were lysed with either 5% trichloroacetic acid (TCA) supplemented with 250 µg/ml IP_6 for inositol phosphate extraction or with Mammalian Protein Extraction Reagent (M-PER) for protein quantification (**Paper II**).

3.9 STATIC INCUBATION ASSAY

This assay was used as a basis to measure different parameters such as IP_7, ATP/ADP and protein expression (**Paper I** and **II**) in β-cell lines and islets. For experiments involving IP_7 measurements in HIT-T15 cells, the cells were pre-labelled with [^{3}H] *myo*-inositol. On the day of the experiment the cells (~ 1.6 x 10^5 cells/cm^2) were treated with or without the given inhibitor for 30 min or 2 h or 4 h in a modified KREBS buffer containing 0.1 mM glucose. In other experiments on this cell line, after 30 min of preincubation the cells were stimulated with 10 mM glucose or 5 mU/ml insulin (Actrapid, Novo Nordisk, Denmark). The cells were lysed by 5% TCA (for IP_7 and ATP/ADP measurements) or modified RIPA buffer (for immunoblotting). RIPA buffer contained 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 50 mM Tris, pH 7.6, complete mini protease inhibitor cocktail and PhosSTOP phosphatase inhibitors (Roche Diagnostics, Stockholm, Sweden).

This static incubation assay was also used as the basis to measure glucose-stimulated increases in IP_7 and ATP/ADP levels in islets (**Paper II**). For these experiments the basal and stimulatory glucose concentrations used were 3 mM and 16.7 mM, respectively.

In addition, this kind of assay was used with MIN6m9 cells in a number of different configurations. In all cases the cells were preincubated for 1 h with KREBS buffer containing 0.5 mM glucose followed by stimulation with respective secretagogue for 3 min (**Paper II** and **III**). Stimuli included glucose (10 mM), sodium pyruvate (10 mM), L-leucine (10 mM) and KCl (25 mM). Following stimulation, various parameters such as IP_7, ATP/ADP and insulin secretion were measured. In **Paper III**, this static incubation assay was also used to obtain protein samples for measuring Akt/PKB and IRS phosphorylation by immunoblotting, as well as samples for measuring insulin release. For experiments involving the treatment with TNP, the inhibitor was added during the last 30 min of preincubation (**Paper III**).

3.10 MEASUREMENTS OF IP6K ACTIVITY

IP6K activity was measured using purified recombinant IP6K1 or pancreatic islet lysates in **Paper I** and **II,** respectively. The recombinant IP6K1 protein was added to the assay buffer

containing 100 mM KCl, 3 mM $MgSO_4$,1 mM Na_2ATP, 1 mM Na_2EDTA, 2 mg/ml BSA, 20 mM HEPES, pH 7.2 with KOH, approximately 20,000 d.p.m. [^{3}H] IP_6 (Perkin Elmer, MA, USA) and 10 μM IP_6 (Merck Millipore), in the presence of the given inhibitor or vehicle (DMSO) as described in **Paper I**. After 15 min the assay was stopped and the inositol phosphates were extracted using ice-cold 2 M perchloric acid supplemented with 0.2 mg/ml IP_6 as described in **Paper I**.

Approximately 1000 islets per condition were used to measure IP6K activity in islets from lean and ob/ob mice. Islets were lysed with a buffer containing 150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, 1 mM EDTA, pH 7.4, protease and phosphatase inhibiting cocktail tablets. Protein levels were quantified using Pierce BCA protein assay kit (ThermoFisher Scientific, Stockholm, Sweden). The proteins (15 μg) from the islet lysate were added to 25 μl of the assay buffer so that the final concentrations of the assay buffer components were 50 mM KCl, 20 mM HEPES pH 7.2 with KOH, 12 mM $MgSO_4$, 10 mM Na_2ATP, 1 mM Na_2EDTA, 20 mM phosphocreatine, 15.5 Sigma Units/ml phosphocreatine kinase, 10 mM sodium fluoride, approximately 20,000 d.p.m. [^{3}H] IP_6, 4 μM IP_6 and 1 protease inhibitor tablet/10 ml. The enzyme reaction was terminated after 20-40 min and the inositol phosphates were extracted using perchloric acid as described in **Paper II**. The extracted inositol phosphates were then analyzed using a Partisphere SAX HPLC, as previously reported [102].

3.11 EXTRACTION OF INOSITOL PHOSPHATES AND ADENINE NUCLEOTIDES

Cells or islets lysed with TCA were left on ice for 30 min with 0.5 M EDTA. The cells were then scraped off and centrifuged at 5040 *g* and 4 °C. The supernatant and the pellet were used for inositol phosphate and total lipid extraction respectively. The inositol phosphates containing supernatant was then washed twice with diethylether and neutralized by 0.5 M EDTA as described in **Paper I** and **Paper II**.

3.12 QUANTIFICATION OF INOSITOL PHOSPHATES

In brief, the extracted inositol phosphates were separated in an ion-exchange HPLC using a SAX column (250 x 4.6 mm Partisphere 5 μm, Hichrom, Berkshire, United Kingdom) with mobile phases: 1 mM EDTA and 1.25 M $(NH_4)_2HPO_3$ in 1 mM EDTA, pH 4.4 with H_3PO_4. HPLC elution profile was essentially as described in **Paper I** and **II**. Scintillant (Perkin Elmer) was added to the separated fractions and the radioactivity was measured using scintillation counter (Perkin Elmer). Peaks corresponding to inositol phosphates of interest were quantified. A schematic representation of this protocol is illustrated in Figure 4.

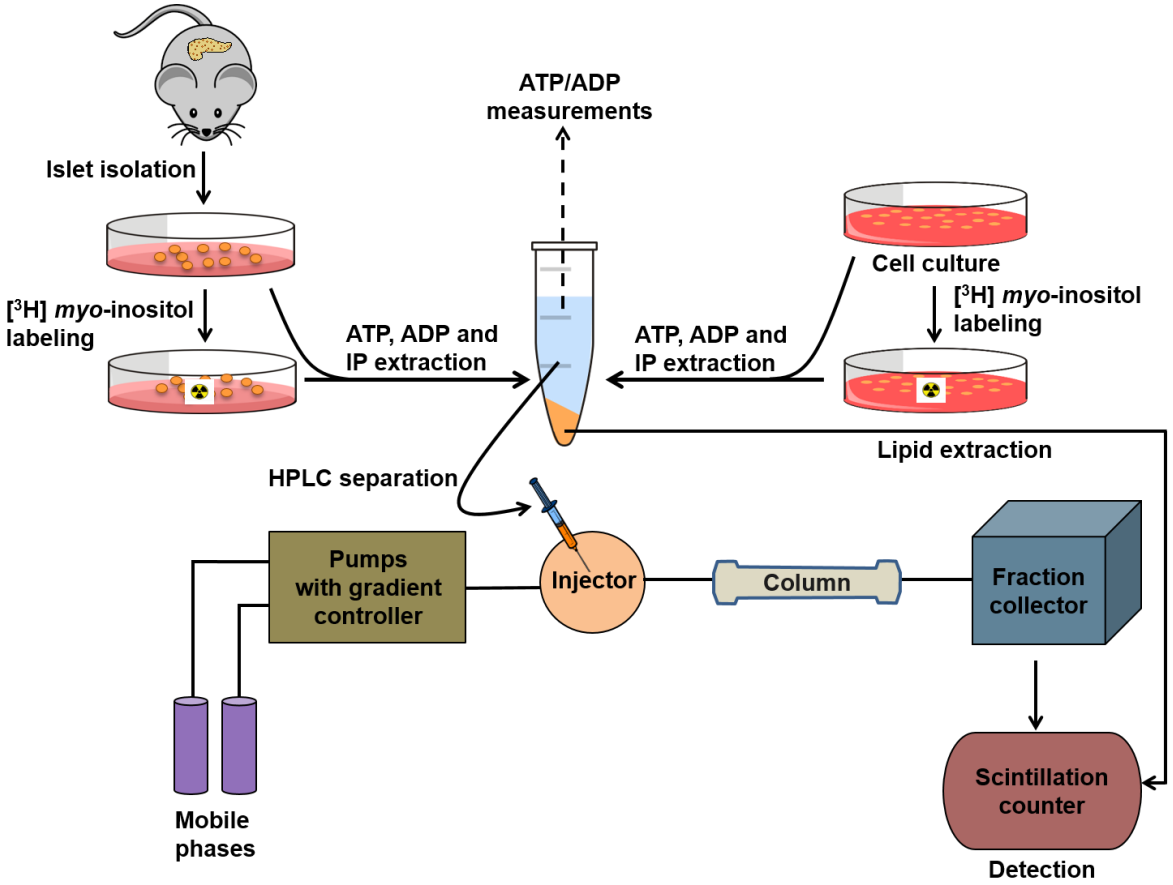

Figure 4. Schematic workflow of [^{3}H] *myo*-inositol labeling, extraction of inositol phosphates (IP), adenine nucleotides and inositol lipids. Extracted inositol phosphates were analysed using HPLC and scintillation counter. Total inositol lipids, detected with the scintillation counter, were used for normalizing inositol phosphate levels. ATP/ADP was measured using a bioluminescence assay.

3.13 ATP/ADP MEASUREMENTS

An aliquot (10 to 15 µl) of acid extracted sample, corresponding to ~ 3000-7500 cells (Figure 4), was used to quantify ATP/ADP using either the ApoSENSOR Kit (Biovision, Milpitas, California) or as previously described [97] in an EnVision 2103 Multilabel Plate Reader (Perkin Elmer) (**Paper I** and **II**).

3.14 EXTRACTION AND QUANTIFICATION OF TOTAL INOSITOL LIPIDS

The pellet obtained from the TCA extraction was used to further extract total inositol lipids by use of a modified Bligh and Dyer method [103]. The pellet was resuspended using a combination of water and a mixture of $CHCl_3$:CH_3OH:HCl (100:200:1). After 30 min, the phases were split by adding equal portion of $CHCl_3$ and a buffer containing 2 M KCl, 0.3 M HCl and 1 mM inositol. The samples were then centrifuged for 3 min at 5040 *g*. The lipid

sample from the lower phase was dried and the radioactivity was quantified using scintillation counting (Figure 4). This was used for normalizing inositol pyrophosphate levels.

3.15 INSULIN MEASUREMENTS

In **Paper II** and **III**, insulin secretion was measured using either the ArcDia two-photon fluorescence excitation microparticle fluorometry (TPX) assay (ArcDia Group, Turku, Finland) [101] or the AlphaLISA detection kit (Perkin Elmer).

3.16 CAPACITANCE MEASUREMENTS

In **Paper II**, cell capacitance was measured as a readout of exocytosis using the perforated patch configuration of the patch-clamp technique. Islet cells were patched in an extracellular medium containing 16.7 mM glucose, 118 mM NaCl, 20 mM tetraethylammonium-Cl, 1.2 mM MgCl, 5.6 mM KCl, 2.6 mM $CaCl_2$ and 5 mM HEPES, pH 7.4. A glucose concentration of 16.7 mM was used to mimic the glucose-stimulated state of the cells. Capacitance was measured upon stimulating the cells with a train of four 500-ms depolarizations (1 Hz) as previously described [76].

3.17 RECORDING OF CYTOPLASMIC FREE CALCIUM CONCENTRATION

Changes in $[Ca^{2+}]_i$ upon glucose stimulation were measured in MIN6m9 cells treated with control or IP6K1 siRNA, using Fura-2-acetoxymethyl ester (Fura-2/AM) and fluorescent microscopy (**Paper II**). This highly lipophilic dye crosses the cell membrane. After being taken up by the cell, the acetoxymethyl ester is cleaved by cellular esterases and the dye is available to bind Ca^{2+} [104]. This binding changes the peak absorbance of the Fura-2, allowing detection of both the Ca^{2+} bound and unbound dye by exciting it with alternating wavelengths (340 nm and 380 nm respectively). MIN6m9 cells grown on coverslips were loaded with 2 μM Fura-2/AM for 1 h in KREBS buffer containing 2.56 mM $CaCl_2$ and 0.5 mM glucose. After loading, the coverslips were mounted in a chamber maintained at 37 °C and perfused with KREBS buffer containing 0.5 or 10 mM glucose. Cells were excited alternatively with 340 nm and 380 nm to measure the fluorescence intensity of Ca^{2+} bound and unbound forms at 510 nm in an Olympus ix 71 microscope. The $[Ca^{2+}]_i$ level was then represented by the ratio of the fluorescence signals obtained from 340 and 380 nm excitation as described in **Paper II**.

3.18 FACS ANALYSIS OF ISLET CELLS

Islets isolated from 12 h fasted ob/ob and lean mice were dissociated into single cells using Accutase [105] to evaluate the proportion of β-cells (**Paper II**). The dispersed cells were

permeabilized and stained overnight at 4 °C with an allophycocyanin conjugated antibody against insulin (R&D system, Abingdon, United Kingdom) in Flow Cytometry Permeabilization/Wash Buffer I (R&D system). Then the cells were labelled with Hoechst (ThermoFisher Scientific) to stain the DNA. Fluorescently labelled cells were analyzed using FACS as described in **Paper II**.

3.19 IMMUNOCYTOCHEMISTRY

MIN6m9 cells (12,500 cells/cm^2) seeded on coverslips were cultured for 3 days in complete DMEM. These cells were fixed using 4% paraformaldehyde (PFA) and immunostained with an antibody against IP6K2 to detect the endogenous protein. IP6K1 localization was visualized by overexpressing a myc-tagged IP6K1. Cells were transfected with a plasmid containing the myc-tagged IP6K1 using lipofectamine 3000, fixed with PFA and immunostained with a primary antibody against c-Myc as described in **Paper II**. The coverslips were then incubated with Alexa Fluor 488 secondary antibodies and the nuclear staining dye, 7-aminoactinomycin D, before mounting them on the glass slides using Vectashield mounting medium. Immunofluorescence was detected by imaging the cells using a Leica SP2/SP5 confocal microscope as described in **Paper II**.

3.20 RNA ISOLATION AND REAL-TIME RT-PCR ANALYSIS

The RNeasy Mini Kit (Qiagen, Sweden) was used to isolate total RNA from HIT-T15 cells according to the manufacture's instruction. Reverse transcription of total RNA was carried out at 37 °C with the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Expression of genes such as IP6K1, IP6K2, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified by real-time semi-quantitative PCR with PowerUp SYBR Green Master Mix (ThermoFisher Scientific) using specific primers on an ABI7300 instrument (Applied Biosystems, USA) as described in **Paper I**. GAPDH expression was used an endogenous control for normalization.

3.21 IMMUNOBLOTTING

MIN6m9 and HIT-T15 cells were lysed with RIPA buffer. After protein quantification using the Pierce BCA kit, equal amount of proteins (10-20 µg/lane) were separated by NuPAGE 4-12% Bis-Tris gel and transferred to nitrocellulose membrane using a dry blot (iBlot, ThermoFisher Scientific). Membranes were blocked and incubated overnight in primary antibodies against IP6K1, phospho-Akt (T308), phospho-Akt (S473), Akt, phospho-IRS1 (Y608), IRS1 and β-actin as described in **Paper I-III**. The membranes were then incubated with specific HRP-conjugated secondary antibodies followed by detection of

immunoreactivity using either SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher Scientific) or Amersham ECL prime western blotting reagent (GE Healthcare, Uppsala, Sweden). The chemiluminescence signal was detected by ChemiDoc Imaging System and analyzed by densitometry using either Image Lab or Image J software as described in **Paper I-III**.

3.22 STATISTICAL ANALYSIS

Statistical analyses were performed with GraphPad Prism 5 or 7. Student's t-test, 95% confidence interval, one-way ANOVA (Tukey's post-test) or two-way ANOVA (Bonferroni's post-test) were used when appropriate. All results were expressed as means ± standard error of the mean (SEM), p values < 0.05 were considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Several inhibitors of signal transduction reduce IP_7 levels

Basal IP_7 levels are kept high in β-cells (~ 6 μM) compared to yeast and other mammalian cells, whose concentration range is from 0.5 to 5 μM [1, 3, 68]. Our research group has previously shown that IP_7 is important for the exocytotic capacity of the β-cell [76], but the precise mechanisms regulating IP_7 levels are unknown.

In order to understand IP_7 regulation we used a number of pharmacological inhibitors against different inositide signaling pathways that might impact on IP_7 levels. In this study, we used insulin secreting HIT-T15 cells as they have similar levels of IP_7 to those observed in primary β-cells [76]. Cells were treated with inhibitors against the insulin receptor, PI3K and PLC pathways under basal glucose conditions. The choice of interrogating these pathways was motivated by (1) studies showing increased IP_7 levels upon insulin stimulation, which activates the PI3K pathway [60] and (2) the fact that PLC mediated break down of PIP_2 is the main pathway for the generation of IP_3 and other higher inositol phosphates such as IP_7 [73-75]. The inhibitors tested were HNMPA (insulin receptor); wortmannin (PI3K); LY294002 (PI3K); PAO (phosphatidylinositol 4-kinase, PI4K); TBB (CK2), U73122 (PLC) and U73343 (negative control for PLC inhibitor).

To our surprise, all the relevant inhibitors examined, targeting different signaling pathways (e.g. insulin receptor/PI3K pathway and PLC pathway), decreased IP_7 levels. This raised suspicion that the impact on IP_7 was mediated by off-target effects of these inhibitors. There are two known properties of cellular systems that favor this thought. Firstly, most of the kinase inhibitors are targeted against the ATP binding site. Thus, achieving high specificity is difficult because of the similarity between the ATP binding sites in different proteins. Secondly, IP_7 generating IP6Ks have a high K_m for ATP [65, 68, 83, 84, 96], making them vulnerable to depletion of ATP levels. Therefore, we investigated if the inhibitors decreased IP_7 levels by non-specifically compromising the bioenergetic status of the cell.

4.1.2 Inhibitors that reduced IP_7 levels also decreased ATP/ADP levels

Interestingly, all the inhibitors at concentrations that decreased IP_7 levels also reduced ATP/ADP levels. This suggested that the reduction in IP_7 could have been mediated by ATP/ADP decrease. However, IP_7 levels can also be influenced by other factors such as the availability of its precursor (IP_6) or the activity and protein levels of IP6K. In order to

understand the contribution of these factors in the reduction of IP_7 levels, we measured all the above parameters. Measuring IP6K activity upon inhibitor treatment by using purified IP6K1 as a surrogate showed that only LY294002, PAO and U73122 decreased IP6K activity. Therefore, for these three inhibitors, in addition to the decreased ATP/ADP ratio, the disruption in IP6K activity could also contribute to the reduction in cellular IP_7. For the rest of the inhibitors, the effect on IP_7 can mostly be attributed to the decrease in cellular ATP/ADP levels. We also determined that the decrease in IP_7 levels was not mediated by an actual decrease in IP6K1 protein amount. Furthermore, the levels of IP_7´s precursor IP_6 were not altered by treatment with any of the inhibitors. This showed that the effect of various inhibitors on inositol phosphates were specific to IP_7. This could be due to the difference in the K_m (for ATP) of IP_6 generating inositol pentakisphosphate 2-kinases (IP5Ks) and IP_7 generating IP6Ks. IP5K's K_m for ATP is 62.8 µM [106] whereas IP6K has a much higher K_m value of 1 mM [107], which is the free concentration of ATP in β-cells [85].

4.1.3 Correlation between IP_7 levels and ATP/ADP ratio

Since the decrease in IP_7 caused by the inhibitors was paralleled with a reduction in cellular ATP/ADP levels, we performed a correlation analysis to statistically examine the relationship between IP_7 and ATP/ADP levels. Data acquired from all the inhibitors were pooled together and included in the analysis. Pearson correlation analysis showed a significant positive correlation between ATP/ADP and IP_7 ($R = 0.7835$, $R^2 = 0.6139$, $p = 0.0003$). The exclusion of the inhibitors that directly affected the IP6K activity from the analysis enhanced the correlation coefficient ($R = 0.9129$, $R^2 = 0.8334$, $p = 0.0006$). Overall, these results suggest ATP/ADP to be the main regulator of IP_7 in β-cells. For inhibitors such as LY294002, PAO and U73122 both kinase activity inhibition and ATP/ADP reduction may contribute to the cellular IP_7 decrease.

This study demonstrates for the first time a correlation between endogenous ATP/ADP and IP_7 in an intact cell context. The protein kinase and lipase inhibitors tested indirectly affected IP_7, predominantly through a decrease in cellular ATP/ADP levels, even though some of them compromised IP6K activity. Furthermore, it demonstrates that IP_7 levels are largely dependent and possibly regulated by the cellular bioenergetic status of the β-cell. These data are in alignment with a previous publication where it has been shown that metabolic poisons such as sodium azide decrease IP_7 levels [97]. One recent study showed an increase in IP_7 levels following the artificial delivery of ATP into cells [108]. However, it is only our study (**Paper I**) that has directly correlated endogenous intracellular ATP/ADP to IP_7.

In addition, this work questions the validity of the studies that have backed their conclusion only by the usage of these inhibitors. For example, Beith et al., [109] used HNMPA and LY294002 to show the role of insulin receptor and PI3K in cell proliferation, whereas, taking into account our study, IP_7 could have also been involved. Thus it is necessary to complement future studies with either more specific inhibitors or with siRNA against the enzyme of interest. Furthermore, measurement of the impact of an inhibitor on the cellular ATP/ADP will also serve as a simple cross-check whether IP_7 depletion may be playing a role in a given cellular system.

4.2 PAPER II

In **Paper I** we showed that IP_7 levels are dependent on ATP/ADP levels under basal glucose conditions. This relationship was revealed by artificially decreasing ATP/ADP levels following treatment of β-cells with pharmacological inhibitors. In **Paper II** we investigated the influence of a physiologically relevant stimulus, glucose, on IP_7 concentrations in β-cells. We used glucose as it is known to both raise ATP/ADP and drive insulin exocytosis [15, 110]. The role of IP_7 in modulating the β-cell exocytotic capacity had been previously demonstrated [76], but its regulation by glucose was not examined. As mentioned earlier, because of the high K_m for ATP of the IP6Ks [65, 68, 83, 84, 96], we hypothesized that these enzymes could act as metabolic sensors in pancreatic β-cells. The specific aims were to investigate (1) if the glucose-mediated increase in ATP/ADP can further increase the existing high IP_7 concentration and (2) the role of IP6Ks in translating the metabolic signal (ATP/ADP) into IP_7 generation and insulin secretion. We used insulin secreting MIN6m9 and HIT-T15 cells together with pancreatic islets from human, normal mice and diabetic ob/ob mice as systems to address the hypothesis.

4.2.1 IP6K1 decodes glucose-mediated increase in ATP/ADP to increases in IP_7 and first phase insulin secretion

In **Paper II** we showed that IP_7 production was acutely increased upon glucose stimulation in both cell lines and islets from mice and human. This IP_7 increase ranged from 24 to 66% (Table 1). Studies on the MIN6m9 cells demonstrated that glucose-stimulated increases in IP_7 level were transient, peaking at 3 minutes. This transient increase in IP_7 was accompanied by a parallel change in the ATP/ADP ratio. Furthermore, the IP_7 increase was not exclusive to glucose but was also found in response to other secretagogues that increased ATP/ADP, such as pyruvate and leucine. These data, together with results from **Paper I**, substantiate the tight relationship between ATP/ADP and IP_7 levels. **Paper I** demonstrated that artificially

imposing ATP/ADP reduction is paralleled by a drop in IP_7 levels and **Paper II** shows that a physiological increase in ATP/ADP elevates IP_7 production.

We used RNA interference to identify the IP6K isoforms responsible for the glucose-induced IP_7 generation in MIN6m9 cells. These experiments revealed that the increase in IP_7 upon glucose stimulus was solely mediated by IP6K1. Although both the isoforms have a similar K_m, the specificity for one isoform over the other could be attributed to the difference in cellular localization of the enzymes. Supporting this idea IP6K1 was mainly found to be present in the cytoplasm and IP6K2 predominantly in the nucleus.

After elucidating that IP6K1 regulates the glucose-mediated increase in IP_7, we wanted to investigate its role in glucose-stimulated insulin secretion. A dynamic insulin release assay utilizing MIN6m9 cells treated with siRNA against IP6K1 and IP6K2 revealed that only IP6K1 silencing decreased first phase insulin exocytosis. This role of IP6K1 was also confirmed in primary β-cells from NMRI mice using capacitance measurements performed on cells treated with siRNA. This demonstrates that the glucose-stimulated increase in IP_7 is driven by IP6K1 and is required for proper first phase insulin secretion. Overall, these data show that IP6K1 acts as a metabolic sensor in β-cells, exerting a key role downstream of bioenergetic changes.

Table 1. IP_7 increase induced by glucose-stimulation in insulin secreting β-cell lines and pancreatic islets.

Cell/islet types	IP_7 increase (%)
Hamster, HIT-T15 cells	30
Mouse, MIN6m9 cells	34
C57BL/6 mouse islets	66
Lean mouse islets	27
Human islets	24

4.2.2 IP_7 generation and insulin secretion reflect perturbed metabolism in islets from diabetic mice

After establishing IP6K1 as a metabolic sensor under physiological condition, a natural question was to investigate its function under pathophysiological conditions. During early and mid-stage progression of diabetes, pancreatic islets display two hallmark symptoms, that

is, elevated basal insulin secretion and compromised first phase secretion [34, 111-113]. Furthermore, there is, for example, an altered metabolism in islets from diabetic humans and mice, where the basal levels of ATP are elevated [39-41].

To test the metabolic sensing property of the IP6K, we chose as a model the diabetic ob/ob mouse islets [98]. They exhibit an altered metabolism, that is increased basal metabolism and a compromised response to glucose [39, 40]. The results reported in **Paper II** showed that the higher basal ATP/ADP observed in ob/ob islets was reflected in increased basal IP_7 levels, compared to islets from control lean mice. Interestingly this was also associated with increased basal insulin secretion in ob/ob islets, compared to control islets. These data support the relationship between ATP/ADP and IP_7 in regulating insulin secretion. When ob/ob islets were stimulated with glucose there was a compromised increase in ATP/ADP and thus an attenuated increase in IP_7 levels and insulin secretion. Results obtained under both basal and high glucose conditions in ob/ob and control islets highlight the tight metabolic coupling between ATP/ADP and IP_7. The activity of the IP6Ks was not altered in ob/ob islets compared to controls, emphasizing the normal functioning of IP6Ks even under diabetic condition. Thus under a pathophysiological setting IP6K, acts as a translator of disrupted metabolism into improper insulin exocytosis. Since IP6K1 helps promote first phase insulin secretion and loss of this is one of the earliest signs of diabetes, our data suggest that translation of defective β-cell metabolism by IP6K may play a role in disease development.

4.2.3 IP6K1 as a new metabolic sensor in β-cells

We are proposing that IP6K1 is an important metabolic sensor in β-cells. However, there are other metabolic sensors that have been considered dominant in these cells. The most important example in terms of sensing ATP/ADP is the K_{ATP} channel [114]. What might the cell gain by having an additional sensor? A clue may come from another metabolic sensor, which is also generating an inositol derivative, namely PI4K [115]. This enzyme is inhibited by ADP and a drop in ADP, arising from an increased ATP/ADP ratio, stimulates its activity. This leads to generation of PI4P, a limiting step in the production of PIP_2, which is important in the final stages of exocytosis. Both the PI4K and IP6K1 affect the process of exocytosis directly, whereas the effect of the K_{ATP} channel on exocytosis is indirect and is responsible for the main driving force of insulin release, the local elevation of $[Ca^{2+}]_i$. Since IP6K1 affects the pool of releasable granules it may modulate the degree of the response that can be driven via the depolarization of the cell induced by ATP/ADP on the K_{ATP} channel. In this

way one can see that these two ATP/ADP modulated proteins could act in partnership to integrate metabolic sensing.

4.3 PAPER III

4.3.1 Regulation of Akt/PKB by inositol pyrophosphates in β-cells

IP_7 has been shown to negatively regulate Akt/PKB signaling by binding to the PH domain of this kinase and thus interfering with its recruitment to the membrane bound PIP_3. This recruitment is required for Akt/PKB activation [60, 93]. The negative regulation of Akt/PKB by IP_7, upon insulin stimulation, has been suggested in insulin sensitive tissues such as liver, adipose tissue and skeletal muscle from IP6K1 KO mice [60] and muscle from humans [61]. Furthermore, global deletion of IP6K1 or pharmacologically inhibiting IP6Ks in an animal model resulted in the mice exhibiting resistance to high fat diet, because of the enhanced insulin sensitivity in peripheral tissues. This suggests a beneficial aspect of deleting IP6K1 in peripheral insulin signaling [94, 116]. However, **Paper II** described the positive role of IP6K1-generated IP_7 in insulin secretion *in vitro* and this conclusion is supported by the low serum insulin levels observed when IP6K1 is inhibited *in vivo* [116]. Thus an important question is the relevance of the IP_7 mediated inhibition of Akt/PKB in pancreatic β-cells, where IP_7 both drives insulin secretion but also potentially inhibits the downstream signaling of the insulin feedback loop mediated by Akt/PKB. This autocrine signaling has been described to be important for β-cell function, including insulin release [44, 45, 101]. A further question is whether the inhibition of Akt/PKB by IP_7 is the means by which IP_7 can promote exocytosis. This last possibility is less likely as most publications suggest that Akt/PKB is a positive regulator of insulin secretion [101, 117, 118]. To resolve this complexity, we investigated the impact of silencing IP6K1 on β-cell Akt/PKB signaling under conditions where IP_7 is maximally stimulated.

In **Paper II** we showed that glucose-stimulated increase in IP_7 and insulin secretion peaked at 3 min in MIN6m9 cells. Hence we chose this time point to interrogate Akt/PKB signaling in MIN6m9 cells. Activation of Akt/PKB signaling was determined by monitoring the phosphorylation of Akt/PKB at T308 and S473 positions using immunoblotting.

Glucose stimulation of MIN6m9 cells induced a 2.46-fold increase in Akt/PKB phosphorylation at T308. Under glucose-stimulated condition, when MIN6m9 cells were silenced with siRNA against IP6K1, Akt/PKB phosphorylation at both T308 and S473 was decreased. This was a surprising result, since either knock down or decreased expression of IP6K1 in other tissues (e.g. muscle) had been shown to increase Akt/PKB activity [60, 61].

However, our data suggest a reduction in Akt/PKB activity in β-cells, implying a positive regulation of IP_7, rather than a negative one as observed in other insulin sensitive cell types [60]. The role of IP6K1 in positively regulating Akt/PKB was confirmed using a pan-IP6K inhibitor, TNP, which also reduced glucose-stimulated Akt/PKB phosphorylation on T308.

Thus our findings from β-cells differ from previous published studies [60, 61]. In order to investigate this apparent contradiction, we focused on the insulin stimulating property of IP_7 in β-cells. Since IP_7 is known to stimulate insulin secretion, we investigated if the positive effect on Akt/PKB signaling is a secondary one, mediated by the insulin feedback loop. The first step after the binding of insulin to the insulin receptor is phosphorylation of IRS, followed by activation of PI3K and Akt/PKB [45]. We evaluated phosphorylation of IRS1 at Y608 in MIN6m9 cells stimulated with glucose for 3 min in the presence or absence of TNP. Glucose stimulation increased both insulin secretion and IRS1 phosphorylation in MIN6m9 cells, whereas in the presence of TNP both of them were reduced. Hence, we propose that in β-cells IP_7 activates Akt/PKB indirectly through insulin exocytosis and the concomitant insulin feedback loop.

These data do not exclude an inhibitory effect of IP_7 on Akt/PKB in β-cells. They rather suggest that, in balance, the positive driving force of IP_7 in insulin secretion and the feedback loop is stronger than any possible direct inhibitory effect on Akt/PKB. Hence it does not necessarily contradict the existing view of IP_7's property to inhibit Akt/PKB signaling, instead it exposes a differential inositol pyrophosphate signaling in a complex biological setting. Thus IP6K1 knock out in β-cells could lead to an insulin resistance phenotype, which could then affect β-cell function. Therefore, strategies to knockdown IP6K1for its beneficial aspect in other tissues [60, 94] would be counter-productive in β-cells.

4.4 FUTURE PERSPECTIVES

Results from **Paper I**– **III** represent valuable progress made in understanding the regulation and role of the inositol pyrophosphate IP_7 in pancreatic β-cells. Several aspects merit further investigation. Key questions that remain are the mechanisms through which IP_7 regulates insulin exocytosis and the role of IP_7 derived inositol pyrophosphate, IP_8, in β-cells. These are areas future work should focus on.

In order to establish the mechanisms through which IP_7 regulates insulin secretion insights can be gained from other studies, especially from those that relate to vesicle trafficking. For example, IP_7 has been shown to pyrophosphorylate the β-subunit of the adaptor protein complex AP-3 (AP3B1), which changes the association of AP3B1 with the motor protein Kif3A, an important component of vesicle transport [119]. Recent work has exposed the involvement of IP6K1/IP_7 in regulating the actin cytoskeleton dynamics [120, 121]. A potential role of IP_7 in modulating the vesicle trafficking of insulin granules and cytoskeleton rearrangement is consistent with its function in modulating β-cell insulin secretion [122, 123]. Future studies in this area could aid in unraveling how IP_7 mechanistically promotes insulin release. A current approach that we are undertaking to address the mechanism of IP_7 action is the co-immunoprecipitation of IP6K1 followed by proteomics. This will identify the interacting partners of IP6K1, thus establishing the potential ways through which it can exert its function. Although this will provide some insights on IP6K1 and possibly IP_7 action in β-cells, a more appropriate approach would be to investigate the direct partners of IP_7 itself. This is challenging, since endogenous IP_7 is labile and thus does not lend itself to the construction of an affinity ligand. A synthetically produced non-hydrolysable IP_7 analogue is required. We are currently in collaboration with chemists to address this.

Our current work on IP_7 has focused on the main function of pancreatic β-cells, i.e. secretion. However, IP_7 is involved in diverse cellular functions some of which may also be relevant to β-cell regulation. One such area to investigate would be the role of IP_7 generated by IP6K2 in β-cell apoptosis.

The other major direction that future work should take is the study of the IP_7 metabolite, IP_8 [2, 3, 27]. This thesis has addressed some of the functions of IP_7 in β-cells, such as in insulin secretion and Akt/PKB regulation. However, the existence of IP_8 and the kinases that produce it is completely unexplored in these cells. IP_8 has been described to be involved in influencing mitochondrial oxidative phosphorylation through unclear mechanisms in the HCT116 colon cancer cell line [108] and to affect microtubule dynamics in fungi [124]. These processes are important for β-cell exocytosis [31, 33]. Future work directed towards

the understanding of IP_8 may expose potentially interesting functions related to β-cell secretion.

5 CONCLUSIONS

Inositol pyrophosphates are ubiquitous and evolutionarily conserved molecules. They have been implicated in virtually every aspect of cell biology [3, 68, 96]. Much of this knowledge on function was obtained in non-β-cells. However, one of the earlier reports to identify a cellular function for the inositol pyrophosphate IP_7, was in β-cells [76]. That study, while demonstrating a role for IP_7 in increasing the immediate β-cell exocytotic capacity, left many questions unanswered, such as IP_7 regulation by glucose. In this thesis we investigated the regulation, role and functional interaction of IP_7 in pancreatic β-cells and thus substantially expanded the earlier observations. We showed that IP_7 accurately reflects the bioenergetic status of the β-cell converting it into the final output of insulin secretion. Thus we have confirmed in a physiological context the often speculated hypothesis [27, 66, 107, 125] that IP_7 generating IP6Ks can act as metabolic sensors. We have also exposed a difference in physiological regulation of Akt/PKB by IP_7 in β-cells compared to other cellular systems such as liver, muscle and fat cells. This reveals again the unique position the β-cell occupies in our understanding of inositide function (Figure 5).

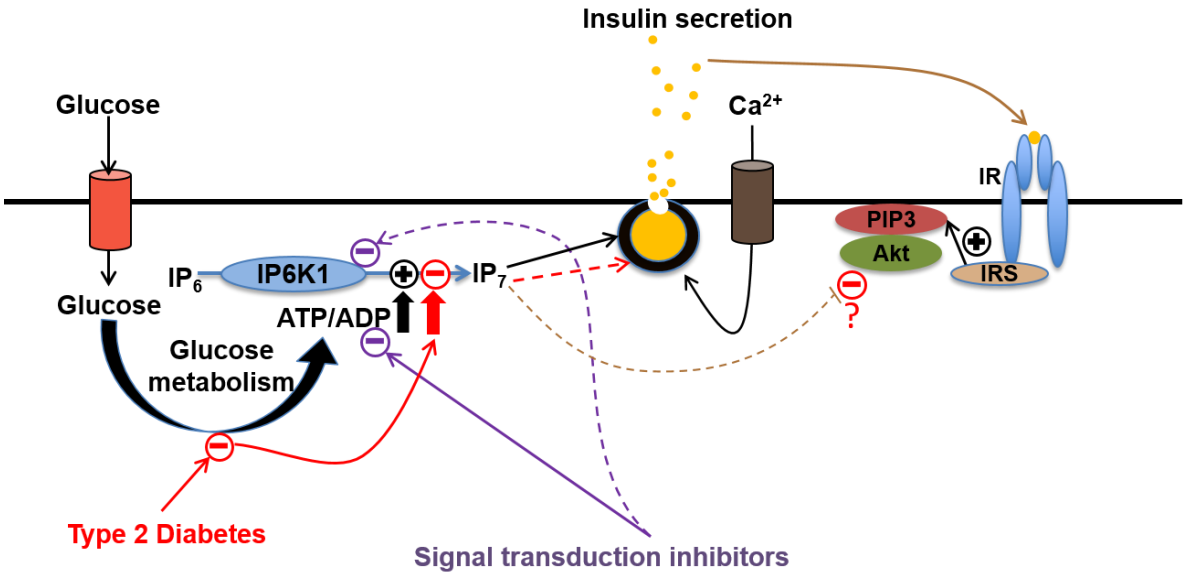

Figure 5. Graphical summary of the thesis describing the proposed role of IP6K1 and IP_7 in pancreatic β-cells.

This Ph.D. work has also led to the following specific conclusions:

- In **Paper I**, a selection of protein- and lipid kinase inhibitors related to inositide signaling pathways was shown to decrease IP_7, predominantly through a reduction in cellular ATP/ADP levels and in a minority of cases by a direct inhibition of the IP6Ks. A significant positive correlation between IP_7 and ATP/ADP suggests that IP_7 levels are regulated by the bioenergetics status of the cell. Thus modulating ATP/ADP ratio by any means will impact the cellular IP_7 levels and the processes that IP_7 regulates.
- The transient increase in IP6K1-generated IP_7, that is induced by glucose, is important for proper first phase insulin secretion. IP6K1 likely serves as a metabolic sensor, under both physiological and diabetic conditions, to decipher metabolic changes in the cell into IP_7 levels and insulin release. Thus disrupted metabolism in the diabetic islets is reflected in deranged IP_7 levels and improper basal and first phase insulin exocytosis. Overall, the studies in **Paper I** and **II** suggest that IP_7 plays important roles in cellular functions and signaling pathways that are dependent on cellular bioenergetic levels.
- In pancreatic β-cells, unlike other insulin sensitive cell types, IP6K1-generated IP_7 positively regulates Akt/PKB. This regulation is mainly mediated through the IP_7 dependent increase in insulin secretion and thus the subsequent insulin feedback signaling. Owing to this difference, pharmacological inhibition of IP6K1/IP_7 as an approach to treat whole body insulin resistance may not have a positive effect on β-cells.

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