The Role of Phosphodiesterase 3B in the Regulation of Insulin Secretion

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Results in this thesis demonstrate that mice with a specific increase in beta-cell PDE3B activity (RIP-PDE3B mice) have, in comparison to control mice, a reduced insulin response to glucose as well as to glucose in combination with GLP-1, glucose intolerance and altered islet morphology. Moreover, when metabolically challenged RIP-PDE3B mice develop severe obesity and insulin resistance. The insulin secretory capacity of isolated islets from RIP-PDE3B mice was studied and a specific reduction in the first phase of glucose-stimulated insulin release was identified. An important role of beta-cell PDE3B for exocytosis and release of insulin was further demonstrated by overexpression of PDE3B and by selective inhibition of the enzyme in both insulinoma cell lines and rat pancreatic islets. Of specific interest was the marked decrease of glucose-stimulated cAMP levels and concomitant decrease in insulin release observed in cells overexpressing PDE3B.

In summary, this thesis has contributed to an increased understanding for the role of beta-cell PDE3B in the regulation of insulin secretory processes. Results suggest that PDE3B regulates cAMP pools important for exocytosis of insulin-containing granules responsible for the first phase of insulin release. Also, these studies bring forward the role of cAMP in nutrient-induced insulin release. Finally, the work in this thesis demonstrates for the first time a functional role for beta-cell PDE3B in the maintenance of whole body energy homeostasis in mice.

Key words: PDE3B, cAMP, insulin secretion, islet, beta-cell, diabetes

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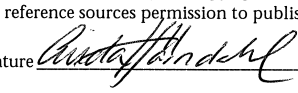
Signature  Date 21th of October 2003

The Role of Phosphodiesterase 3B in the Regulation of Insulin Secretion

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CONTENTS

ABSTRACT

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LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Härndahl, L., Jing, XJ., Ivarsson, R., Degerman, E., Ahrén, B., Manganiello, V.C., Renström, E. and Stenson Holst, L. **Important Role of Phosphodiesterase 3B for the Stimulatory Action of cAMP on Pancreatic β-Cell Exocytosis and Release of Insulin.** J Biol Chem. 2002 Oct 4;277(40):37446-55

II. Härndahl, L., Wierup, N., Enerbäck, S., Mulder, H., Manganiello, V.C., Sundler, F., Degerman, E., Ahrén, B. and Stenson Holst, L. **β-Cell-Targeted Overexpression of Phosphodiesterase 3B in Mice Causes Impaired Insulin Secretion, Glucose Intolerance and Deranged Islet Morphology.** Under revision for publication in J Biol Chem.

III. Härndahl, L., Wierup, N., Zmuda-Trzebiatowska, E., Manganiello, V.C., Sundler, F., Degerman, E., Ahrén, B. and Stenson Holst, L. **Diet-Induced Diabetes in Mice Overexpressing β-Cell PDE3B is Associated with Early Developed Obesity, Severe Islet Dysfunction and Adipose Tissue Insulin Resistance.** Manuscript

ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
Ach	acetylcholine
ADP	adenosine diphosphate
AKAP	A-kinase-anchoring protein
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cAMP-GEF	cAMP-regulated guanine nucleotide exchange factor
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related polypeptide
CNG	cyclic nucleotide-gated
CSP	cysteine string protein
DAG	diacylglycerol
Epac	exchange protein directly activated by cAMP
GDM	gestational diabetes mellitus
GLP-1	glucagon-like peptide-1
GLUT2	glucose transporter-type 2
GRP	gastrin releasing polypeptide
GTP	guanosine triphosphate
IAPP	islet amyloid polypeptide
IBMX	isobuthylmethylxanthine
IDDM	insulin-dependent diabetes mellitus
IGF-1	insulin-like growth factor-1
INS	insulinoma
IP_3	inositol trisphosphate
IR	insulin receptor
IRS	insulin receptor substrate
IVGTT	intravenous glucose tolerance test
KATP	ATP-sensitive K^+-channel
MODY	maturity-onset diabetes of the young
NADPH	nicotineamide adenine dinucleotide phosphate
NIDDM	non-insulin-dependent diabetes mellitus
NPY	neuropeptide Y
PACAP	pituitary adenylyl cyclase-activating polypeptide
PDE	phosphodiesterase
PI	phosphoinositide
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A

PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PP	pancreatic polypeptide
PP-2B	calcium/calmodulin protein phosphatase -2B
RIP2	rat insulin promotor 2
RT	reverse transcriptase
SNAP	soluble NSF (N-ethylmaleimide sensitive factor) attachment protein
SUR1	sulfonylurea receptor-1
VDCC	voltage-dependent calcium channel
VIP	vasoactive intestinal polypeptide

GENERAL INTRODUCTION

Glucose serves as one of the main energy sources in the human body. The dynamic uptake and release of glucose, depending on energy demand, regulates blood glucose levels and in healthy individuals the fasting blood glucose level is maintained at ~ 5 mM glucose. Insulin is the major hormone controlling energy homeostasis. After a meal when the levels of nutrients rise, insulin is secreted from the pancreatic β-cells and stimulates the lowering of nutrients in the blood. Thus, in muscle, insulin stimulates the uptake and storage of glucose as glycogen. In adipose tissue, insulin stimulates the uptake of glucose and fatty acids and their conversion to intracellular triglycerides, as well as inhibits the breakdown of stored triglycerides. In liver, insulin stimulates the storage of glycogen, as well as inhibits the production of glucose and breakdown of glycogen.

Diabetes mellitus is a heterogeneous disease that is defined by chronically high plasma glucose levels. Today diabetes is recognized as a world health problem. In 2001 it was estimated by the *I*nternational *D*iabetes *F*ederation (IDF) that around 177 million people (~ 5%) have diabetes in the adult population. Approximately 85-95% of all patients with diabetes suffer from type 2 diabetes. The prevalence of the disease has reached epidemic levels in many parts of the world, especially in the western society where it has been coupled to dietary habits and low physical activity. Type 2 diabetes is a complex metabolic disorder that involves insulin secretion abnormalities and defects in the action of insulin on its target tissues. Understanding the underlying molecular mechanisms of insulin secretion and insulin action is therefore of crucial importance.

The pancreatic β-cell is the sole producer of insulin and as such insulin biosynthesis and secretion are highly regulated processes. The main stimulus of insulin release is glucose. However, depending on the demand of the body numerous factors, e.g., hormones and neuropeptides modulate glucose-stimulated insulin release. The increased formation of the second messenger cyclic AMP (cAMP) has been demonstrated to be of crucial importance in mediating the intracellular effects of several insulin secretagogues. The amount of cAMP is determined by the synthesis but also by its degradation, which is catalyzed by cyclic nucleotide phosphodiesterases (PDEs).

In this thesis, the role of PDE3B in insulin release and overall energy homeostasis was studied. To provide a background to this work a short introduction to the field of diabetes is given, which is followed by a more detailed description of the current knowledge on the role of cAMP in insulin release. Moreover, the PDE family in general, and PDEs in relation to regulation of insulin release, will be described.

BACKGROUND

DIABETES MELLITUS

The word "Diabetic" comes from the ancient Greek, meaning siphon or fountain. "Mellitus" refers to honey and sweetness. Diabetes mellitus has been known since time immemorial. The Egyptians wrote about diabetes on papyrus scrolls already 100-200 B.C. Much later, in 1679, diabetes mellitus was described by Thomas Willis (Oxford), but not until 100 years later (1776) the physician Matthew Dobson (Liverpool) speculated that glucose was lost before it could be used in nutrition. It took another 100 years before Paul Langerhans (Berlin) in 1869, identified the islets of Langerhans in the pancreas. It was thought that these cells contained a hormone that would counteract diabetes. This was later confirmed by Mehring and Minkowski (Austria), who removed the pancreas from a laboratory dog that as a result developed diabetes. In 1923, the Nobel price was awarded to Banting and Macleod (Toronto) for their discovery and purification of insulin.

Diabetes mellitus is a generic name for several diseases which are all characterised by chronically high levels of blood glucose. Diabetes can be classified, as suggested by the *W*orld *H*ealth *O*rganization (WHO), according to the clinical stages of the disease or etiologically. The four main etiological groups are:

Type 1 (formerly designated *I*nsulin-*d*ependent *d*iabetes *m*ellitus (IDDM)) accounts for 5-15% of all diabetic individuals. Type 1 diabetes is characterised by an autoimmune destruction of β-cells, which often leads to absolute insulin deficiency.

Type 2 (formerly designated *N*on-*i*nsulin-*d*ependent *d*iabetes *m*ellitus (NIDDM)) is the most common form and occurs in ~ 85-95% of all diabetic patients. Type 2 diabetes is a highly heterogeneous disorder, associated with genetic and environmental factors that involve insulin secretion abnormalities and defects in the action of insulin on its target tissues.

Other specific types include types of diabetes that are uncommon but due to specific causes such as genetic defects (for example MODY (maturity-onset diabetes of the young)), disease of the exocrine pancreas and disease due to infection or chemicals.

Gestational diabetes mellitus (GDM) has an onset of glucose intolerance during pregnancy. GDM patients often return to normal glucose tolerance but are more susceptible to the development of diabetes later in life.

Type 2 diabetes

The prevalence of type 2 diabetes differs greatly depending on region and race but strikes women and males the same. According to IDF, the prevalence of the disease is ~5% in the adult population (3.4 billion people between the ages of 20-79 years). The lowest prevalence is found in countries in Africa, and the highest in the Eastern Mediterranean and Middle East Region, and North America (IDF, 2001). In Sweden, ~4% of the population suffer from diabetes (Farnkvist and Lundman, 2003).

Most of the available evidence favours the view that the type 2 diabetes is a heterogeneous disorder reflected by the interrelated defects of resistance to the action of insulin in target tissues and defective endogenous insulin secretion. The specific mechanisms for reduced insulin sensitivity of the peripheral tissues have been studied extensively but are still not fully understood, although at a molecular level several defects in intracellular insulin signalling have been identified. Furthermore, insulin resistance has been linked to obesity, especially visceral adiposity (Kissebah and Krakower, 1994; Kahn B B and Flier, 2000). Patients with type 2 diabetes often have increased levels of serum free fatty acids (Reaven *et al.*, 1988) and there are compelling evidence that elevated free fatty acids inhibit normal glucose utilization in tissues such as muscle and liver (Randle, 1998). Moreover, long-term exposure of islet β-cells to fatty acids has been shown to impair the insulin secretory response to glucose (Sako and Grill, 1990; Paolisso *et al.*, 1995).

As a result of reduced insulin sensitivity, the β-cells initially compensate by hypersecreting insulin and thereby the plasma glucose levels are maintained at normal levels (Kahn S E *et al.*, 1993). However, with time β-cells fail to produce and secrete insulin to the increased demand, blood glucose levels rise and symptoms of diabetes appear (Kahn B B, 1998). Nutrient-induced insulin secretion is normally biphasic, consisting of a rapid first but transient phase of insulin release, followed by a second phase, which is seen as a slowly developing, and sustained release of insulin. Type 2 diabetes is associated with loss of the first phase of insulin secretion, thought to be of crucial importance for postprandial glucose homeostasis (Luzi and DeFronzo, 1989).

ISLETS OF LANGERHANS

Morphology and cell types

The larger part of the pancreas (98%) consists of exocrine tissue, the main function of which is to produce enzymes for digestion of nutrients. The endocrine part of the human pancreas (2% of the pancreas) weighs only a few grams but consists of at least a million groups of endocrine cells, i.e. the islets of Langerhans. The islets of Langerhans, named after the discoverer Paul Langerhans in 1869, are small groups of endocrine cells (~0.4 to 4 μm in diameter) scattered throughout the exocrine pancreas. The islets are highly vascularized through the pancreatic artery which is drained into the portal vein, thus delivering secreted hormones into the liver. The islets are also innervated by parasympathetic, sympathetic and sensory nerves on or near secretory cells.

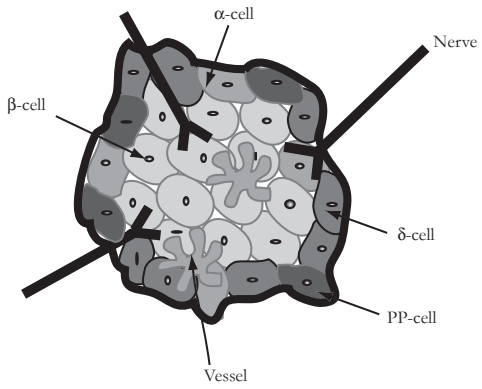

Figure 1. Schematic illustration of the morphology of a pancreatic islet, showing the core of central β-cells and the surrounding α-, δ- and PP-cells, vessels and various nerves.

As illustrated in figure 1, the islet is separated from the exocrine parenchyma by a fine capsule of connective tissue. The size of the human islet varies considerably, ranging from tens of cells to several thousand cells. Four major cell types have been identified; the glucagon-producing α-cell, the insulin-producing β-cell, the somatostatin-producing δ-cell and the pancreatic polypeptide-producing (PP)-cells (also called F-cells). The α-, δ- and PP-cells

are located in the periphery of the islet while the β-cells are situated at the core of the islet and represent ~70% of the islet cells. Apart from the four classical islet hormones, several other peptides, for which the physiological role is not yet fully understood, are produced in the islet cells. For example, the β- and δ-cells produce *i*slet *a*myloid *p*oly*p*eptide (IAPP) (Mulder *et al.*, 1993) and the α-cells produce peptide YY (PYY) (Bottcher *et al.*, 1993). Ghrelin, a newly described peptide produced in the stomach and suggested to affect insulin secretion, was recently also found to be expressed in islets (Wierup *et al.*, 2002).

Insulin biosynthesis

Insulin is transcribed from one single gene in humans while rat and mouse have two functional insulin genes. The expression of insulin is specific for the adult pancreatic β-cells, ascertained by a unique combination of transcription factors which are regulated by several factors such as glucose, growth hormone, GLP-1 and insulin itself (reviewed in (Leibiger *et al.*, 2002; Melloul *et al.*, 2002)). Apart from transcriptional regulation, insulin biosynthesis is further regulated at the level of translation, conversion of preproinsulin to insulin, the vesicular transport to the Golgi and finally the exocytotic machinery.
In short, insulin is synthesised as a preprohormon and the cleavage of the N-terminal signal peptide (directing the molecule to the endoplasmic reticulum) produces proinsulin (Fig 2). Proinsulin is composed of an A-chain (21 amino acids), a B-chain (30 amino acids) and a C-peptide proregion (varies in length from 26-38 amino acids). The A- and B-chains are connected via two disulfide bridges and a third intrachain disulfide bridge is found in the A-chain. Proinsulin is transported via vesicular trafficking through the Golgi and sorted into immature β-cell granules in the cytosol (Goodge and Hutton, 2000). Upon granule maturation, the proinsulin molecules are cleaved yielding biologically active insulin and C-peptide. Insulin-secretory granules have been suggested to almost entirely (>99%) be transported via a regulated secretory pathway to the plasma membrane (Rhodes and Halban, 1987). The final exocytosis of insulin is a highly regulated process and involves several different stages (reviewed in (Barg, 2003)).

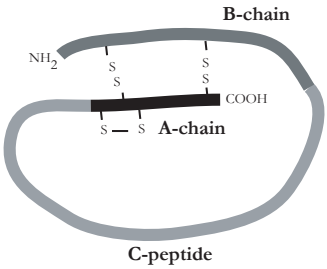

Figure 2. The proinsulin molecule, consisting of the disulfide connected α- and β-chain and the linking C-peptide chain.

INSULIN SECRETION

The complexity of insulin secretion

Insulin secretion from pancreatic islet β-cells is a strictly regulated process (Fig 3). The release of insulin is triggered by intracellular signals derived from the metabolism of nutrients, the most important of which is glucose. Both glucose and fatty acids stimulate insulin release acutely. However, chronic exposure of increased levels can lead to effects described as "glucotoxicity" and "lipotoxicity" (reviewed in (Poitout and Robertson, 2002)).
Nutrient-induced insulin release can be radically modified by a wide variety of extracellular stimuli. In the islet cells, several peptides and hormones are produced some of which have been shown to affect the nutrient-induced insulin release. For example, insulin release is stimulated by glucagon but inhibited by somatostatin. Whether these effects are due to paracrine signalling or cell-to-cell interactions, via gap junctions, is not fully understood (Halban *et al.*, 1982; Meda, 1996). Incretins or gut hormones that are released after meal ingestion potentiate insulin secretion. In humans, the most important incretins known are glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) (Ahrén, 2003). Furthermore, pancreatic islets are innervated by parasympathetic, sympathetic and sensory nerves and, these nerves contain several neurotransmitters or neuropeptides that upon release can modulate nutrient-induced insulin release (reviewed in (Ahrén, 2000)). One

neuropeptide found to potentiate glucose-induced insulin secretion is *p*ituitary *a*denylyl *c*yclase-*a*ctivating *p*olypeptide (PACAP) (Kawai *et al.*, 1992; Filipsson *et al.*, 1999).

In the β-cell, the modulation of nutrient-induced insulin release is mediated by several intracellular second messengers such as cAMP, cGMP, inositol triphosphate (IP3), diacylglycerol (DAG), nitric oxide (NO) and arachidonic acid (AA) (Howell *et al.*, 1994; Ashcroft S J, 1997). In this section I will in short introduce the signalling pathways of glucose-stimulated insulin release and the intracellular pathways involving the second messenger cAMP.

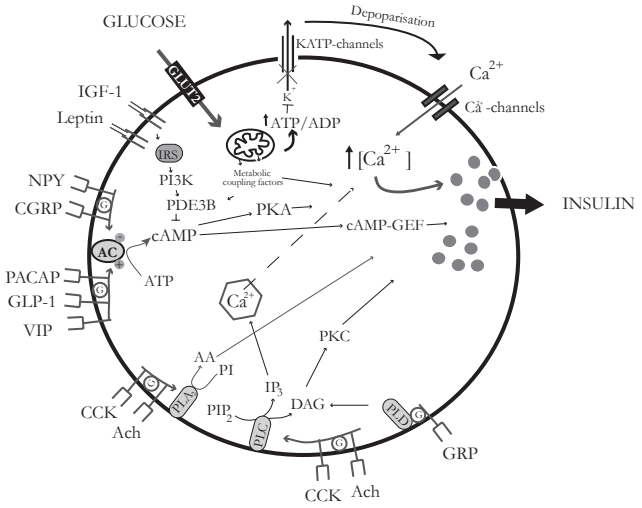

Figure 3. Schematic overview of the complexity of insulin secretion. The illustration shows the glucose-induced signalling pathway which is described in detail in the text. Other pathways illustrated are the AC/cAMP pathway (described in text), PLA_2 -, PLC and PLD-pathway, and the IRS/PI3K-pathway. For abbreviations see page 9. The overview is a modified illustration from Ahrén (Ahrén, 2000).

Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion involves several intracellular signalling pathways that converge to induce pulsatile exocytosis of insulin-containing secretory granules. So far two main pathways have been described, the KATP-dependent- and KATP-independent pathways (Fig 4) (reviewed in (Henquin, 2000; Straub and Sharp, 2002)). KATP is the abbreviation for the ATP-sensitive K^+-channels and in case of the KATP-dependent pathway, the propagation of the signal relies on the closure of this channel.

The KATP-dependent pathway, also called the triggering pathway, is thought to be responsible for the rapid and transient first phase of glucose-stimulated insulin secretion. Glucose enters the cell by facilitated diffusion through the glucose transporter-type 2 (GLUT2) and is phosphorylated by glucokinase, the first enzyme of the glycolytic pathway. The conversion of glucose to glucose-6-phosphate by glucokinase is suggested to be the rate-limiting step of β-cell glucose utilization (Matschinsky, 2002). Glucose is further metabolised through glycolysis and converted to pyruvate which then enters the mitochondria and the tricarboxylic acid cycle. This is followed by the electron transport chain and generation of ATP. The increase in the ATP-to-ADP ratio results in closure of the ATP-sensitive K^+-channels and subsequent depolarisation of the plasma membrane (Ashcroft F M *et al.*, 1984). As a consequence, voltage-dependent L-type Ca^{2+}-channels are opened, resulting in an increase in cytoplasmic Ca^{2+}. The elevated intracellular concentration of Ca^{2+} triggers, by mechanisms still not fully understood, exocytosis of insulin-containing granules. Insulin granules are believed to be present in distinct functional pools (reviewed in (Bratanova-Tochkova *et al.*, 2002)) and the triggering of exocytosis involves several highly regulated processes.

The second suggested pathway by which glucose stimulates insulin release was first demonstrated in 1992 and termed the KATP-independent or amplifying pathway (Gembal *et al.*, 1992; Sato *et al.*, 1992). The amplifying pathway augments the glucose-stimulated secretory response in the presence of elevated intracellular calcium concentrations, independent of the KATP-channels. The pathway can be investigated by the use of diazoxide to activate the KATP channels, and KCl to depolarize the plasma membrane. Under such conditions, glucose augments KCl-induced insulin secretion independent of the KATP channels. The molecular mechanisms underlying the amplifying pathways are not fully understood. However, data indicate that mitochondrial coupling factors are of importance and several candidates have been presented. For example nucleotide messengers such as ATP, GTP and NADPH have all been suggested as possible mediators of glucose-induced insulin release (Gembal *et al.*, 1993; Detimary *et al.*, 1996; Eliasson *et al.*, 1997). Moreover, malonyl-CoA/long-chain acyl-CoA, citrate and glutamate have also been suggested to be

of importance (Corkey *et al.*, 1989; Prentki *et al.*, 1992; Maechler and Wollheim, 1999; Farfari *et al.*, 2000; Rubi *et al.*, 2001). However, the role of glutamate and LC-CoA as mediators of insulin secretion has been questioned (MacDonald and Fahien, 2000; Mulder *et al.*, 2001; Yamada *et al.*, 2001). A KATP-independent pathway not dependent on calcium has also been demonstrated (Komatsu *et al.*, 1995; Komatsu *et al.*, 1997), although a physiological role for a pathway that can operate without a rise in intracellular calcium has not yet been proven. Furthermore, it has been suggested that the second phase of glucose-induced insulin release is the result of the amplifying pathways (Rutter, 2001; Straub and Sharp, 2002) and the rate of the second phase of insulin release is thought to depend on the movement and refilling of the readily releasable pool. A review on insulin granules dynamics has recently been published (Rorsman and Renström, 2003)

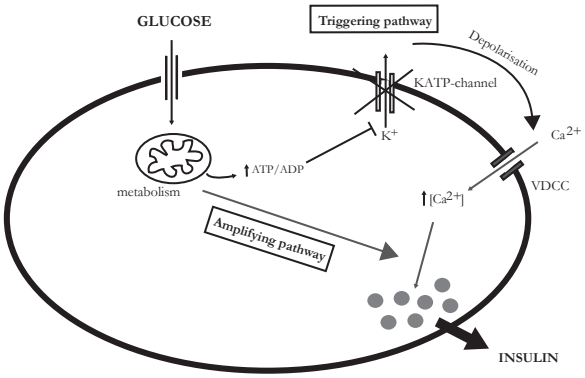

Figure 4. A schematic drawing illustrating the KATP-dependent/triggering and KATP-independent/amplifying pathways of glucose-stimulated insulin secretion in the pancreatic β-cell. The triggering pathway depolarises the β-cell via closure of the ATP-dependent K^+ (KATP)-channel, leading to opening of voltage-dependent Ca^{2+}-channels (VDCC), an increase in intracellular level of Ca^{2+} and stimulation of exocytosis of insulin. The amplifying pathway augments the rate of Ca^{2+}-induced insulin secretion.

Other nutrients such as amino acids and fatty acids are also involved in the regulation of nutrient-induced insulin secretion. Most amino acids need to be metabolised in the β-cells in order to stimulate insulin secretion. However, L-arginine acts directly, by causing depolarisation of the plasma membrane. The role of fatty acids in the regulation of insulin release has gained considerable amount of interest during the last years and their effects are complex (reviewed

in (Prentki *et al.*, 1997; McGarry and Dobbins, 1999; Poitout and Robertson, 2002; Yaney and Corkey, 2003)). In short, fatty acids have been shown to acutely stimulate insulin release, whereas long-term exposure impairs glucose-stimulated insulin secretion.

Release of insulin is pulsatory (reviewed in (Gylfe *et al.*, 2000)). β-cells exhibit oscillations in electrical activity, cytoplasmic Ca^{2+} concentration, and insulin release upon glucose stimulation (Larsson *et al.*, 1996). One suggested explanation for oscillation of insulin secretion is that it results from the pulsatility of the glycolytic pathway, so-called metabolic oscillations (Tornheim, 1997). Interestingly, the pulsatility of insulin secretion has been found to be impaired in patients with diabetes, thus demonstrating the importance of the oscillatory nature of insulin release (Lang *et al.*, 1981; Gumbiner *et al.*, 1996; Porksen *et al.*, 2002).

Role of cAMP in insulin secretion

The cAMP signalling network

cAMP has pivotal roles in signal transduction initiated by a wide variety of extracellular stimuli such as hormones, growth factors, cytokines and neurotransmitters. The level of cAMP is determined by synthesising adenylyl cyclases and cAMP hydrolysing phosphodiesterases (PDEs). When extracellular stimuli bind to and activate transmembrane receptors coupled to G-proteins, adenylyl cyclases are activated or inhibited leading to increased or decreased formation of cAMP. An increased formation of cAMP is the initiation point for several intracellular signalling cascades.
Until recently protein kinase A (PKA) was the only known target for cAMP but now at least two other effectors have been identified, namely, *c*yclic-*n*ucleotide-*g*ated (CNG) ion channels and a family of cAMP-regulated binding proteins. The very first type of CNG channel to be identified was a cGMP-gated ion channel, which was found in retinal photoreceptors already in 1985 (Fesenko *et al.*, 1985; Yau and Nakatani, 1985) This was followed by the discovery of similar CNG channels in olfactory sensory neurons (Nakamura and Gold, 1987) and the pineal gland (Dryer and Henderson, 1991). Since then the family of CNG channels (cAMP and cGMP-gated) has expanded and have been identified in several chemosensory cells, in the brain and also in hepatocytes (Kaupp and Seifert, 2002).
The cAMP-regulated *g*uanine nucleotide *e*xchange *f*actor (cAMP-GEF) also named *e*xchange *p*rotein directly *a*ctivated by *c*AMP (Epac) was identified

simultaneously by two groups in 1998 (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). cAMP-GEF proteins have been identified in several tissues and have so far been implicated in intergrin-mediated cell adhesion, mitosis, neurite outgrowth and in incretin-potentiated insulin secretion (Ozaki *et al.*, 2000; Kashima *et al.*, 2001; Qiao *et al.*, 2002; Bos *et al.*, 2003; Christensen *et al.*, 2003).

In summary, cAMP signalling is complex and involves a number of receptors, cAMP-synthesizing/degrading enzymes, adaptor proteins and other effector proteins (e.g. the *A-k*inase-*a*nchoring *p*roteins (AKAPs)). Thus, in one and the same cell cAMP is believed to be compartmentized and specific cAMP-pools are suggested to regulate different events (Steinberg and Brunton, 2001). A drawing illustrating intracellular cAMP signalling is shown in figure 5.

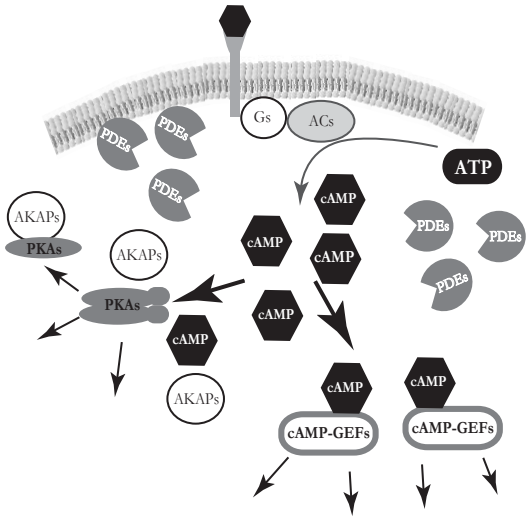

Figure 5. Schematic illustration of subcellular compartmentation of cAMP. Upon binding to G-protein coupled receptors adenylyl cyclases (ACs) are activated leading to the production of cAMP from ATP. cAMP is then either degraded by different members of the phosphodiesterases (PDEs) family or binds to different types of protein kinase A (PKAs) and cAMP-regulated *g*uanine nucleotide *e*xchange *f*actors (cAMP-GEFs). The specificity of PKA action is mediated by a family of *A-k*inase-*a*nchoring *p*roteins (AKAPs). The signal is then propagated via various signalling pathways in the cell.

cAMP-potentiated insulin release

The stimulatory action of several neurotransmitters, hormones and incretins such as PACAP, glucagon and glucagon-like petide-1 (GLP-1) on glucose-mediated insulin secretion are mainly mediated by cAMP (Moens *et al.*, 1996; Filipsson *et al.*, 2001). Most known effects of increased cAMP are mediated by PKA and subsequent phosphorylation of signalling components of the secretory machinery. For example, PKA has been shown to phosphorylate GLUT2 and the KATP-channel subunits Kir6.2 and SUR1 (Thorens *et al.*, 1996; Beguin *et al.*, 1999; Light *et al.*, 2002). Several AKAPs have been identified in β-cells that target PKA to specific subcellular locations, assuring its specific action. For example, PKA phosphorylation of L-type Ca^{2+}-channels has been suggested to rely on the proper location of PKA directed by AKAP (Lester *et al.*, 1997; Fraser *et al.*, 1998). Interestingly, recent data suggest that AKAP co-targets PKA and the calcium/calmodulin protein phosphatase-2B (PP-2B) as a complex, suggesting a strict regulation of reversible phosphorylation events involved in insulin secretion (Lester *et al.*, 2001).
cAMP has been found to stimulate exocytosis via PKA-independent as well as PKA-dependent pathways (Renström *et al.*, 1997; Bode *et al.*, 1999). Renström et al demonstrated rapid and PKA-independent effects of cAMP on exocytosis and speculated about the existence of a "cAMP receptor" in the granule membrane (Renström *et al.*, 1997). Recently, cAMP-binding protein cAMP-GEFII was shown to be a direct target of cAMP in regulated PKA-independent exocytosis of insulin in β-cells (Ozaki *et al.*, 2000; Kashima *et al.*, 2001). Moreover, studies performed in islets from SUR1 knock out mice identified the SUR1 subunit as an important regulator of incretin-potentiated PKA-independent glucose-stimulated insulin secretion (Nakazaki *et al.*, 2002; Eliasson *et al.*, 2003). The SUR1 subunit has been suggested to interact with cAMP-GEFII (Ozaki *et al.*, 2000) and, Eliasson et al hypothesized that cAMP-induced, PKA-independent granule priming is facilitated by the interaction of the SUR1 subunit with cAMP-GEFII (Eliasson *et al.*, 2003).

Role of cAMP in glucose-stimulated insulin secretion

An increase in cAMP in response to glucose was reported in the late 1960s and early 1970s using isolated islets (Malaisse, 1969; Charles *et al.*, 1973; Grill and Cerasi, 1973), and it was suggested that cAMP was a prime mediator of glucose-stimulated insulin release (Grill and Cerasi, 1973; Sharp, 1979). However, since the capacity of glucose to induce insulin release could not be mimicked by cAMP-increasing agents (i.e. adenylyl cyclase activators and non-

specific PDE inhibitors) (Charles *et al.*, 1973; Hellman *et al.*, 1974), the idea of cAMP as an insulin-releasing signal in glucose-stimulated insulin release was discarded. It was instead suggested that glucose could modulate its own action by elevating cAMP. The increase in cAMP in response to glucose was further suggested to be linked to the metabolic activity of glucose since it was found that other substances fed into the glycolytic cycle also increased cAMP (Charles *et al.*, 1973; Hellman *et al.*, 1974). The suggested amplifying function of cAMP in glucose-stimulated insulin secretion has however also been questioned since it was demonstrated that inhibition of PKA, the established target for cAMP, did not impair glucose-stimulated release of insulin (Persaud *et al.*, 1990; Harris *et al.*, 1997). Thus, these results implied that glucose-stimulated insulin release occurs independently of increases in cAMP. However, recent findings have identified new targets for cAMP that are independent of PKA. In β-cells, binding of cAMP to cAMP-receptor proteins, i.e. cAMP-GEFFII, was recently demonstrated to mediate the potentiating effects of incretins on glucose-stimulated insulin release (Nakazaki *et al.*, 2002; Eliasson *et al.*, 2003).

Although the role of cAMP in the regulation of glucose-induced insulin secretion is not established, several groups have coupled impaired glucose-stimulated insulin release to a reduced capacity of glucose to increase cAMP (Holz and Habener, 1992; Laychock, 1995; Dachicourt *et al.*, 1996; Schuit, 1996; Björklund and Grill, 2000). Thus, it is of great importance to further elucidate and establish the potential and seemingly important role of cAMP in glucose-stimulated insulin release.

CYCLIC NUCLEOTIDE PHOSPODIESTERASES

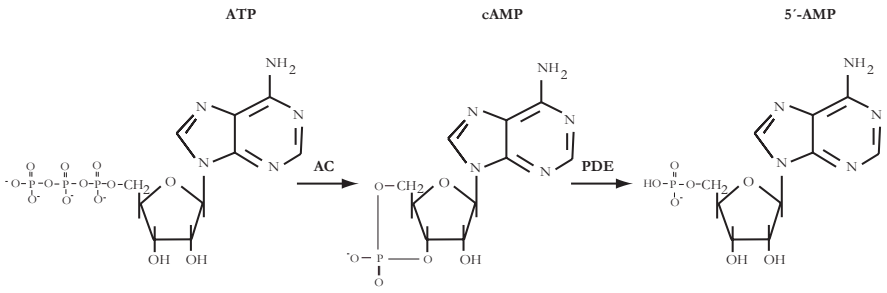

Figure 6. Synthesis and degradation of cyclic adenosine 3′, 5′-monophosphate (cAMP). The cellular content of cAMP is defined by the balance of synthesising enzymes adenylyl cyclases (AC) and by cyclic AMP-degrading phosphodiesterases (PDEs).

The PDE superfamily

PDEs catalyze the hydrolysis of cAMP and cGMP by cleavage of the 3′-phosphodiester bond, generating AMP and GMP, respectively (Fig 6). After the discovery of cAMP and cGMP degrading enzyme-activities in the late 1950s, it was soon evident that PDE represented many different enzymes that could be divided into different families (Butcher and Sutherland, 1962; Thompson and Appleman, 1971). Today at least 11 distinct PDE-families have been identified (Manganiello V.C. and Degerman E., 1999; Fawcett *et al.*, 2000). Most families consist of several subfamilies, in some cases encoded by different genes. The genes encode several protein products generated by alternative transcription and splicing. Thus, PDEs represent a superfamily of enzymes that in concert with other proteins strictly regulate the level of cyclic nucleotides in the cell.

The PDE families differ with respect to their primary structure, sensitivity to specific inhibitors, mechanism of regulation, tissue distribution and subcellular localisation (Manganiello V.C. and Degerman E., 1999). PDEs have in common a catalytic domain. The catalytic domain is conserved between different PDEs and is located in the C-terminal part of the protein. The N-terminal regions show no homology between different PDEs and contain regions that give each PDE unique regulatory properties and intracellular localisation. For example, binding sites for cGMP, Ca^{2+}-calmodulin and proteins are found in this region. Also membrane association regions and sites phosphorylated by various kinases in response to different hormones are located N-terminally.

The discovery of selective inhibitors of PDEs has been a valuable tool for the understanding of the role of specific PDEs in modulating specific biological responses (Manganiello V.C. and Degerman E., 1999) and for the design of drugs. For example, the PDE3 family has been demonstrated to be involved in regulation of insulin secretion, lipolysis and smooth muscle relaxation (Shafiee-Nick *et al.*, 1995; Degerman *et al.*, 1997). PDE4 and PDE5 inhibitors have been used to show an important role for PDE4 and PDE5 in inflammatory responses (Essayan, 2001) and vasorelaxation (Corbin *et al.*, 2002), respectively. Recently, the development of selective PDE5 inhibitors (Sildenafil (Viagra®)) has gained much attention due to its use for the treatment of male erectile dysfunction (Corbin *et al.*, 2002).

In summary, the PDE members present with highly regulated, complex and unique properties.

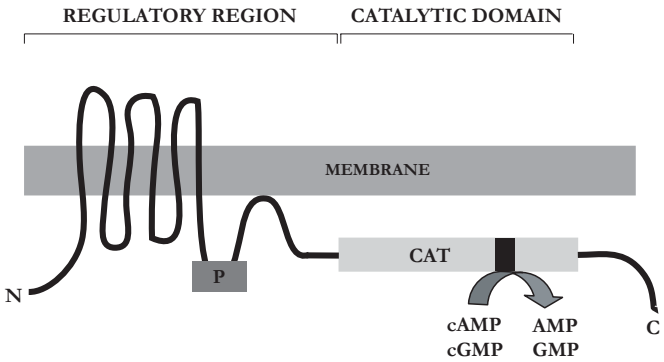

Figure 7. Structural organization of phosphodiesterase 3B (PDE3B). PDE3B contains a region of six transmembrane segments in the N-terminus, phosphorylation-sites (P), a second hydrophobic region and a catalytic (CAT) core near the C-terminus. An insert of 44 amino acids (black box) is found in the catalytic core, specific for the PDE3-family.

The PDE3 family

The PDE3 family, formerly designated cGMP-inhibited (cGI)-PDE, consists of two members; PDE3A and PDE3B. These enzymes are products of two separate genes in humans located on chromosome 12 and 11, respectively (Meacci *et al.*, 1992; Taira *et al.*, 1993; Kasuya *et al.*, 1995). The complementary DNA (cDNA) for both PDE3A and PDE3B have been cloned from various sources (Meacci *et al.*, 1992; Taira *et al.*, 1993; Lobbert *et al.*, 1996; Miki *et al.*, 1996; Choi *et al.*, 2001; Shitsukawa *et al.*, 2001). Although cDNA from PDE3 predicts molecular weights of 120-125 kDa, proteins of 130-135 kDa and 105-110 kDa have been identified.

The overall structural organisation of PDE3 is similar to that of the other known families (Fig 7). However, an insertion of 44 amino acids is found within the conserved catalytic domain of both PDE3A and PDE3B that is specific for the family although it differs between the two members (Degerman *et al.*, 1997). The N-terminal region of both PDE3A and PDE3B contains similar hydrophobic regions believed to be important in membrane association and downstream of these regions are found several consensus sequences for phosphorylation by PKA and protein kinase B (PKB). The role for serine residues localised within these sequences has been most studied in the insulin-induced regulation of adipocyte PDE3B. The sites in PDE3B thought to be

involved in the activation process are (for the rat sequence) Ser 302 (Rahn *et al.*, 1996) and Ser 279 (Kitamura *et al.*, 1999).
PDE3 has high affinity for both cyclic nucleotides (K_m values of 0.1-0.8 μM). However, the V_{max} for cAMP is ~ 4-10-fold higher than for cGMP (Beavo, 1995; Manganiello V. C. *et al.*, 1995). Another defining characteristic of the PDE3 family is its sensitivity to a number of selective inhibitors such as cilostamide, milrinone and imazodan (Komas, 1996). These compounds have IC_{50} values 10 to 100 fold lower for PDE3 compared to other PDE families. By use of such inhibitors, PDE3 has been implicated in the regulation of several cAMP-mediated processes.
The PDE3 isoforms are expressed in several different cell-types such as adipocytes, hepatocytes, smooth muscle cells and pancreatic β-cells (Degerman *et al.*, 1997; Zhao *et al.*, 1997). However, PDE3A and PDE3B exhibit cell-specific different protein expression and subcellular localisation. PDE3B expression is relatively high in cells important for energy homeostasis and localised to the membrane fraction of cells while PDE3A expression is high in the cardiovascular system and usually found in the cytosol (Degerman *et al.*, 1997).

Role of β-cell PDEs in glucose-stimulated insulin secretion

Before and during the production of this thesis, a number of studies have been published with the aim to identify PDE expression in β-cells and to identify the role of different PDEs in the regulation of insulin secretion.
Thus, by use of selective PDE1 inhibitors, several reports have shown the presence of PDE1 activity in islets and cells (Parker *et al.*, 1995; Shafiee-Nick *et al.*, 1995; Han *et al.*, 1999; Ahmad *et al.*, 2000). Although the expression of the PDE1 protein has not been demonstrated, PDE1C mRNA has been identified in insulinoma β-TC3 cells (Han *et al.*, 1999). Results regarding the role of PDE1 in the regulation of glucose-stimulated insulin release differ. Inhibition of PDE1 in mouse islets (Han *et al.*, 1999) but not in human and rat islets (Parker *et al.*, 1995; Shafiee-Nick *et al.*, 1995) has been suggested to potentiate glucose-induced insulin secretion. In cell lines, inhibition of PDE1 in β-TC3 (Han *et al.*, 1999) but not in BRIN-BD11 cells (Ahmad *et al.*, 2000) have been shown to augment glucose-stimulated insulin secretion. Moreover, a combined PDE1 and PDE5 inhibitor has also been used to demonstrate the presence of PDE1/PDE5 activity in insulinoma cells and islets. However, the inhibitor was without effect on glucose-stimulated insulin secretion (Shafiee-Nick *et al.*,

1995; Ahmad *et al.*, 2000). Expression of PDE5 in β-cells has so far not been identified.

The expression of PDE3B protein has been shown in HIT-T15 cells and rat islets (Zhao *et al.*, 1997) and PDE3B mRNA has been shown in BRIN-BD11 cells (Ahmad *et al.*, 2000). Moreover, co-localisation of PDE3B with insulin has been demonstrated in rat islets (Zhao *et al.*, 1997). The role of PDE3 in the regulation of insulin release in response to glucose has been studied using human, rat and mouse islets, and the BRIN-BD11 and βTC3 cell lines (Parker *et al.*, 1995; Shafiee-Nick *et al.*, 1995; El-Metwally *et al.*, 1997; Han *et al.*, 1999; Ahmad *et al.*, 2000). All of these studies except for the studies performed in the β-TC3 cell line, have demonstrated a role for PDE3 in the potentiation of glucose-stimulated insulin release. Moreover, data from *in vivo* experiments have indicated that PDE3 inhibitors act as insulin secretagogues both in rodents and in humans (El-Metwally *et al.*, 1997; Parker *et al.*, 1997; Okada *et al.*, 2002)

The expression of PDE4 in β-cells has only been demonstrated using RT-PCR, showing the presence of PDE4A and 4D in β-TC3 cells (Han *et al.*, 1999). Results on the role of PDE4 in the regulation of insulin in response to glucose vary depending on the cell model used. Thus, in islets inhibition of PDE4 has been shown not to significantly augment insulin release in response to glucose (Parker *et al.*, 1995; Shafiee-Nick *et al.*, 1995) whereas in both β-TC3 and BRIN-BD11 cells, inhibition of PDE4 results in a potentiation of glucose-stimulated insulin release (Han *et al.*, 1999; Ahmad *et al.*, 2000).

In summary, it appears that varying results are obtained depending on model systems, inhibitors and experimental conditions used. However, using selective inhibitors it has become clear that different PDEs are involved in the potentiation of insulin in response to glucose and it seems as if the PDEs involved, so far, include the PDE1, PDE3 and PDE4 families.

PRESENT INVESTIGATION

AIMS

The overall aim of this thesis was to study the importance of β-cell PDE3B in insulin secretion and overall energy homeostasis. Thus, the specific aims were to:

- characterise PDE3 expression, localisation and activity in β-cells.
- evaluate the role of PDE3B in the regulation of β-cell insulin release.
- investigate the physiological importance of β-cell PDE3B in the regulation of insulin release and overall energy homeostasis.

PDE3 expression and role of PDE3B in insulin release (paper I)

Model systems

As model systems in this study, we chose insulinoma cell lines and isolated rat islets. The two cell lines used were INS-1 and INS-1(832/13) cells. INS-1 cells are insulin-producing cells originally isolated from an x-ray-induced rat transplantable insulinoma (Asfari *et al.*, 1992). These cells respond with increased insulin release in response to increased glucose within the physiological range and can be cultured through multiple passages. One drawback with these cells is, however, that they respond heterogeneously to glucose and that the amount of insulin secreted in response to glucose is far less than that of isolated islets. For these reasons we have also used an INS-1-derived clone named INS-1(832/13) (Hohmeier *et al.*, 2000). These cells have been stably transfected with the human proinsulin gene. The INS-1(832/13) cells exhibit markedly enhanced and stable responsiveness to glucose and several other secretagogues. Another important and attractive feature of these cells is that they retain both the glucose-stimulated KATP-dependent and KATP-independent pathway of insulin release. The KATP-independent pathway of glucose-stimulated insulin release is lost in several insulinoma cell lines (Straub and Sharp, 2002). We have also used freshly isolated islets from rat. The method used for isolation of islets is based on a method described by Lacy and Kostianovsky (Lacy and Kostianovsky, 1967). Pancreatic islets represent a more physiological model as compared to insulinoma cells, primarily since they consist of native β-cells but also since the interactions and paracrine effects of the surrounding islet cells are kept intact. One limiting aspect is, however, that islets can not be maintained in culture for longer periods of time.

Expression of PDE3 in β-cells and islets

Endogenous expression of PDE3B in insulinoma cells and isolated rat islets was demonstrated in homogenates using western blot analysis (Fig 1A and B, paper I). The apparent molecular weight of the β-cell PDE3B protein was 135 kDa, identical with the size of the rat adipocyte form of PDE3B. Furthermore, the expression of PDE3B mRNA was detected in insulinoma cell and islets (Fig 1C, paper I). In agreement, in insulinoma BRIN-BD11 cells, an RT-PCR amplified PDE3B cDNA product was reported (Ahmad *et al.*, 2000) and the predicted amino acid sequence of this fragment showed >97% homology with rat adipocyte PDE3B. The PDE3A isoform could not be identified by western blot analysis in neither insulinoma cells nor isolated rat islets. However,

PDE3A mRNA expression was detected in islets but not in insulinoma cells by RT-PCR (Fig 1C, paper I), indicating that PDE3A mRNA expression identified in islets reflects expression in non β-cells.
To study the cellular localisation of PDE3, we prepared membrane and cytosol fractions of insulinoma cells and isolated rat islets. The protein expression of PDE3B was solely found in the particulate fraction (Fig 1D, paper I) and this is in agreement with the previously known location of PDE3B in cells of other tissues (Degerman *et al.*, 1997; Liu and Maurice, 1998). Also, PDE3 activity was found in the particulate fraction in insulinoma cells (~35% of the total membrane PDE activity) (Fig 1D, paper I). However, in islets PDE3 activity was measurable in both membrane and cytosol fractions. PDE3 activity represented ~ 50% of the total membrane-bound PDE activity and 30% of total cytosolic PDE activity (unpublished data). It is possible that the PDE3 activity found in the cytosol of islets represents PDE3A activity present in non β-cells and blood vessels of the islet. In addition to PDE3 activity, also PDE1, PDE4 and non-IBMX inhibited PDE activities were identified in membrane and in cytosol fractions of insulinoma cells and rat islets (unpublished data). In insulinoma cells, PDE1 activity was estimated to constitute ~ 20%, PDE3 activity ~ 35%, PDE4 ~ 30% and non-IBMX inhibited PDE activity ~ 20% of total membrane-bound PDE activity. In the cytosol ~ 60% was represented by PDE1 activity, ~ 30% PDE4 activity and ~ 10% non-IBMX inhibited PDE activity.

Role of PDE3B in insulin secretory processes

To investigate the importance of PDE3B for the stimulatory action of cAMP on insulin release, we increased the specific activity of PDE3B in β-cells and isolated rat islets. To produce overexpression of PDE3B we used recombinant adenovirus. The adenovirus is relatively easily manipulated, generates high titers and infects both quiescent and proliferating cells with high efficiency. In insulinoma cells the estimated efficiency of infection is almost 100% and for isolated islets ~ 70% (Becker *et al.*, 1994; Ferber *et al.*, 1994; Noel *et al.*, 1997). Recombinant adenovirus housing mouse PDE3B cDNA (AdPDE3B) or β-galactosidase cDNA (Adβ-gal, control virus) were generated. By employing adenovirus-mediated gene transfer, PDE3B was efficiently overexpressed in insulinoma cells and isolated rat islets which resulted in enzymatically active and membrane localised overexpression of PDE3B (Fig 2, paper I). We therefore concluded that this was an appropriate model to study effects of increased PDE3B activity on β-cell function in insulinoma cells and islets.

Different levels of overexpression of PDE3B protein and PDE3 activity could be obtained by using different amounts of recombinant adenovirus (Fig 2A and B, paper I). We chose to use an amount of AdPDE3B resulting in a 6-8 fold overexpression of PDE3B activity in insulinoma cells and rat islets as compared to Adβ-gal infected cells and islets. Glucose stimulation of Adβ-gal-infected insulinoma cells and islets resulted in increased insulin release as compared to non-stimulatory concentrations of glucose (Fig 8A). This response was significantly reduced in AdDPE3B-infected islets and in AdPDE3B-infected cells (Fig 8A), indicating that cAMP is involved in the regulation of glucose-stimulated insulin secretion. Measuring cAMP in Adβ-gal-infected cells after glucose stimulation revealed an increased in cAMP concentration (50%). However, in AdPDE3B-infected cells, the cAMP level was decreased (by 60%) as compared to Adβ-gal-infected insulinoma cells (Fig 8B). These results suggest that cAMP is involved in the regulation of glucose-stimulated insulin release and regulated by PDE3B. A role for cAMP in nutrient-induced insulin release was first suggested for more than 30 years ago. This hypothesis was, however, discarded on the basis that cAMP-elevating agents were found not to mimic the effect of glucose and since PKA was found not to influence the stimulatory action of glucose on insulin secretion. Our results again bring forward the role of cAMP in glucose-stimulated insulin secretion.

Furthermore, we studied the effect of the selective PDE3-inhibitor OPC3911 on glucose-stimulated insulin release in insulinoma cells. Stimulation of cells with glucose in combination with OPC3911 resulted in ~ 2-fold increase in insulin release as compared to glucose alone (Fig 7, paper I). This is in agreement with the previously reported potentiating effects on glucose-stimulated insulin release when using PDE3-inhibitors and insulinoma cells (Ahmad *et al.*, 2000).

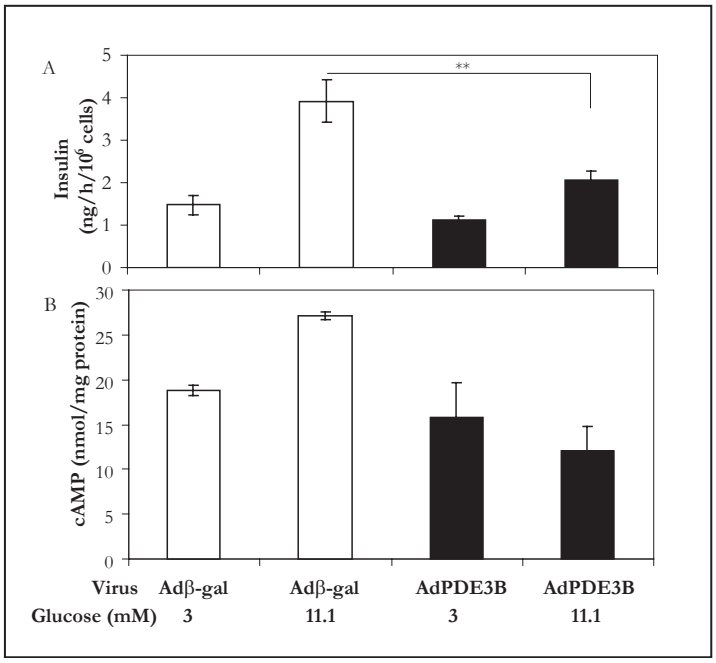

Figure 8. Glucose-stimulated cAMP increase and release of insulin in insulinoma cells overexpressing PDE3B. Insulinoma cells, infected with recombinant adenovirus expressing PDE3B (AdPDE3B) or β-galactosidase (Adβ-gal) were stimulated with glucose at the indicated concentrations for 1 h followed by determinations of insulin secreted into the buffer (A) and of the cAMP content in cell homogenates (B). ** $p<0.01$

GLP-1 is known to enhance glucose-induced insulin release mainly via increased formation of cAMP. We therefore studied the ability of GLP-1 to stimulate cAMP formation and concomitant insulin release in AdPDE3B-infected cells and islets. The potentiating effect of GLP-1 on glucose-induced insulin response was significantly reduced in both AdPDE3B-infected cells and islets compared to control (Adβ-gal infected) cells and islets (Fig 4, paper I). Also, the increase in cAMP in response to GLP-1 was inhibited. These results suggest that PDE3B regulates GLP-1-induced cAMP formation and glucose-stimulated insulin secretion.

Moreover, the stimulatory action of cAMP on Ca^{2+}-elicited exocytosis was investigated in single AdPDE3B-infected β-cells. This was performed in patch clamp experiments using the standard whole-cell configuration (Barg *et al.*, 1999). The basis for this method is that an increase in the cell surface of the

plasma membrane upon exocytosis can be measured as an increase in cell capacitance. Intracellular dialysis of the Ca^{2+}- and cAMP-containing pipette solution induced exocytosis in control (Adβ-gal) cells, whereas in AdPDE3B-infected cells this response was inhibited (Fig 5A and B, paper I). Moreover, when substituting cAMP for the non-hydrolysable cAMP-analogue, Sp-cAMPS, the observed inhibitory effect on exocytosis in AdPDE3B-infected cells was lost (Fig 5C and D, paper I), strongly suggesting that the inhibition observed was due to the overexpressed PDE3B. Furthermore, the effect after the addition of the selective PDE3 inhibitor OPC3911 on cAMP-enhanced Ca^{2+}-elicited exocytosis was investigated. The addition of OPC3911 increased exocytosis by >40% while no effect was detected when replacing cAMP for the analogue Sp-cAMPS (Fig 6A and B, paper I), suggesting that the observed increase in insulin release was due to the inhibition of PDE3. We also investigated the effect of PDE3 inhibition on exocytosis in intact single β-cells. This was done by the perforated patch whole-cell configuration (Ämmalä *et al.*, 1993). Depolarisation-evoked exocytosis was increased by over 200% in the presence of OPC3911 (Fig. 6E and F, paper I) and was not due to a further increase in Ca^{2+} through the plasma membrane (Fig. 6F, paper I), indicating that the main action of cAMP is exerted on the level of the exocytotic machinery.

Main conclusion

- ✓ β-cell PDE3B is involved in the regulation of glucose-stimulated insulin secretion as well as GLP-1 mediated potentiation of glucose-stimulated insulin secretion.

Physiological role of β-cell PDE3B studied in mice (paper II)

Generation of mice with a β-cell-specific overexpression of PDE3B

To study the physiological importance of β-cell PDE3B, mice with a β-cell-targeted overexpression of PDE3B were generated. A transgene construct for β-cell-specific expression of PDE3B was produced by placing full-length mouse PDE3B cDNA under the control of the rat insulin promoter (RIP)2 (Fig 9). Pronuclear microinjection of the construct into one-cell stage zygotes of C57Bl/6 x CBA females was performed (at the Transgenic Core Facility of Karolinska Institute, Stockholm, Sweden), generating the C57BL6xCBA-Tg(RIP-PDE3B)Lsh mice (RIP-PDE3B). In total, the pronuclear injection resulted in eleven founder mice with islet PDE3B protein overexpression ranging from 2-10 fold. We established two lines, one with a 2-3 fold increase (RIP-PDE3B/2 mice) and another with a 7-10 fold increase (RIP-PDE3B/7 mice) in islet PDE3B protein expression and activity. To study the β-cell-specificity of the β-cell-targeted overexpression of PDE3B, we isolated several tissues from RIP-PDE3B mice. By western blot analysis and PDE3 activity measurements (Table 1, paper II) we could conclude that the overexpression of PDE3B was specific for the pancreatic islets, and not found in tissues such as skeletal muscle, liver and adipose tissue. The initial phenotypic characterisation of the RIP-PDE3B mice did not reveal any abnormalities in reproductive capacity or behaviour. Furthermore, there was no difference in food intake or body weight as inspected from birth to adult life. Thus, RIP-PDE3B mice were seemingly normal and healthy.

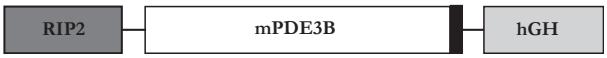

Figure 9. The RIP-PDE3B transgene construct. cDNA for full-length mouse phosphodiesterase 3B (mPDE3B) was placed under the control of the rat insulin promoter (RIP)2, a flag epitope for transgene detection was inserted in the C-terminal end (black box), and to promote transcription, a genomic fragment was included (hGH).

Characterisation of RIP-PDE3B mice

Intravenous glucose tolerance tests (IVGTT) were performed to assess the impact of the β-cell-specific overexpression of PDE3B on insulin release and glucose tolerance. Blood samples taken immediately before the injection of glucose, revealed no difference in plasma glucose or insulin in RIP-PDE3B/2 mice. However, in fasted male RIP-PDE3B/7 mice the basal plasma glucose level was significantly increased compared to wild-type littermates (Fig 10D). Interestingly, the acute insulin response to glucose was markedly reduced in male mice of both transgenic lines (Fig 10). However, no difference in insulin secretory capacity was observed in female mice. In male RIP-PDE3B/2 mice, the acute insulin response was reduced by 70% and in RIP-PDE3B/7 mice, the insulin response was completely abolished as compared to control mice. The reduced level of insulin was associated with impaired glucose tolerance in male RIP-PDE3B mice (Fig 10 B and D). In agreement with the unchanged insulin response to glucose in female transgenic compared to female wild-type mice, no difference in glucose disposal was observed. IVGTTs were performed repeatedly in mice during 6-25 weeks of age and the results were similar, indicating that the differences observed were not age-dependent. Thus, initial IVGTTs revealed a difference in response to glucose between male RIP-PDE3B and wild-type mice but no difference was observed between female RIP-PDE3B and wild-type mice. Gender differences in transgenic mice have been reported in several studies, often leaving the female mice unaffected (Gebre-Medhin *et al.*, 1998; Reisz-Porszasz *et al.*, 2003). We do not yet have an explanation for the gender differences observed in our transgenic mice. In the present investigation all studies were performed on male RIP-PDE3B mice.

Moreover, GLP-1 in combination with glucose was injected intravenously to RIP-PDE3B/7 and wild-type mice (Fig. 4, paper II). In wild-type mice the combination of glucose and GLP-1 resulted in an increased insulin secretory response and increased glucose elimination. While RIP-PDE3B/7 mice were unable to acutely increase the level of insulin in response to glucose, they did respond to glucose in combination with GLP-1. However, the acute insulin response was only half of that observed in wild-type littermates and despite the increase in insulin glucose disposal was not improved as compared to RIP-PDE3B/7 mice injected with glucose alone.

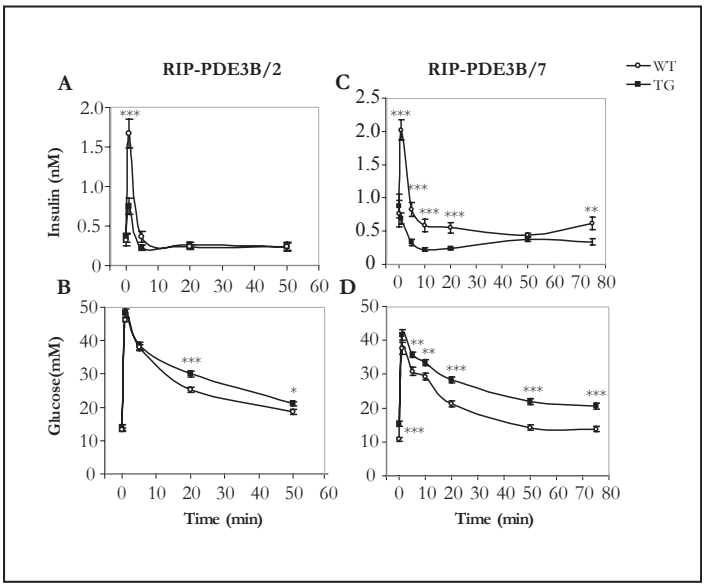

Figure 10. Intravenous glucose tolerance test performed in RIP-PDE3B (TG) and wild-type (WT) mice. Mice were given an intravenous injection of glucose, blood samples were taken before and during the test at the indicated time-points and plasma insulin and glucose concentrations were determined. p*<0.05, **p<0.01 and ***p<0.001.

Characterisation of islet insulin secretion and morphology

Insulin secretory capacity of isolated islets from RIP-PDE3B mice

Islets from transgenic and wild-type mice were isolated and the secretory performance in response to glucose, GLP-1 and the PDE3-selective inhibitor OPC3911 was assessed in static incubations of size matched islets. In wild-type islets, insulin secretion was stimulated by glucose and further potentiated with GLP-1, OPC3911 and GLP-1 in combination with OPC3911, respectively (Fig 5, paper I). In accordance with the reduced insulin response to glucose identified in transgenic mice, the secretory response to glucose and glucose in combination with GLP-1 was significantly reduced in isolated transgenic islets (exemplified by RIP-PDE3B/2 islets in paper I, figure 5A). The observed

reduction in release of insulin in response to glucose and glucose plus GLP-1 was reversed by the addition of OPC3911.
Furthermore, the kinetics of glucose-stimulated insulin release was studied in perifusion experiments with groups of isolated islets from RIP-PDE3B/7 mice. These results demonstrated that there was a marked reduction in the first phase of glucose-stimulated insulin release (Fig. 5C, paper II). However, since mice have a poor second phase of insulin we can not exclude that cAMP also is involved in the second phase of insulin, possibly also regulated by PDE3B.

Morphology of islets from RIP-PDE3B mice

Insulin content was increased in RIP-PDE3B/2 islets as compared to wild-type and RIP-PDE3B/7 islets (Fig. 5B, paper II). Morphological examinations of pancreatic sections revealed that both pancreases of RIP-PDE3B/2 and RIP-PDE3B/7 mice contained mixed populations of large islets. Immunostainings for insulin (Fig 6, paper II) revealed that the expression of insulin was high but unevenly distributed in RIP-PDE3B/2 islets. In islets from RIP-PDE3B/7 mice, a weaker expression of insulin was seen which was even more irregularly distributed than that seen in RIP-PDE3B/2 islets. Moreover, the cytoarchitecture of the transgenic islets was changed (Fig 6, paper II). In RIP-PDE3B/2 islets several glucagon-immunoreactive cells were found in the centre of the islet, and in RIP-PDE3B/7 islets most glucagon-cells were centrally located. Also, the GLUT2 expression was changed in the transgenic islets. Instead of the normal plasma membrane localisation of GLUT2, its expression was to a large extent also found in the cytoplasm. Immunostainings revealed an uneven and reduced expression of GLUT2 which was more pronounced in islets of RIP-PDE3B/7 mice.

Main conclusion

- ✓ β-cell PDE3B is involved in the regulation of whole body energy homeostasis.
- ✓ β-cell PDE3B has a role in the regulation of the first phase of glucose-stimulated insulin secretion.

Impact of high-fat feeding studied in RIP-PDE3B mice (paper III)

To study the impact of overexpressed β-cell PDE3B in the development of obesity and insulin resistance, RIP-PDE3B/2 and wild-type mice were studied after the challenge of a high-fat diet. C57BL/6J mice have an increased susceptibility to high-fat diet-induced obesity and insulin resistance (Surwit *et al.*, 1988; Ahrén *et al.*, 1997). After only one week on high-fat feeding, these mice exhibit hyperglycemia accompanied by hyperinsulinemia, which is indicative of insulin resistance (Ahrén *et al.*, 1997). Thus, these mice are considered a valuable model in the study of high-fat diet-induced insulin resistance.

From 8 weeks of age, RIP-PDE3B/2 and wild-type mice were fed a control diet (11% fat by energy) or a diet rich in fat (58% fat by energy) for 7 months. In RIP-PDE3B/2 mice weight gain was more pronounced already after one week of high-fat diet treatment as compared to wild-type mice, and after 24 weeks of treatment RIP-PDE3B/2 mice had developed severe obesity (Fig 1, table 1, Paper III). The increase in body weight observed in RIP-PDE3B/2 mice as compared to wild-type mice fed high-fat diet was not due differences in food intake. Moreover, high-fat fed RIP-PDE3B/2 mice developed fasting hyperglycaemia and hyperinsulinemia after 8 weeks of treatment (Table 1, paper III). This development was slower in wild-type mice fed high-fat diet and after 24 weeks of high-fat feeding wild-type mice had developed hyperinsulinemia but maintained euglycemia. This result is in contrast to the rapid development of hyperglycemia and hyperinsulinemia reported in C57BL/6J mice (Ahrén *et al.*, 1997). One possible explanation for this discrepancy is that the wild-type mice used in our study are a crossbreed of C57BL/6 mice and CBA mice and, CBA mice do not carry a genetic predisposition to develop high-fat diet-induced type 2 diabetes as is suggested for C57BL/6J mice.

To assess the insulin secretory response and glucose tolerance in wild-type and RIP-PDE3B/2 mice after high-fat feeding, IVGTTs were performed. Intravenous administration of glucose to wild-type mice after 10 weeks of high-fat feeding clearly demonstrated an inability to increase insulin levels in response to glucose which was associated with elevated glucose (Fig 11 A and C). This is in agreement with the previous report in C57BL/6J mice (Ahrén *et al.*, 1997). In contrast, RIP-PDE3B/2 mice fed high-fat diet exhibited an acute insulin response to glucose and kept their insulin level high throughout the test (Fig 11B). Despite the high amounts of insulin, glucose levels were increased in high-fat fed RIP-PDE3B/2 mice as compared to high-fat fed wild-type and control fed RIP-PDE3B/2 mice (Fig 11D). Thus, these results demonstrate that high-fat fed RIP-PDE3B/2 mice have a rapid development of obesity, exhibit basal as well as stimulated hyperinsulinemia and glucose intolerance.

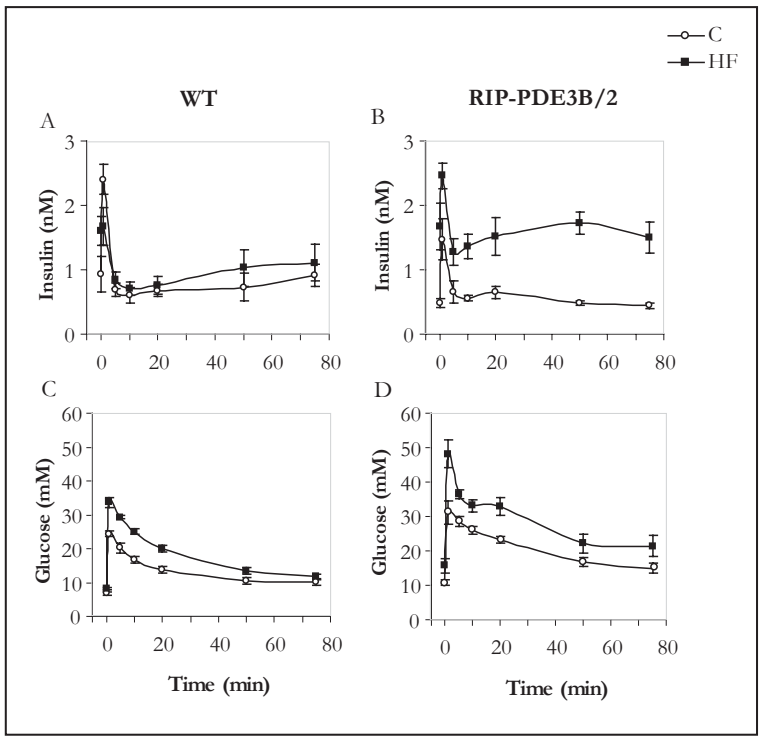

Figure 11. Intravenous glucose tolerance test performed in RIP-PDE3B/2 and wild-type (WT) mice fed control (C) and high-fat (HF) diet. Mice were given an intravenous injection of glucose, blood samples were taken before and during the test at the indicated time-points and plasma insulin (A and B) and glucose (C and D) concentrations were determined.

To study insulin sensitivity in mice fed high-fat diet, we performed insulin tolerance tests. Mice were injected intraperitoneally with a fixed amount of insulin that resulted in comparable insulin levels in transgenic and wild-type mice fed the respective diets (Fig 12 A). Under these conditions, RIP-PDE3B/2 mice fed high-fat diet were incapable of reducing their glucose levels (Fig 12 B). Calculating the glucose elimination rate and the efficiency to eliminate glucose clearly demonstrated an insulin resistant state in the high-fat fed transgenic mice (Table 3, Paper III). Furthermore, we have studied insulin sensitivity of adipose tissue from high-fat fed mice and, preliminary data suggest that insulin-stimulated lipogenesis is reduced in isolated adipocytes of transgenic mice

compared to wild-type mice after high-fat feeding (Fig 7B, paper III). Interestingly, initial experiments also indicate increased insulin sensitivity in adipose tissue from transgenic mice fed control diet compared to wild-type mice fed the same diet (Fig 7A, paper III).

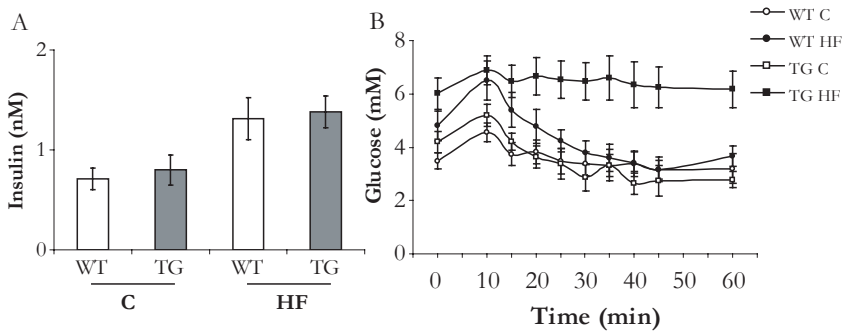

Figure 12. Intraperitoneal insulin tolerance test performed in high-fat fed RIP-PDE3B/2 and wild-type mice. Mice were given an intraperitoneal injection of insulin, blood samples were taken before and during the test. Plasma insulin at 10 minutes post-injection (A) and glucose (B) levels at the indicated time points were determined. WT, wild-type mice; TG, RIP-PDE3B/2 mice; C, control diet; HF, high-fat diet.
.

Islets were isolated from transgenic and wild-type mice after high-fat feeding and the secretory capacity was examined in randomly picked islets. Insulin secretion was increased in transgenic islets already at non-stimulatory glucose levels as compared to wild-type islets, and insulin secretion was further markedly higher in transgenic islets when stimulated with glucose and glucose in combination with GLP-1 (Fig 4A, paper III). Thus, results are in agreement with the observed increased levels of insulin in response to glucose in high-fat fed transgenic mice. Islet insulin content of RIP-PDE3B/2 mice was significantly higher than in wild-type islets when fed a normal control diet and, the high-fat diet did not induce any further change in insulin content (Fig 4B, paper III). However, islet size of transgenic and wild-type mice was increased after the high-fat diet treatment. Wild-type islets from high-fat fed mice were now similar in size to the ones seen in transgenic mice fed control diet, while the transgenic islets from high-fat fed mice were abnormally large. Previously, immunostainings for insulin revealed that the expression of insulin was high

but unevenly distributed in RIP-PDE3B/2 islets. After high-fat feeding, the expression of insulin was further impaired with larger areas lacking insulin immunoreactivity (Fig 5, paper III). Moreover, transgenic mice fed control diet and wild-type mice fed high-fat diet exhibited altered cytoarchitecture with a few centrally located glucagon-cells. These alterations were significantly more pronounced in islet of transgenic mice after high-fat feeding, disclosing accumulation of centrally located glucagon-immunoreactive cells in the vast majority of islets. Furthermore, GLUT2 immunoreactivity was reduced and showed weak membrane staining in transgenic islets from mice fed control diet and in wild-type mice fed high-fat diet. In islets from transgenic mice fed high-fat diet the majority of the β-cells exhibited almost no immunoreactivity for GLUT2.

Main conclusion

- ✓ β-cell PDE3B is involved in high-fat diet-induced development of obesity and insulin resistance.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The main findings in this thesis are that β-cell PDE3B is of importance for the regulation of exocytosis and release of insulin, glucose disposal and islet morphology. These findings raise several questions.

We show that a specific overexpression of β-cell PDE3B activity significantly reduces cAMP and impairs glucose-stimulated insulin secretion, thus bringing forward the role of cAMP in nutrient-induced insulin release. Since insulin release in response to glucose has been suggested not to involve activation of PKA (Persaud *et al.*, 1990) it is therefore possible that cAMP mediates its effect via the newly described cAMP-GEF proteins (Ozaki *et al.*, 2000) or other so far unidentified cAMP activated/binding proteins. How the increase in cAMP in response to nutrients or that of cAMP-elevating agents relates spatially or functionally to β-cell PDE3B is not established. We show that PDE3B is membrane-bound but so far we have not identified the specific location of the enzyme.

Interestingly, the β-cell-specific overexpression of PDE3B in mice (RIP-PDE3B mice) had detrimental effects on *in vivo* insulin release, glucose tolerance and islet morphology. The reduced insulin response to glucose and glucose in combination with GLP-1 and, subsequent reduction in glucose elimination are in line with the previous and present *in vitro* findings of an inhibitory role of PDE3B on insulin secretion. The reduction in glucose-stimulated insulin secretion in isolated islets of RIP-PDE3B/2 mice was demonstrated to be the result of a marked reduction in the first phase of glucose-stimulated insulin release. First phase insulin release is believed to result from the release of a distinct set of insulin-containing granules that are primed and docked for exocytosis, but the mechanisms are not yet fully understood.

Also, when metabolically challenged, RIP-PDE3B mice exhibited an early development of obesity associated with insulin resistance and severe islet dysfunction. The connections between adipose tissue and cAMP-deficient β-cells need to be further explored to reach full understanding. Furthermore, we have preliminary data suggesting reduced insulin sensitivity in adipose tissue of these mice. However, the contribution of muscle and liver to the insulin resistant state of RIP-PDE3B mice after high-fat feeding is so far not known and will be further evaluated.

Finally, whether β-cell PDE3B is involved in β-cell functions other than insulin secretion is not known. The observed alterations in islet morphology are distinct and could be a secondary effect due to insulin deficiency but it could also involve alterations in other signalling pathways relying on cAMP signalling regulated by PDE3B.

To address some of the questions raised by the results of my studies near future goals are to:

- identify the subcellular localisation of PDE3B in β-cells.

- study if PDE3B specifically regulates cAMP pools important for the first phase of glucose-stimulated insulin secretion.

- evaluate if other cAMP-signalling pathways are regulated by PDE3B in the β-cell.

- study the insulin sensitivity of muscle and liver after high-fat feeding of RIP-PDE3B mice.

- investigate possible changes in gene-, mRNA- or protein expression profiles in β-cells from RIP-PDE3B mice as compared to β-cells from wild-type littermates.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Denna avhandling är en s.k. sammanläggningsavhandling och innehåller tre delarbeten. Delarbete I är publicerat, delarbete II är under revision inför publikation och delarbete III är i manuskriptform. Syftet med mina studier har varit att undersöka regleringen av insulinsekretion från de insulinproducerande cellerna i bukspottkörteln med fokus på ett specifikt protein - **fosfodiesteras 3B (PDE3B).**

Bakgrund

Diabetes mellitus, i dagligt tal också kallad sockersjuka, är en globala folksjukdom och förekomsten av sjukdomen har ökat dramatiskt under senare år. Antalet diabetespatienter beräknades år 2001 vara drygt 150 miljoner och beräknas fördubblas till år 2025. Detta enligt siffror från den internationella diabetesfederationen (IDF).
Diabetes är ett samlingsnamn på ett flertal sjukdomar som uppstår till följd av flera olika orsaker men som samtliga slutligen leder till förhöjda nivåer av socker (glukos) i blodet. Diabetes kan delas in i två huvudgrupper; typ 1 diabetes, också kallad insulinberoende diabetes eller ungdomsdiabetes, och typ 2 diabetes, också kallad icke insulinberoende diabetes eller åldersdiabetes. Typ 1 diabetes drabbar cirka 10% av diabetespatienterna och orsakas av brist på hormonet insulin. Insulin är det hormon som signalerar till kroppens vävnader att ta upp och lagra näringsämnen. Typ 1 diabetes uppstår till följd av att vårt eget immunförsvar bryter ner de insulinproducerande cellerna. Typ 2 diabetes drabbar cirka 90% av alla diabetespatienter. Antalet typ 2 diabetiker ökar snabbt i västvärlden, vilket har kopplats samman med ärftliga faktorer, fysisk inaktivitet och övervikt. Orsaken till typ 2 diabetes är inte känd, men symtomen är att utsöndringen av insulin minskar och att de vävnader, muskel, fettväv och lever, som normalt svarar på insulinsignalen blir okänsliga. Denna oförmåga att svara på insulin kallas insulinresistens.
Insulin produceras i bukspottkörteln. Bukspottkörteln är belägen bakom magsäcken och består av två delar: en exokrin och en endokrin. Den exokrina delen producerar pankreassaft som innehåller enzymer vilka är viktiga för nedbrytningen av intagen föda till små beståndsdelar. Den endokrina delen består av små så kallade Langerhanska cellöar. Namnet kommer av dess upptäckare Paul Langerhans (1869). Dessa öar består av ansamlingar av olika typer av celler varav beta-cellerna är de som producerar insulin. Beta-cellerna är de enda cellerna i kroppen som kan producera och utsöndra insulin. Insulin utsöndras som svar på ökade nivåer av näringsämnen i kroppen, som till exempel efter en måltid. Insulin signalerar i sin tur till de insulinkänsliga

målvävnaderna att ta upp och lagra glukos, fettsyror och aminosyror i form av kolhydrater, fett och protein.
Det huvudsakliga stimulit för insulinutsöndring är glukos. Glukosmolekylen tas upp av beta-cellen där den bryts ner, vilket genererar energi i form av ATP. ATP binder till och stänger ATP-känsliga kaliumkanler i cellens cellmembran. Det minskade utflödet av kalium höjer membranpotentialen och leder till öppnandet av spänningskänsliga kalciumkanaler i cellmembranet. Detta leder till ökad koncentration av kalcium inne i cellen som i sin tur leder till insulinutsöndring.
Denna glukosstimulerade insulinutsöndring påverkas av ett stort antal utifrån kommande signaler. Gemensamt för många av de signaler som kan öka den glukosstimulerade insulinutsöndringen är att de verkar via en ökning av cykliskt AMP (cAMP). cAMP är en så kallad ”second messenger” eller budbärare som för signalen vidare in i cellen och påverkar andra proteiners aktiviteter, vilket slutligen leder till en ökad glukosstimulerad insulinutsöndring. Detta kallas cAMP-potentierad glukossstimulerad insulinutsöndring. cAMP nivåerna i cellen regleras av de protein (enzym) som producerar respektive bryter ner cAMP. Enzymer som bryter ner cAMP kallas fosfodiesteraser (PDE). Det finns elva olika familjer av PDE:er och inom varje familj finns det flera subfamiljer.
I min avhandling har jag studerat rollen av PDE3B i regleringen av insulinutsöndring från beta-cellerna.

Resultat

I **delarbete I** studerades uttrycket av PDE3 i odlade beta-celler och i isolerade cellöar från råtta. Vi kunde visa att i beta-celler är PDE3B det huvudsakliga enzymet av de två existerande PDE3 medlemmarna (PDE3A och PDE3B), och att PDE3B återfinns specifikt i membraner av beta-celler. Vidare undersöktes rollen av PDE3B i regleringen av glukosstimulerad och cAMP-potentierad insulinutsöndring. Dessa processer studerades i beta-celler och isolerade öar från råtta som manipulerats så att de hade en ökad mängd PDE3B. Vi kunde visa att en ökad mängd av PDE3B leder till minskad glukosstimulerad- och cAMP-potentierad insulinutsöndring i beta-celler och öar och har direkt hämmande effekter på den proccess som reglerar själva utsläppet av insulin (exocytos). Vi använde oss också av hämmare av PDE3 för att minska mängden aktivt PDE3 och kunde då visa att insulinutsöndringen istället ökade.

I **delarbete II** studerade vi rollen av beta-cell-PDE3B för reglering av energibalansen i möss som manipulerats så att de har ett ökad mängd av PDE3B endast i beta-cellerna (transgena möss). Intravenösa glukostoleranstester utfördes vilket innebär att sövda möss belastas med glukos och under cirka en timme tas flera blodprov för bestämning av insulin- och

glukosnivåer i blodet. I arbete II visade vi att mängden insulin som svar på glukos var minskad i de transgena mössen, vilket var kopplat till en försämrad förmåga att sänka blodsockret. Öar isolerades från dessa möss och vi kunde visa att den minskade mängden insulin var kopplad till en minskad utsöndring av insulin som svar på glukos. Vidare visade vi att den stimulerande effekten av cAMP på glukosstimulerad insulinutsöndring också minskade i både mössen och i de isolerade öarna. Vi fann även att många öar från transgena mössen var större än normalt och att deras cellstruktur var förändrad.

I **delarbete III** studerade vi möss efter att de fått äta en kost rik på fett under en längre tid och så jämfördes transgena och normala kontroll möss. Det var känt sedan tidigare att kontrollmössen utvecklar fetma och insulinresistens som svar på fet kost. Våra transgena möss utvecklade fetma snabbare än kontrolldjuren trots att de inte åt mer än dessa. Vi kunde visa att de transgena mössen utsöndrade höga nivåer av insulin (hyperinsulinemi) men trots detta var glukosnivåerna ändå höga. Detta är ett tecken på att vävnader i kroppen utvecklat en lägre känslighet för insulin, det vill säga insulinresistens. Vi utförde ett så kallat intraperitonealt insulintoleranstest på mössen. Detta innebär att sövda möss ges en injektion av insulin i magen och blodprover tas under en timme för bestämningar av insulin- och glukosnivåer i blodet. Vi kunde visa att de transgena mössen var oförmögna att sänka glukosnivåerna trots insulininjektionen. Detta är en indikation på utveckling av insulinresistens. Vi studerade också öarnas storlek och struktur och fann att öarna från de transgena mössen efter fetkostbehandlingen nu var ännu större och förändringar av cellernas struktur var ännu tydligare än de vi tidigare sett i de transgena djuren på normal kost. Sammantaget tyder dessa resultat på att våra trangena möss med en ökad mängd PDE3B är mer benägna att utveckla fetma och insulinresistens än de normala mössen.

Slutsats

Det övergripande syftet med mina studier var att öka förståelsen för hur insulinutsöndring regleras. Detta är av betydelse för att förstå de defekter som ligger till grund för minskad utsöndring av insulin vid typ 2 diabetes och också för utvecklingen av nya läkemedel som specifikt kan öka utsöndringen av insulin. Slutsatsen av dessa studier är att mängden PDE3B är viktig för regleringen av de cAMP nivåer som påverkar insulinutsöndringen och därmed regleringen av energibalansen i kroppen. Mot bakgrunden av dessa resultat i möss vore utvecklingen av en hämmare mot PDE3B i beta-cellerna en strategi för utvecklandet av nya diabetesläkemedel.

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