Regulation of exocytosis by Ca2+ and cAMP - A study on pancreatic beta- and alpha-cells

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Abstract

Type-2 diabetes is characterized by impaired insulin secretion associated with excess glucagon release. Exocytosis of insulin- or glucagon-containing granules is initiated by Ca^{2+}-influx through voltage-dependent Ca^{2+}-channels and is modulated by the second messengers such as cAMP. Membrane capacitance measurements were used in this study to investigate the cellular mechanisms behind cAMP-regulated Ca^{2+}-dependent exocytosis in pancreatic β- and α-cells.

In pancreatic β-cells, the secretory granules have been demonstrated to co-localize with L-type Ca^{2+}-channels. Experiments using photorelease of caged Ca^{2+} revealed that exocytosis of insulin-containing granules requires an increase in $[Ca^{2+}]_i$ with a K_d of 17 μM, which is most likely achieved in the vicinity of the Ca^{2+}-channels. It was further proved that a close association between the α_{1c} L-type Ca^{2+}-channels and the secretory granules is a prerequisite for rapid exocytosis and ensures maximum usage of Ca^{2+} influx in a cell with few Ca^{2+}-channels (~500) as is the case in the β-cell.

Pharmacologically, L-type Ca^{2+}-channels are suppressed by organic Ca^{2+}-channel blockers such as dihydropyridines. Glacontryphan- M was isolated from the venom of *Conus Marmoreus* and was detected to reduce L-type Ca^{2+} currents and associated exocytosis. It was discovered that this novel antagonist needs the binding of Ca^{2+} to the Gla-residues for its function.

Ca^{2+}-dependent exocytosis in the β-cell is enhanced by cAMP through both PKA-independent granular priming and PKA-dependent recruitment of granules from a reserve pool. This study revealed a concentration-dependent activation of the two cAMP-signalling pathways and that 5-fold higher $[cAMP]_i$ was needed for the PKA-independent mechanism. Interestingly, the sulfonylurea receptor SUR1 was required in the regulation of cAMP-stimulated PKA-independent exocytosis. This is an additional function of SUR1 apart from being a subunit of the K_{ATP} channel.

Enhancement of exocytosis by cAMP in α-cells was discovered to involve similar mechanisms as in the β-cell. In the present study glucagon was detected to elevate cAMP resulting in an enhanced Ca^{2+}-dependent exocytotic response by binding to the glucagon-receptors in the plasma membrane of the α-cell. The stimulatory effect of glucagon was mainly mediated by PKA-dependent mechanisms, possibly due to the inability of glucagon to elevate $[cAMP]_i$ to the sufficient level needed for activation of the PKA-independent pathway.

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Reports Constituting This Thesis

This thesis is a summery of the following papers, which will be referred to by their Roman numerals.

I. Sebastian Barg, Xiaosong Ma, Lena Eliasson, Juris Galvanovskis, Sven O.Göpel, Stefanie Obermüller, Josef Platzer, Erik Renström, Michel Trus, Daphne Atlas, Jörg Striessnig and Patrik Rorsman (2001). Fast exocytosis with few Ca^{2+} channels in insulin-secreting mouse pancreatic B cells. *Biophys J* 81: 3308-3323

II. Karin Hansson, Xiaosong Ma, Lena Eliasson, Eva Czerwiec, Bruce Furie, Barbara C. Furie, Patrik Rorsman and Johan Stenflo (2004). The first γ-carboxyglutamic acid-containing contryphan: A selective L-type calcium ion channel blocker isolated from the venom of conus marmoreus. *J Biol Chem* 279: 32453-32463

III. Lena Eliasson*, Xiaosong Ma*, Erik Renström*, Sebastian Barg, Per-Olof Berggren, Juris Galvanovskis, Jesper Gromada, Xingjun Jing, Ingmar Lundquist, Albert Salehi, Sabine Sewing and Patrik Rorsman (2003). SUR1 regulates PKA-independent cAMP-induced granule priming in mouse pancreatic B-cells. *J Gen Physiol* 121: 181-197

IV. Xiaosong Ma, Yang Zhang, Jesper Gromada, Sabine Sewing, Per-Olof Berggren, Karsten Buschard, Albert Salehi, Jenny Vikman, Patrik Rorsman and Lena Eliasson (2004). Glucagon stimulates exocytosis in mouse and rat pancreatic α cells by binding to glucagon receptors. *Submitted*

* Equal contribution to the study

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	cytosolic free calcium concentration
$[cAMP]_i$	intracellular adenosine 3',5'-cyclic monophosphate concentration
cAMP-GEF II	cAMP-guanosine nucleotide exchange factor II
Gla	γ-carboxyglutamic acid
GLP-1	glucagon-like peptide-1
GLUT-2	glucose transporter 2
G-protein	GTP-binding protein
IRP	the immediately releasable pool
K_{ATP}-channel	ATP-regulated potassium channel
PKA	protein kinase A
RRP	the readily releasable pool
SUR1	sulfonylurea receptor protein 1

1. Introduction

1.1. Regulation of blood glucose

Carbohydrates and fat serve as the main fuels to maintain normal metabolism and to respond to stress, starvation and exercise. Glucose constitutes the major energy supplier for most tissues, most notably the brain. Normally, the blood-glucose level is balanced by the uptake of glucose into peripheral tissue and the entry of glucose into the bloodstream. The main hormones responsible for the control of blood glucose are insulin and glucagon released from the islets of Langerhans, the endocrine part of the pancreas. Insulin reduces blood glucose by facilitating glucose uptake into muscle and fat and by suppressing glycogenolysis and gluco-neogenesis in the liver. Glucagon has a hyperglycemic action by increasing glucose output from the liver. Any alteration in the islet hormone secretion, especially insulin from β-cells, has a profound impact on glucose homeostasis: excessive secretion of insulin causes hypoglycemia and insufficient secretion leads to diabetes. Diabetes can be primarily divided into two main classes (National Diabetes Data Group, 1979), "insulin-dependent diabetes" (IDDM or type-1) and "non-insulin-dependent diabetes" (NIDDM or type-2). The former arises from an autoimmune attack on the pancreatic β-cells that leads to their complete destruction; the latter is because of impaired insulin secretion and insulin resistance in peripheral tissues. Increased obesity and a Western life style have led to an accelerating increase in the number of people with type-2 diabetes and it is predicted to affect 300 million people worldwide by 2005 (King et al., 1998; Zimmet et al., 2001). Abnormalities in insulin secretion occur at an early stage of type-2 diabetes (Kahn, 2003; Ashcroft & Rorsman, 2004). Glucose-stimulated insulin secretion normally has two phases: a rapid first phase which last ≤ 10 minutes followed by a sustained second phase. The disturbed insulin secretion in type-2 diabetic patients is characterized by the absence of the first phase of insulin release and a reduced second phase (Cerasi, 1992). Type-2 diabetes is also associated with an excessive glucagon-secretion that exacerbates the hyperglycemia (Unger, 1971). Knowledge of the molecular and cellular mechanisms behind normal secretion of the pancreatic hormones is therefore central to the understanding of the pathogenesis of type-2 diabetes.

1.2. Interactions in the islet of Langerhans

The islet of Langerhans is a complex microorgan that contains ~1000 cells. A majority (60-80%) of these are insulin-secreting β-cells situated in the islet core. The core of β-cells is surrounded by a mantle that consists of the less abundant glucagon-secreting α-cells (20%) together with somatostain-secreting δ-cells (5-10%) and pancreatic polypeptide secreting PP-cells (1%). Nutrients, hormones, and neurotransmitters control islet secretion. The ability of circulating hormones and nutrients to reach their target cells is facilitated by the abundant vascular supply in the islet. Neurotransmitters are released from parasympathetic, sympathetic and various peptidergic nerves. In addition, secretion from the islet cells is regulated by paracrine and autocrine influences (Fig.1); i.e. secretion from a cell act on neighboring cells or affects its own secretion, respectively. Insulin suppresses both glucagon and somatostatin secretion. Somatostatin inhibits secretion from the α- and the β-cell. Conversely, glucagon from the α-cell stimulates both somatostatin and insulin secretion. It has been demonstrated that the β-cell can modulate its own release. The autocrine effect on β-cells remains controversial and there are reports in favour for both stimulation (Aspinwall *et al.*, 1999) and inhibition (Khan *et al.*, 2001) of insulin secretion. A possible stimulatory autocrine mechanism mediated by glucagon in the α-cell is discussed in paper IV.

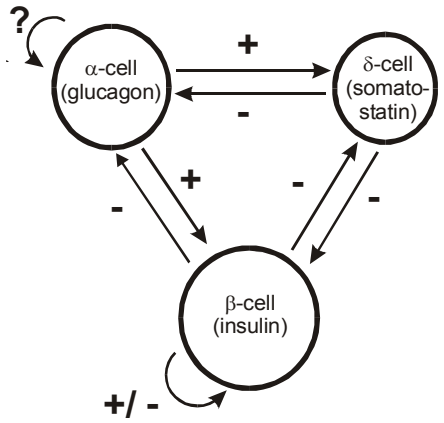

Figure 1. Effects of islet hormones on α-, β-, and δ-cells. Paracrine and autocrine regulation within the islet. Stimulation is marked by + and inhibition by −. The question mark indicates the possibility of autocrine regulation of glucagon secretion as discussed in paper IV.

1.3. Glucose-stimulated insulin secretion

Insulin is produced in the pancreatic β-cells where it is stored in the secretory granules until released into the bloodstream by regulated exocytosis. The primary stimulator of insulin release is glucose. The β-cells are electrically active and use this property to couple elevated blood glucose to stimulated insulin secretion. The electrical activity in mouse β-cells is generated at glucose-concentrations ≥7 mM and consists of slow oscillations in membrane potential upon

which bursts of Ca^{2+}-dependent action potentials are superimposed (Henquin & Meissner, 1984). The changes in electrical activity lead to oscillations in the cytoplasmic Ca^{2+}-concentration (Gilon & Henquin, 1992), which drives pulsatile insulin secretion (Gilon *et al.*, 1993; Bergsten, 1995; Barbosa *et al.*, 1998).

The transduction pathway linking changes in glucose concentration to electrical activity and insulin secretion is well established (Fig. 2). Glucose is taken up by the β-cell via the glucose transporter GLUT-2 and is metabolized in the mitochondria to produce ATP at the expense of ADP. The increased ATP/ADP ratio leads to the closure of ATP-sensitive K^+-channels (K_{ATP} channels) in the plasma membrane. These channels are octamers consisting of two subunits (the inward rectifier K^+-channel Kir6.2 subunit and the sulfonylurea receptor subunit SUR1) assembled at 4:4 stoichiometry (Ashcroft & Gribble, 1998). The K_{ATP} channels are responsible for maintaining the resting membrane potential at ~ -70 mV. Closure of the K_{ATP} channels leads to depolarisation of the β-cell. This is followed by activation of voltage-dependent L-type Ca^{2+}-channels and influx

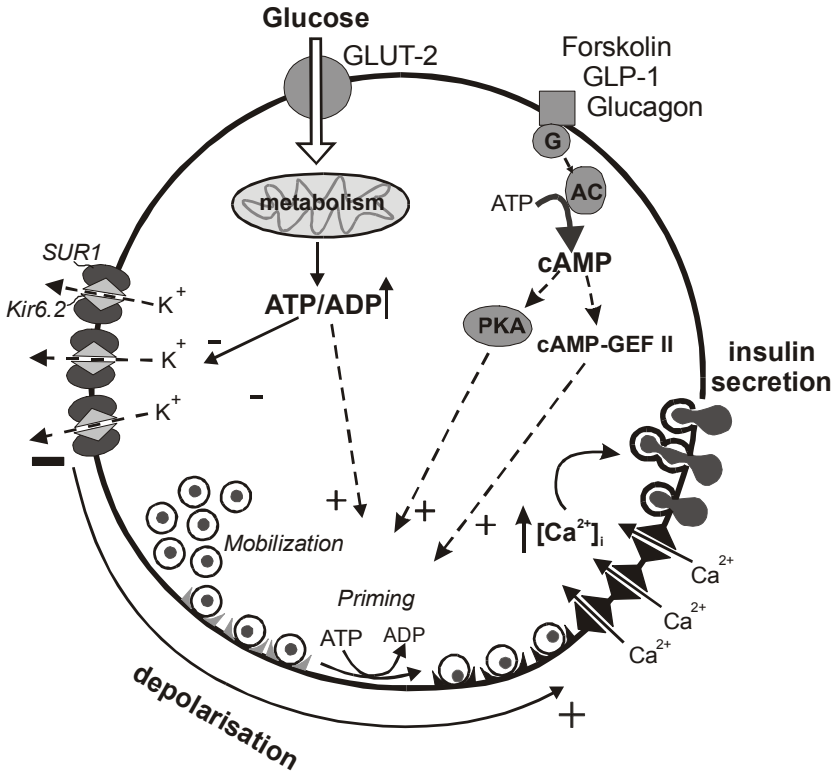

Figure 2. Interplay of signal transduction pathways in pancreatic β-cells. Demonstration of the triggering (filled arrows) and amplifying (open arrows) pathways in the β-cells. Indicated are also the signaling pathways by which cAMP enhances exocytosis. These include activation of PKA (protein kinase A) and cAMP-GEFII (cAMP-guanonine nucleotide exchange factor II). Abbreviations used: GLUT-2: glucose transporter 2; SUR1: sulfonylurea receptor 1; Kir 6.2: inward rectifier K^+ channel 6.2; G: G-protein coupled receptor; AC: adenylate cyclase;.

of Ca^{2+}. The resultant increase in $[Ca^{2+}]_i$ stimulates exocytosis of the insulin-containing granules and release of insulin (Ashcroft & Rorsman, 1989; Lang, 1999; Rorsman & Renström, 2003).

The mechanism outlined above is regarded as the K_{ATP}-channel dependent or triggering pathway of glucose-induced insulin secretion (Henquin, 2000). During the last ten years, increasing studies have suggested that K_{ATP} channels are not the sole site for regulation of insulin secretion by glucose (Gembal *et al.*, 1992; Sato *et al.*, 1992; Chan & MacPhail, 1996). Under conditions as when K_{ATP} channels are kept open by diazoxide combined with high K^+ to stimulate Ca^{2+}-influx (Gembal *et al.*, 1992), or when K_{ATP} channels are completely closed by maximally effective concentration of sulfonylureas (Sato *et al.*, 1999), glucose is still able to increase insulin secretion independently of further associated changes in membrane potential or $[Ca^{2+}]_i$. This K_{ATP}-channel independent pathway by which glucose augments insulin release is now often referred to as the amplifying pathway of glucose-induced insulin secretion (Aizawa *et al.*, 1992; Henquin, 2000). Its activations is dependent on a rise in $[Ca^{2+}]_i$ and therefore it remains silent until the triggering pathway depolarises the membrane and elevates $[Ca^{2+}]_i$. The significance of the amplifying pathway is to optimize the secretory response induced by the triggering signals and it has been suggested to be responsible for the second phase of glucose induced insulin secretion, while the triggering pathway contributes to the first phase of insulin release (Henquin, 2000).

Nucleotides play crucial roles for the two pathways (Fig. 2). Except for the important reciprocal roles of ATP and ADP on the K_{ATP} channel, these nucleotides are able to directly modulate the exocytotic process (Eliasson *et al.*, 1997; Rorsman & Renström, 2003). Capacitance measurements using flash photolysis of the caged nucleotides have demonstrated that ATP and ADP have opposite actions on exocytosis (Eliasson *et al.*, 1997; Barg *et al.*, 2002a). It has been suggested that the suppression of the amplifying action by ADP is the more important one and that ATP is a permissive factor (Rorsman & Renström, 2003). This is based on the observation that changes in ADP within the physiological range exert stronger effects on exocytosis than variations of ATP-concentration (Olsen *et al.*, 2003).

Incretins, such as GLP-1 and glucagon, enhance glucose-stimulated insulin secretion by the generation of intracellular cAMP. This is accomplished by G-protein-coupled activation of adelylate cyclase. The intracellular

concentration of cAMP in the cell represents the balance between production through adenylate cyclase and breakdown by the cyclic nucleotide phosphor-diesterases (PDE). Cyclic-AMP is involved in multiple signalling pathways in the β-cell, among which one is the activation of protein kinase A (PKA). Several activities in the β-cell are stimulated by activation of this pathway, including enhancement of electrical activity (Henquin & Meissner, 1983), increasing influx of Ca^{2+} through voltage-dependent Ca^{2+}-channels (Ämmälä *et al.*, 1993a) and potentiation of exocytosis (Ämmälä *et al.*, 1993a; Renström *et al.*, 1997). Another pathway by which cAMP can increase insulin secretion is through the cytosolic cAMP-binding protein, cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFII or Epac; see Holz, 2004). This PKA-independent interaction involves enhancement of the exocytotic process (Renström *et al.*, 1997; Ozaki *et al.*, 2000; Paper III). It has also been proposed that Epac modulates Ca^{2+}-signalling by promoting mobilization of Ca^{2+} from intracellular stores by activation of Ca^{2+}-induced Ca^{2+} release in INS-1 cells and human β-cells (Kang *et al.*, 2001; Kang *et al.*, 2003). By contrast, cAMP has been demonstrated to regulate Ca^{2+}-induced Ca^{2+} release by activation of PKA in rodent β-cells (Dyachok & Gylfe, 2004).

1.4. Regulation of glucagon release

Although studies of the mechanisms by which nutrients stimulate insulin secretion have made great progress in β-cells, much less is known about the mechanism by which nutrients modulate glucagon secretion. Like β-cells, α-cells are electrically excitable and they generate action potentials in the absence of glucose. In Fig. 3, a suggested model explains the stimulus-secretion coupling in the α-cell. The glucose transporter GLUT-1 (Heimberg *et al.*, 1996) mediates the uptake of glucose into the cell. The electrical activity in α-cells is mainly dependent on Na^+-currents but T-, N-, L-type Ca^{2+}channels are also involved

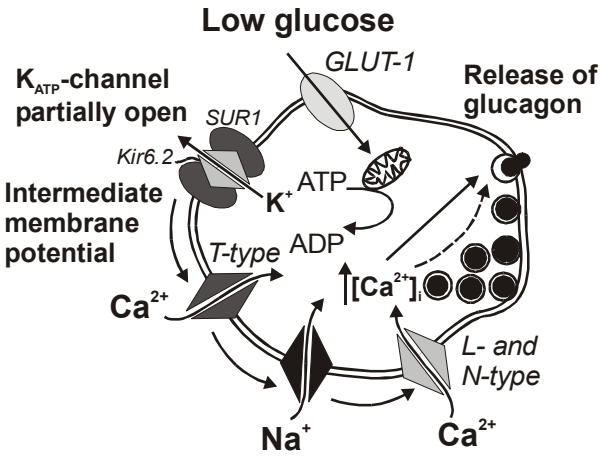

Figure 3. Model of stimulus secretion coupling in α-cells. At low glucose concentration, the K_{ATP}-channels are partially open resulting in an intermediate membrane potential and generation of Ca^{2+}- and Na^+-dependent action potentials.

(Göpel *et al.*, 2000). Unlike the L-type Ca^{2+}-channels, the Na^{+}-channels undergo voltage-dependent inactivation, i.e. they enter a non-conducting state when the membrane is depolarised. The presence of inactivating Na^{+}-channels in the mouse α-cell is central to this model.

In addition, the α-cell is also equipped with K_{ATP} channels of the same type as in the β-cell (Ronner *et al.*, 1993; Bokvist *et al.*, 1999; Suzuki *et al.*, 1999). Already at low glucose, the ATP/ADP ratio in α-cells has been reported to be relatively high (Detimary *et al.*, 1998), thus allowing only partial activation of the K_{ATP} channels. As a result, the membrane potential is kept sufficiently depolarised to allow regenerative electrical activity (Gromada *et al.*, 2004) whilst preventing voltage-dependent inactivation of the Na^{+}-channels. The generation of action potentials leads to influx of Ca^{2+} through L- and N-type Ca^{2+}-channels, which stimulate exocytosis of glucagon secreting granules. When glucose is increased, the K_{ATP} channels become fully inhibited. This results in a stronger depolarisation followed by inactivation of the Na^{+}-channels and reduction of the electrical activity (Göpel *et al.*, 2000).

Several other hypotheses of the metabolic regulation of the α-cell have been suggested. These include paracrine inhibition by somatostatin by neighbouring δ-cells (Cejvan *et al.*, 2003) and Zn^{2+} co-secreted with insulin (Ishihara *et al.*, 2003). Another model, based on measurements on rat α- and β-cells, suggests that GABA secreted from synaptic-like microvesicles in the β-cell exerts an inhibitory action on the α-cell (Rorsman *et al.*, 1989; Braun *et al.*, 2004; Wendt *et al.*, 2004). Finally, it has been proposed from measurements of $[Ca^{2+}]_i$ in mouse α-cell that the regulation of α-cell electrical activity by glucose involves the modulation of a store-operated current (Liu *et al.*, 2004b). This current is activated at low glucose leading to a small depolarisation and activation of action potentials. Once the glucose concentration increases the intracellular Ca^{2+} stores are refilled, the store-operated channel is closed, the membrane is repolarised and glucagon-secretion is inhibited.

1.5. Ca^{2+}-dependent exocytosis

Secretion of insulin and glucagon from pancreatic β- and α-cells proceed through Ca^{2+}-dependent regulated exocytosis (Wollheim & Sharp, 1981; Jones *et al.*, 1985; Pipeleers *et al.*, 1985a; Rorsman *et al.*, 1991; Ämmälä *et al.*, 1993b). This is similar to the situation in neurons and neuroendocrine cells (Neher, 1998). It is believed that Ca^{2+} influx through voltage-

gated Ca^{2+}-channels in the plasma membrane is the most important determinant of the elevated $[Ca^{2+}]_i$ needed for exocytosis in α- and β-cells (Ämmälä *et al.*, 1993b; Gromada *et al.*, 1997a).

1.5.1. Different types of Ca^{2+}-channels

Multiple types of Ca^{2+} currents have been identified by their physiological and pharmacological properties (Tsien *et al.*, 1988; Bean, 1989; Hess, 1990). The initial purification studies revealed that the Ca^{2+}-channel is composed of a pore-forming α_1-subunit; an intracellular β subunit; a transmembrane disulfide-linked complex of α_2- and δ-subunits and a transmembrane γ-subunit (Curtis & Catterall, 1984; Hosey *et al.*, 1987; Leung *et al.*, 1987; Striessnig *et al.*, 1987; Takahashi *et al.*, 1987). Based on the primary structure of α-subunits, the voltage-gated Ca^{2+}-channels have been divided into three subfamilies (Catterall, 2000). The Ca_v1 family of α_1-subunits gives rise to L-type Ca^{2+} currents. Electrophysiologically, these types of currents are named L-type because they have a large conductance and long-lasting currents. L-type Ca^{2+}currents regulate muscle contraction, hormone secretion and gene transcription. This class of Ca^{2+} currents can be selectively blocked by dihydropyridines, phenylakylamines and benzothiazepines. Recently, venom toxins have been identified to affect L-type currents (de Weille *et al.*, 1991; Mintz *et al.*, 1991; Paper II). The Ca_v2 family of α_1-subunits conducts N-type, P/Q-type and R-type Ca^{2+} currents, which mostly exist in neurons. This class of Ca^{2+} currents elicits rapid synaptic transmission and is sensitive to peptide toxins. The Ca_v3 family of α_1-subunits conducts T-type Ca^{2+} currents, which are named by their transient kinetics and (usually) tiny amplitude. They are activated and inactivated more rapidly than other Ca^{2+}-current types. T-type currents are involved in the shaping of the action potentials and controlling patterns of repetitive firing in a wide variety of cell types.

Many of the Ca^{2+}-channel subtypes are expressed in the islets of Langerhans as detected by RT-PCR (Vajna *et al.*, 1998) or Northern-blotting (Perez-Reyes *et al.*, 1998). The majority (~50%) of the voltage-dependent Ca^{2+}-current in the mouse β-cells is carried by dihydropyridine-sensitive L-type Ca^{2+} currents (Rorsman & Trube, 1986; Rorsman *et al.*, 1988; Bokvist *et al.*, 1995; Schulla *et al.*, 2003) and the presence of both $Ca_v1.2$ (Paper I, Schulla *et al* 2003) and $Ca_v1.3$ (Yang *et al.*, 1999; Liu *et al.*, 2004a) have been reported. The remaining current is carried by Ca^{2+} through Cav2.1 (P/Q-type) and $Ca_v2.3$ (R-type) Ca^{2+}-

channels (Schulla *et al.*, 2003). The L-type Ca^{2+}-channels have been suggested to be closely linked to the secretory granules in the β-cell (Bokvist *et al.*, 1995; Wiser *et al.*, 1999), an aspect that is discussed further in paper I.

In the α-cell, electrophysiological experiments have revealed the presence of at least two types of Ca^{2+} current, where one of them have the characteristics of the low-threshold T-type current (Rorsman, 1988; Göpel *et al.*, 2000). Other Ca^{2+}-currents described to be present in the α-cell are conducted through N- and L-type Ca^{2+}-channels (Gromada *et al.*, 1997a; Barg *et al.*, 2000). Interestingly, exocytosis in the α-cells is dependent on the influx through L- and N-type Ca^{2+}-channels under different experimental conditions. Data derived from capacitance measurement in rat (Gromada *et al.*, 1997a) and mouse (Ma *et al.*, 2003) α-cells demonstrate that N-type Ca^{2+} currents are more important for basal glucagon release, while L-type Ca^{2+}-channels are mainly responsible for cAMP-stimulated exocytosis in α-cells.

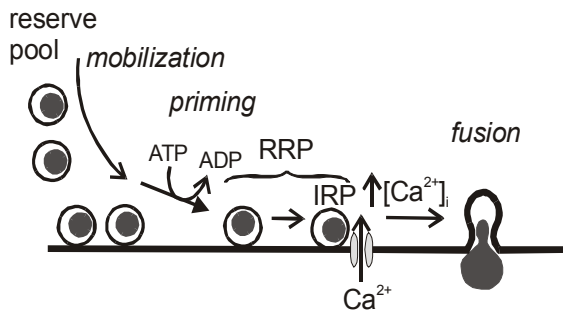

Figure 4. Model of Ca^{2+}-dependent exocytosis. Prior to exocytosis the secretory granules are divided into different pools with different release kinetics. Granules residing in a reserve pool must be translocated to the plasma membrane, docked and primed prior to release. Primed granules belong to a readily releasable pool (RRP). A subpool of RRP, the immediately releasable pool (IRP) is situated close to the Ca^{2+}-channels. Localized increase in $[Ca^{2+}]_i$ triggers the release of primed granules.

1.5.2. Kinetics of exocytosis

Ca^{2+}-induced exocytosis measured as an increase in membrane capacitance from a variety of neural and endocrine cells display at least two kinetic components (Heinemann *et al.*, 1993; Voets *et al.*, 1999). Analyses of these measurements have led to the proposal that granules prior to release belong to different functional pools (Neher, 1998; Voets *et al.*, 1999; Fig. 4). A fraction of the granules (1-5%) reside in a readily releasable pool (RRP). These granules are regarded as release competent because of their ability to undergo exocytosis immediately upon Ca^{2+}-influx. A subpool of RRP, the immediately releasable pool (IRP), is situated in the close vicinity of the Ca^{2+}-channels (Voets *et al.*, 1999). The remaining granules form a reserve pool (RP). These granules need to be mobilized and/or primed prior to fusion with the plasma membrane. Priming involves several ATP-, Ca^{2+}- and temperature-dependent maturation steps (Bittner & Holz, 1992; Renström *et al.*, 1996;

Eliasson *et al.*, 1997). Additionally, in order for fusion to occur, it is necessary that SNARE-complexes assemble during the priming process (Lin & Scheller, 2000; Bruns & Jahn, 2002). The SNARE-complexes consist of the plasma membrane proteins syntaxin 1 and SNAP-25 (synaptosomal protein of 25 kDa), and the vesicular protein VAMP (Vesicular Associated Membrane Protein or synaptobrevin). The complexing of these proteins brings the vesicular membrane in close contact with the plasma membrane analogues to a zipper (Lin & Scheller, 2000; Bruns & Jahn, 2002).

Can biphasic insulin secretion be explained by the existence of distinct functional pools of granules? Ultrastructural, electrophysiological and molecular investigations have indeed revealed the presence of distinct pools in the pancreatic β-cell (Daniel *et al.*, 1999; Barg *et al.*, 2002a; Olofsson *et al.*, 2002). The granules in RRP are released independently of ATP (Eliasson *et al.*, 1997), whereas the subsequent release of insulin granules from the reserve pool requires the metabolic nucleotide. This is reminiscent of the two phases of insulin secretion (Gembal *et al.*, 1993). Thus, initiation of the K_{ATP}-channel dependent (or triggering) pathway discussed above, results in exocytosis of the readily releasable pool that is responsible for the first phase of glucose-stimulated insulin secretion (Rorsman *et al.*, 2000). In addition, activation of the amplifying pathway leads to recruitment of granules from RP that underlies the second phase of glucose-stimulated insulin secretion. RRP can be depleted within a second (paper I), whereas refilling of RRP takes up to more than a minute (Gromada *et al.*, 1999; Barg *et al.*, 2002b). Thus, granule priming, necessary for the conversion of reserve pool granules to readily releasable granules, is the rate-limiting step in second phase of insulin secretion.

What about the kinetics of exocytosis in the α-cells? In α-cells, RRP is larger (~120 granules compared to ~50 granules in the β-cell; Barg *et al.,* 2000; Barg *et al.,* 2002a; Eliasson *et al.,* 1997) and replenishment of RRP granules is faster (Barg *et al.*, 2000). These characteristics result in the faster and more pronounced exocytotic response. In addition, when exocytosis in α-cells is induced by protracted stimulation, a strong secondary acceleration of exocytosis can be observed. This is due to quick depletion of RRP followed by rapid refilling. Possibly these features contribute to the ability of the α-cells to secrete glucagon rapidly during periods of increased glucose demand.

2. Aims

The general objective of this study was to investigate the modulation of Ca^{2+}-dependent exocytosis in single pancreatic α- and β-cells. The specific aims were to:

1. investigate the significance of the interaction between Ca^{2+}-channels and exocytotic granules in β-cells;
2. identify and characterize the properties of a novel Ca^{2+}-channel blocker from conus snail;
3. dissect the cellular and molecular mechanisms by which cAMP enhances Ca^{2+}-dependent exocytosis in β-cells; and
4. examine the effect of glucagon in the regulation of Ca^{2+}-dependent exocytosis in rodent α-cells.

3. Methods

To study exocytosis of pancreatic β- and α-cells, we employed capacitance measurements as an indicator of exocytosis combined with the whole-cell configuration of the patch-clamp technique first described by Hamill (Hamill *et al.*, 1981). This technique was the main tool in our study and is discussed in more detail below. Other techniques used are described in the original papers.

Experiments were performed on isolated α- and β-cells from mouse and rat islets. Rat α- and β-cells were separated by fluorescence-activated cell sorting (FACS; Josefsen *et al.*, 1996). Mouse α- and β-cells were maintained together in the same cell suspension and they were distinguished by their electrophysiological properties as follows: First, β-cells exhibit bursts of electrically activity at high glucose; whereas α-cells are electrical silent under this condition (Göpel *et al.*, 2000). Second, β-cells have a larger whole-cell capacitance (7 pF) while α-cells have lower cell capacitance (3-5 pF; Barg *et al.,* 2000). Third, the inactivation properties of Na^+-current differ between β- and α-cells. Steady-state inactivation of Na^+-currents in the β-cell is half-maximal at -100 mV whereas half-maximal inactivation of Na^+-currents in α-cells occur at -40 mV (Barg *et al.*, 2000; Göpel *et al.*, 2000). In practice, we mainly employed the latter two properties to distinguish the β-cells from the α-cells since our experimental conditions did not permit us to measure electrical activity.

3.1. Patch-clamp technique

The patch-clamp technique offers the possibility to measure ion channel currents under voltage-clamp conditions. This technique has four different recording configurations: cell-attached, whole-cell, outside-out patch and inside-out patch (Hamill *et al.*, 1981). In this study, we mainly used the *whole-cell configuration* (Fig. 5A-B). To establish the whole-cell configuration, a heat-polished glass electrode is pressed against the cell membrane. By applying gentle suction to the interior of the pipette, a high-resistance seal (giga-seal) is formed between the cell membrane and the glass pipette. The membrane enclosed by the pipette can then be ruptured by a pulse of negative pressure to establish direct contact with the cell interior and the *standard whole-cell configuration* is thus obtained. In this configuration, the cytosol is dialyzed by the pipette solution. This provides the means to control the intracellular environment and substances of interest can

be introduced into the cytosol simply by inclusion of the compounds in the pipette solution. One disadvantage of this configuration is that dialysis of the cell interior may lead to the wash-out of important diffusible cytosolic factors. A way to circumvent this problem is to use the *perforated patch configuration* (Horn & Marty, 1988). In this configuration electrical contact is established by insertion of a pore-forming antibiotic amphotericin B into the patch membrane (Rae *et al.*, 1991). This approach allows measurements from metabolically intact cells, since the small pores (8Å) formed by amphotericin B only allow monovalent ions and molecules with a molecular weight of < 200 D to pass through.

3.2. Capacitance measurements

Capacitance measurements (Neher & Marty, 1982; Neher, 1998) monitor the changes in cell capacitance associated with changes in cell surface area that occur when secretory granules fuse with the plasma membrane (Fig 5C). The technique is based on the fact that cell capacitance is proportionally related to the cell surface area with a specific capacitance ($C=\varepsilon * A$, where C stands for cell capacitance, A is the surface area and ε represents the specific membrane capacitance). An increase in membrane capacitance can thus be considered as an indicator of exocytosis. The granules in α- and β-cells have spherical geometry with diameters of 240-280 nm (Barg *et al.*, 2000; Göpel *et al.*, 2004) and 300-360 nm (Olofsson *et al.*, 2002; Göpel *et al.*, 2004), respectively. Assuming a specific capacitance of 9 $fF/\mu m^2$ (Gentet *et al.*, 2000), the fusion of one α-cell granule corresponds to an increase in membrane capacitance of ~2 fF, whereas the fusion of one β-cell granule adds ~3 fF to the capacitance increase.

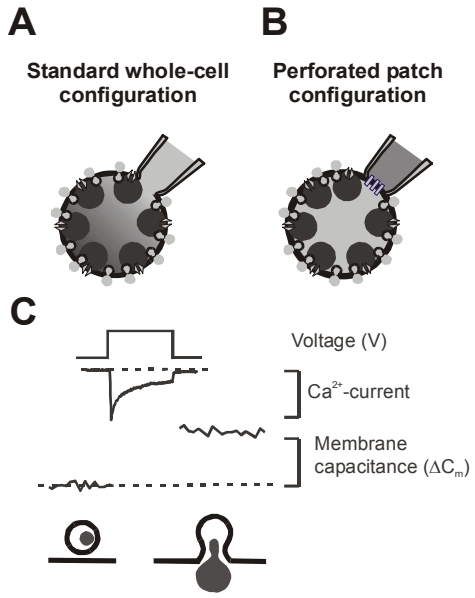

Figure 5. Principles of capacitance measurements. Illustration of the two patch-clamp configurations used for capacitance measurements in this study: **A**. The standard whole-cell configuration **B**. The perforated-patch whole-cell configuration. **C.** Demonstration of how a voltage-clamp depolarisation evokes an inward Ca^{2+} current and concomitant increase in membrane capacitance (ΔC_m). The increase in membrane capacitance reflects the fusion of the secretory granules with the plasma membrane as outlined in the cartoon at the bottom

The Lindau-Neher method to measure changes in membrane capacitance (Lindau & Neher, 1988) was used in this study. Employing this method, a sinusoidal voltage (*V*) is added to the command voltage and the resulting sinusoidal varying current (*I*) is measured using a phase-sensitive detector. *I* has the same frequency, but is phase-shifted with respect to *V*. Changes in current amplitude will reflect changes in admittance (*Y*) since $I=V*Y(\omega)$, which in turn reflects changes in the circuit parameters [membrane capacitance (C_m), membrane conductance (G_m) and series conductance (G_s)] of the recording configuration.

Capacitance measurements have several advantages over traditional biochemical assays of secretions. Thus, exocytosis can be monitored in single cells with millisecond time resolution, making the study of release kinetics available. However, there are some limitations of this technique: First, capacitance measurements only report the net change in cell surface area and do not distinguish exocytosis and concomitant endocytosis. In β-cells, this will not influence the measurement of exocytosis because endocytosis proceeds on a relatively slow time scale (Eliasson *et al.*, 1996a). Moreover, they do not distinguish exocytosis of insulin-containing granules and other processes that can increase cell surface area such as fusion of small synaptic-like microvesicles (SLMVs) that are present in the β-cell. However, the contribution from SLMVs to the increase in cell capacitance elicited by depolarisations is only ~1% of the total capacitance increase (Braun *et al.*, 2004). Finally, an increase in cell capacitance only indicates that membrane fusion has occurred, which does not necessarily equate to the release of insulin. Results from experiments where capacitance measurements were combined with real-time confocal microscopy revealed that the release of peptide cargo is much slower than the increase in membrane capacitance (Barg *et al.*, 2002b). Despite these disadvantages, capacitance measurements remain an important tool to elucidate the cellular mechanism involved in exocytosis.

3.3. How to study rapid exocytosis

3.3.1. Photoliberation of caged Ca^{2+}

In order to be able to investigate fast kinetics of exocytosis, the technique of flash photoliberation of caged Ca^{2+} was employed. In this study, caged Ca^{2+} was applied as a Ca^{2+}/NP-EGTA mixture. NP-EGTA has the same binding affinity to Ca^{2+} as standard EGTA but following exposure to UV-light, the affinity is dramatically decreased, leading to the release of Ca^{2+} (Ellis-Davies & Kaplan,

1994). In principle, a brief flash of UV-light (<1ms) directed at the cell, photoreleases the cage and increases Ca^{2+} uniformly within 1-2 ms. This results in a biphasic increase in membrane capacitance, one rapid component followed by a more sustained component. The capacitance increase during the rapid component represents the release of IRP-granules and the second component is regarded as the recruitment of new granules. In *paper I*, the cytosolic [Ca^{2+}] was monitored simultaneously by ratiometric micro-fluorimetry. The Ca^{2+}-indicator was excited at two different wave-lengths and the resultant emitted light was measured. The ratio of the emitted light was calculated and converted into $[Ca^{2+}]_i$ (Grynkiewicz *et al.*, 1985).

3.3.2.Exocytosis elicited by progressively longer depolarisations

Monitoring changes in membrane capacitance requires the application of a sinousidal command-voltage. This sine-wave is interrupted during a voltage-clamp depolarisation because changes in membrane conductance associated with activation of voltage-dependent currents may invalidate the capacitance measurements. It is therefore not straight forward to measure exocytosis during the voltage-clamp pulse. In order to determine the time course of exocytosis during the first hundred ms, we reconstructed the depolarisation by applying progressively longer depolarising pulses (5-850 ms) and measured the resulting increase in membrane capacitance (Fig. 6A). The

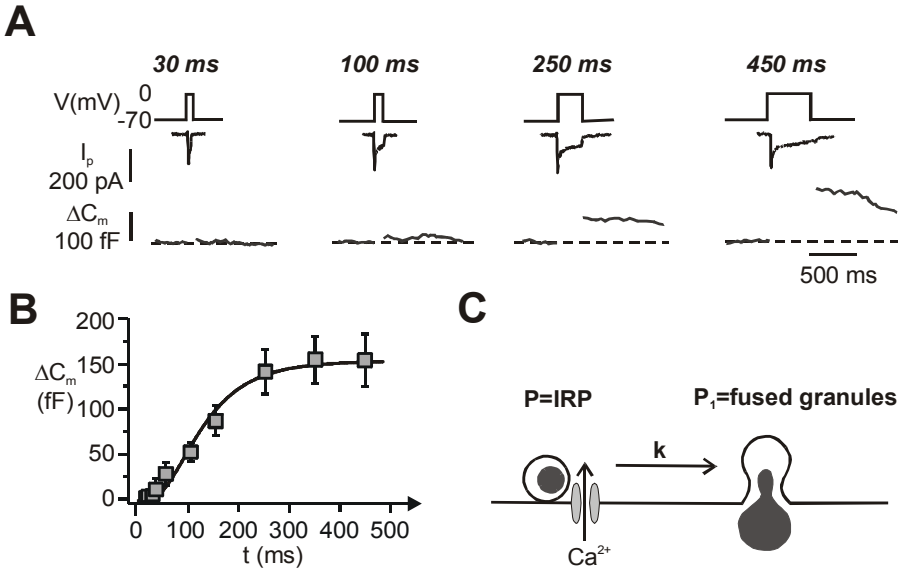

Figure 6. Rapid exocytosis studied using the pulse length protocol. A. Measured Ca^{2+}-currents and increase in membrane capacitance (ΔC_m) elicited by voltage-clamp depolarisations lasting 5-850 ms. For clarity, only the 30 ms, 100 ms, 250 ms and 450 ms are shown. **B**. Relationship between ΔC_m and duration of depolarisation (*t*). The curve is derived by fitting Eq.1 to the data points as described in the text. **C**. Schematic of granule conversion from a readily releasable state (P) to a fused state (P_1).

increase in membrane capacitance can then be plotted against the duration of the depolarisation (Fig. 6B) and the data points can be approximated to a differential equation describing the release kinetics. The kinetic model we have used considers two pools of granules; the immediately readily releasable pool (P) and a pool of fused granules (P1) as described in Fig. 6C. The pool dynamics can then be described by the differential equation:

$$\frac{\delta P}{\delta t} = -kP \qquad \text{(Eq. 1)}$$

Where k is:

$$k = -\alpha_0 (1 - e^{-\frac{t}{\tau}})^3 P \qquad \text{(Eq. 2)}$$

The initial size of P was taken to represent the size of IRP. The constant α_0 describes the decay rate of P and the time constant τ the sequential binding of Ca^{2+}-ions to the Ca^{2+}-sensor of secretion. The third power yielded the best results and suggests that three Ca^{2+}-ions are involved.

4. Results and discussion

4.1. A close connection between Ca^{2+}-channels and insulin-containing granules (*paper I*)

As discussed above, glucose-stimulated insulin secretion is associated with electrical activity consisting of Ca^{2+}-dependent action potentials resulting in an elevation of $[Ca^{2+}]_i$ (Rorsman *et al.*, 1992; Theler *et al.*, 1992). The increase in $[Ca^{2+}]_i$ temporally correlates to exocytosis of insulin-containing granules (Rosario *et al.*, 1986; Pralong *et al.*, 1990; Gilon *et al.*, 1993). Secretion in the β-cell thus echoes Ca^{2+}-channel activity and proceeds only during depolarisation and stops upon repolarisation (Ämmälä *et al.*, 1993b). This suggests a close connection between the Ca^{2+}-channels and the secretory granules, which is further supported by the observation that Ca^{2+}-channels and secretory granules co-localize to the same area in the β-cell (Bokvist *et al.*, 1995). In paper I, we have explored the functional interaction of the L-type Ca^{2+}-channel with the insulin-containing granules in detail.

4.1.1. Ca^{2+}-channels tether to the secretory granules in β-cells

Nonstationary fluctuation analysis showed that a single β-cell contain 450 channels. In a cell with a surface area of 510 μm^2, as expected from the mean value of the cell capacitance of ~5 pF, the Ca^{2+}-channel density was estimated to be 0.9±0.2 channels/μm^2. With so few Ca^{2+}-channels, it seems likely that the elevation of Ca^{2+} concentration to exocytotic levels (as high as 30 μM; Takahashi *et al.*, 1997) can only be achieved in the immediate vicinity of the Ca^{2+}-channels. We reasoned that if exocytosis were determined by the global rather than local $[Ca^{2+}]_i$, then both fast exocytosis elicited by short depolarisations (release of granules close to the Ca^{2+}-channels) and slow exocytosis induced by longer depolarisations (release of granules of further away from Ca^{2+}-channels) should be increased upon enhanced Ca^{2+} influx. This can be achieved either by increasing the driving force of Ca^{2+} or by prolonging the mean Ca^{2+}-channel open time. In the presence of a high extracellular Ca^{2+} concentration (10 mM instead of normally 2.6 mM), exocytosis elicited by short (<15 ms) depolarisations was greatly enhanced by the augmented Ca^{2+} influx whereas exocytosis elicited by longer depolarisations was not affected. Similar observations were achieved in the presence of the L-type Ca^{2+}-channel agonist Bay K8644. Further analysis of the capacitance data obtained with the Ca^{2+}-channel agonist revealed that the effect could

entirely be explained by a larger Ca^{2+} influx and not by a prolonged open-time. Thus, the results from the measurements in presence of a high Ca^{2+}-concentration or the Ca^{2+}-channel agonist Bay K8644 indicated that promotion of Ca^{2+} influx merely stimulated exocytosis of granules close to Ca^{2+}-channels, with little effect on granules situated further away from the Ca^{2+}-channels. These results reinforce the idea that the Ca^{2+}-sensor for exocytosis is situated close to the inner mouth of the Ca^{2+}-channels. The close connection between the secretory granules and the Ca^{2+}-channel was further supported by experiments using the synprint peptide $L_{c753\text{-}893}$. Synprint peptide is an intracellular loop connecting the II domain and the III domain of the α_{1c}-subunit of the Ca^{2+}-channel. In agreement with an earlier report (Wiser *et al.*, 1999), our data revealed that the synprint peptide couples the Ca^{2+}-channels to the exocytotic granules. How are the few Ca^{2+}-channels distributed? Most cell-attached patches either contained no channels at all or three active channels, suggesting that the Ca^{2+}-channels in the β cell are not randomly distributed. Theoretical analysis of these data indicated that the Ca^{2+}-channels form ~115 Ca^{2+}-channel triplets. We proposed that each of the triplets associates with individual secretory granules and thus forms an "exocytotic complex".

4.1.2. Rapid exocytosis at high $[Ca^{2+}]_i$

The interaction between the Ca^{2+}-channels and the insulin-containing secretory granules is important for fast exocytosis. Previous experiments in pancreatic β-cells indicated that $[Ca^{2+}]_i$ did not reach beyond ~2μM upon Ca^{2+} influx through voltage-dependent Ca^{2+}-channels (Ämmälä et al., 1993b; Bokvist et al., 1995; Dryselius et al., 1999). We used flash-photolysis of caged Ca^{2+} to allow the increase of $[Ca^{2+}]_i$ instantaneously and uniformly throughout the entire cell. These results revealed that exocytosis was regulated by Ca^{2+} with a K_d as high as 17 μM, which was similar to that previously reported by Takahashi *et al.,* (1997). As documented above, such high concentration can only be attained in the close vicinity of the Ca^{2+}-channels because of the scarcity of Ca^{2+}-channels in β-cells. It is implicit from these observations that there exist a steep spatial $[Ca^{2+}]_i$ gradient in pancreatic β-cells and that $[Ca^{2+}]_i$ in spacially confined "active zones" triggers exocytosis in spite of low global cytosolic Ca^{2+} concentration being attained (Bokvist *et al*., 1995).

Analysis of exocytotic responses elicited by progressively longer (5-450 ms) voltage-clamp depolarisations suggested

that ~60 granules in the β-cell can be released at a very high speed (>600 granules/s) within 50-100 ms and with minimal latency (≤15 ms). Fast exocytosis was not affected by inclusion of EGTA in the intracellular buffer, which indicated that rapid exocytosis is controlled by local Ca^{2+} concentration and not cytosolic $[Ca^{2+}]_i$. This conclusion was further supported by the observation that rapid exocytosis was suppressed by exogenous synprint peptide. Addition of exogenous synprint peptide competes with the endogenous protein loop for the binding site(s). This leads to the dissociation of the tight complex between the Ca^{2+}-channel and the granules (Wiser *et al.*, 1999). Thus, the mean distance between the granules and the Ca^{2+}-channels is increased and the Ca^{2+} sensor for exocytosis may no longer be exposed to exocytotic levels of $[Ca^{2+}]_i$.

4.1.3. IRP is a subset of RRP

As discussed above, the rapid component of exocytosis results from a close association between triplets of Ca^{2+}-channels and the secretory granules. Like that in chromaffin cells, IRP represents a subset of RRP in pancreatic β cells. To investigate the relationship between IRP and RRP further, we performed capacitance measurement, where exocytosis was evoked by voltage-clamp depolarisations followed by flash-photolysis of caged Ca^{2+} (Fig, 7). We reasoned that both granules in IRP and RRP are primed but that they differ with regard to their relationship with the Ca^{2+}-channels. The granules in IRP are thus assumed to be coupled to the Ca^{2+}-channels, while those in RRP are situated further away from the Ca^{2+}-channels. The voltage-clamp depolarisations would then preferably release the granules in IRP, whereas the uniform increase in $[Ca^{2+}]_i$ by flash photolysis was expected to release all RRP granules. Comparing the increase in membrane capacitance before and after flash release of $[Ca^{2+}]_i$ suggested that IRP

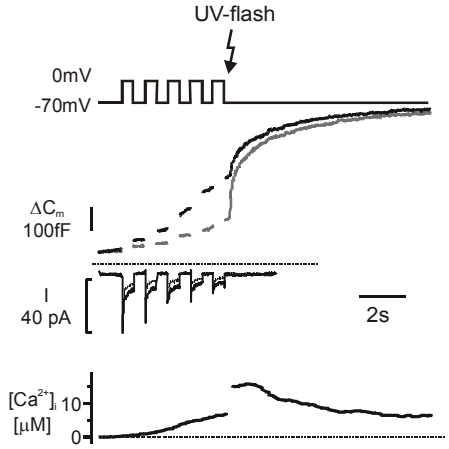

Figure 7. IRP is a subset of RRP. A train of five 500-ms depolarisations from -70 mV to 0 mV (top trace) applied at 1 Hz followed by photoliberation of caged Ca^{2+} (200 ms after the last depolarisation). The evoked whole-cell Ca^{2+} currents (I, second trace from bottom), cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$, bottom) and concomitant increase in cell capacitance (ΔC_m, second from top) are shown. The stimulation protocol was repeated twice. The response to the first stimulation is shown in black, and the response to the second stimulation applied 2 min after the first is displayed in gray.

comprise 10% of RRP in pancreatic β-cells. Application of the same protocol 2 min later, to allow recovery of RRP and $[Ca^{2+}]_i$ to return to basal, revealed some interesting information (Fig. 7). It could be observed that RRP did indeed recover completely and the first and the second stimulations elicited the same total exocytotic response. However, the increase in membrane capacitance during the voltage-clamp depolarisations was much reduced. Thus, IRP needs longer time to recover. Conceivably, replenishment of IRP involves the physical movement of either the secretory granules or the Ca^{2+}-channels to form new Ca^{2+}-channel granule complexes, which are capable of undergoing rapid exocytosis upon stimulation.

4.2. A novel L-type Ca^{2+}-channel antagonist (*paper II*)

As described in the introduction of this thesis, different types of Ca^{2+}-currents have been identified by their distinct electrophysiological and pharmacological properties. In pancreatic β-cells, L-type Ca^{2+}-channels are found to be the predominant channel type. Until now, pharmacological inhibition or stimulation of ion channels has been a primarily method to distinguish different channel types. For example, dihydropyridines, phenylalkylamines and benzothiazepines are found to selectively block the L-type Ca^{2+}-channels, whereas the different polypeptide toxins from snail and spider venoms are specific antagonists for N-type and P/Q-type Ca^{2+}-channels. The cone snail venoms contain a variety of paralyzing peptides (conotoxins) that are injected into the victim when the cone snail preys. These peptides are capable of specific binding to different receptors and ion channels in the neuromuscular system, thus disturbing their functions. One group of conotoxins are the family of contryphans. They are known to contain an unusual density of post-translational modifications including tryptophan bromination and amidation of the C-terminal residue. Contryphans cause the "stiff-tail syndrome" when injected intracranially in rat (Jimenez *et al.*, 1996) and body tremor and secretion of mucous substances when injected into fish (Jacobsen *et al.*, 1999). The γ-carboxyglutamyl residues (Gla) are also found in venom toxin. The Gla-content varies from species to species but it has been found to be abundant in venom from *Conus textile* and *Conus marmoreus* (Hauschka, 1988). In paper II, we report a new member of the contryphan family, Glacontryphan-M. This is the first known

Gla-containing contryphan peptide that selectively inhibits L-type Ca^{2+}-channels.

4.2.1. Inhibition of whole-cell Ca^{2+} currents by Glacontryphan-M

Glacontryphan-M was extracted from the venom of *conus marmoreus* and purified by HPLC separation. The amino acid sequence of the peptide was determined using automated Edman degradation and electrospray ionization mass spectroscopy. Gla-residues have earlier been demonstrated to exhibit a Ca^{2+} binding property that is crucial for their biological activity (Brown *et al.*, 2000). Using fluorescence spectroscopy, it was detected that Glacontryphan-M presented a high Ca^{2+} affinity (K_d=0.63 mM). When the Gla-residues were replaced with Glu residues (Glucontryphan-M), the Ca^{2+}-binding property was lost. These data suggests that the Gla residues confer Glacontryphan-M with its Ca^{2+} binding ability.

To investigate the pharmacological properties of Glacontryphan-M, we next examined the effects of this peptides on Ca^{2+} currents in pancreatic β-cells. Ca^{2+}-currents were elicited by voltage-clamp depolarisations from -70 mV to zero mV. Addition of Glacontryphan-M (1 μM) in the extracellular solution produced a 30% reduction of the inward peak current (I_p). However, contryphan peptides where the Gla-residues had been exchanged with Glu (Glucontrypan-M), failed to interfere with the Ca^{2+} currents. Thus, the Gla residues are necessary for the toxin to suppress the Ca^{2+}-channel current. In order to identify the specificity of Glacontryphan-M on Ca^{2+}-channels, we next applied the dihydropyridine L-type Ca^{2+}-channel antagonist isradipine (5 μM) in the extracellular solution to maximally block L-type currents. Under these conditions, Glacontryphan-M was unable to produce further reduction of the Ca^{2+} current, suggesting that Glycontryphan-M is an L-type channel antagonist.

Structure analysis has revealed a subtle difference in the structure of Glacontryphan-M in the presence and absence of extracellular Ca^{2+} (Grant *et al.*, 2004). We next examined if the Ca^{2+}-induced conformational change of Glacontryphan-M was important for its function. Ca^{2+}-channels are highly permeable to monovalent cations and in the absence of Ca^{2+}, the voltage-currents are mainly carried by monovalent cations (Almers *et al.*, 1984). This makes it possible to study currents flowing through the voltage-dependent Ca^{2+}-channels even in the absence of extracellular Ca^{2+}. We used this approach and found that Glacontryphan-M was unable to block the

inward cation current through the Ca^{2+}-channels. By contrast, a slight stimulatory effect was detected and the peak currents increased by 22%. It is possible that the binding of Ca^{2+} to the Gla-residues, leading to a conformational change, is necessary for the interaction of the antagonist with the L-type Ca^{2+}-channels. Thus, both the Gla-residue and the presence of extracellular Ca^{2+} are required for the inhibitory role of Glacontryphan-M.

4.2.2. Ca^{2+}-dependent exocytosis is depressed by Glacontryphan-M

In a new series of experiments we investigated if Glacontryphan-M interfered with Ca^{2+}-dependent exocytosis. A train of ten voltage-clamp depolarisations at 1 Hz was applied to single β-cells to evoke exocytosis (Fig. 8A). In seven paired experiments, application of Gla-contryphan-M markedly reduced the exocytotic response from 410±153 fF under control conditions to 106±48 fF ($P<0.05$) in the presence of the peptide. From these measurements it was difficult to decide whether the reduced exocytosis was due to direct interference with the exocytotic processes or only a resultant from inhibition of the Ca^{2+} current. To elucidate the mechanism by which Glacontryphan-M inhibited exocytosis, we then clamped the membrane potential at -70 mV to exclude the interference from changes in Ca^{2+}-channel activities. Exocytosis was then induced by dialyzing the cell interior with a Ca^{2+}-EGTA buffer to keep the $[Ca^{2+}]_i$ constant at 1.5 μM. Under these circumstances, addition of Glacontryphan-M failed to reduce the exocytotic response (Fig. 8 B-C). It can be concluded that the inhibitory effects of Glycontryphan-M on exocytosis are secondary to its action on the L-type Ca^{2+}-channels.

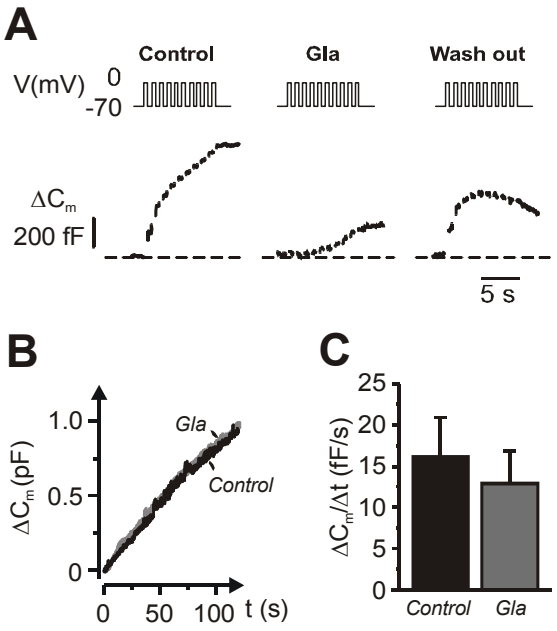

Figure 8. Reduced exocytotic response by Glacontryphan-M is secondary to its block of Ca^{2+} channels. A. A train of ten 500-ms depolarisations (1 Hz) from -70 mV to 0 mV (top trace) was applied to a single β-cell under control conditions, in the presence of 1 μM Glacontryphan-M (Gla) and 10 minutes after wash out of the peptide. The associated increase in cell capacitance was measured and is displayed in the lower trace. **B.** Increase in cell capacitance (ΔC_m) induced by dialysing the cell interior with a Ca^{2+}/EGTA-buffer (free Ca^{2+} concentration of 1.5 μM) in the absence and presence of 1 μM Glacontryphan-M (Gla). The whole cell configuration is established at time zero. **C.** Histogram summerizing the effect of Glacontryphan-M (Gla) on the rate of capacitance changes in β-cells. Data are presented as mean values ± S.E.M. of 6-7 experiments in each group.

4.3. cAMP-stimulated exocytosis in β cells (*paper III*)

Cyclic AMP is one of the most important intracellular signals in regulation of insulin secretion (Pipeleers *et al.*, 1985a; Schuit & Pipeleers, 1985). The stimulatory effects of cAMP on insulin secretion are considered to be mainly mediated by the cAMP-PKA signalling pathway (Wollheim *et al.*, 1987; Gillis & Misler, 1993; Ämmälä *et al.*, 1993a; Gromada *et al.*, 1997b; Renström *et al.*, 1997). However, it is now apparent that PKA is not the only intracellular cAMP-binding protein. Recently, cAMP-GEF II (guanine nucleotide exchange factors) or Epac (exchange protein directly activated by cAMP) was found to be another cAMP-binding protein able to regulate Ca^{2+}-dependent exocytosis (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998; Ozaki *et al.*, 2000; Kang *et al.*, 2001; Nakazaki *et al.*, 2002). In paper III, we have considered the PKA-dependent and PKA-independent effects of cAMP in further detail.

4.3.1. PKA-dependent and PKA-independent pathways

First we explored the kinetics of cAMP-induced exocytosis on single mouse β-cells. Progressively longer depolarisations (5-850 ms) from -70 mV to 0 mV were applied to the cells and the concomitant increase in membrane capacitance was measured. The cAMP-increasing agent GLP-1 enhanced the increase in membrane capacitance both during shorter and longer depolarisations and IRP was enlarged 2.3-fold. The stimulatory action of GLP-1 on exocytosis evoked by longer depolarisations was fully inhibited by the PKA-antagonist Rp-cAMPS. However, the PKA-antagonist was unable to counteract the stimulation of GLP-1 on exocytosis evoked by shorter depolarisations. Similar results were obtained in the presence of forskolin or after intracellular application of cAMP. These data support the previous observations that cAMP stimulates insulin secretion through both PKA-dependent and PKA-independent mechanisms (Renström *et al*., 1997; Nakazaki *et al*., 2001). Close inspection of the capacitance steps revealed that cAMP-stimulated exocytosis

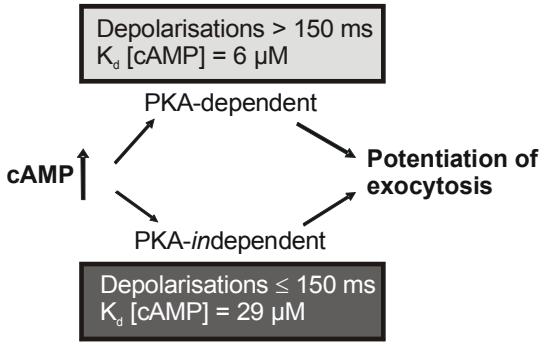

Figure 9. Illustration of dissimilarities between PKA-dependent and PKA-independent exocytosis in the β-cells. The two pathways differ in their $[cAMP]_i$ dependence and the duration of the voltage-dependent depolarisation needed to be activated. K_d is the concentration of cAMP at which stimulation is half-maximal.

can be divided into two distinct pathways (Fig. 9): a fast, Rp-cAMPS resistant (PKA-independent) component elicited by depolarisations ≤ 150 ms and a slow, Rp-cAMPS sensitive (PKA-dependent) component observed at longer stimulations (> 150 ms). The rapid cAMP-dependent PKA-independent action is more important for the priming process, whereas the PKA-dependent mechanism involves the physical recruitment of granules from the reserve pool. The two cAMP-signalling pathways were further found to differ in their concentration-dependence (Fig. 9). The PKA-independent pathway needed a 5-fold higher cAMP-concentration (K_d =29 μM) to be activated than the PKA-dependent pathway (K_d=6 μM).

4.3.2. cAMP-GEF II stimulates rapid exocytosis

The family of cAMP-GEFs are cAMP-binding proteins first identified on the basis of their ability to stimulate the exchange of GDP for GTP at the guanyl nucleotide binding sites of Rap1 and Rap2 (de Rooij *et al*., 1998; Kawasaki *et al*., 1998). There are two isoforms of cAMP-GEF: cAMPGEFI (Epac1) and cAMP-GEFII (Epac 2). Both isoforms are present in the pancreas (Kawasaki *et al*., 1998), in pancreatic islets (Leech *et al.*, 2000) and in insulin secreting cell lines (Leech *et al* 2000). We were able to detect isoform II in single primary β- and α-cells as well as in MIN6 cells (Paper III and IV). Accumulating evidence suggest that cAMP-GEFII serves as a direct intracellular target of cAMP to mediate cAMP-dependent, PKA-independent exocytosis in MIN6 cells (Ozaki *et al*., 2000) and in primary pancreatic β-cells (Kashima *et al.*, 2001).

Several observations indicate that cAMP-GEFII is involved in cAMP-PKA-independent exocytosis in mouse pancreatic β-cells. First, treatment of β-cells with antisense oligonucleotide against cAMP-GEFII reduced the forskolin-induced Rp-cAMPS resistant exocytotic response by 50%. Second, the cAMP-GEFII agonist, 8CPT-2Me-cAMP, selectively promoted rapid PKA-independent exocytosis. In line with these observations, it has been demonstrated that downregulation of cAMP-GEFII expres-sion or overexpression of a dominant-negative mutant of the protein diminishes the stimulatory effects of GLP-1 or the cAMP-analog 8-Br-cAMP on insulin secretion in mouse islets (Kashima *et al*., 2001). Cyclic-AMP-GEFII was first discovered by two-yeast hybrid screening on a MIN6 cell library using the sulfonylurea receptor SUR1 as the bait (Ozaki *et al*., 2000). SUR1 is the

regulatory subunit of the K_{ATP} channel in the plasma membrane and also the target of the sulfonylureas (Ashcroft & Gribble, 1998) that are commonly used as anti-diabetic drugs. We have previously reported that sulfonylureas, such as tolbutamide and glibenclamide, are able to directly stimulate the exocytotic machinery, independent of its actions on the K_{ATP} channel (Eliasson *et al.*, 1996b). It is further discovered that sulfonylurea could exert a direct effect on the priming of the secretory granules (Barg *et al.*, 1999; Barg *et al.*, 2001). Since cAMP-GEFII is involved in rapid exocytosis, we were interested to investigate the significance of this protein for sulfonylurea-stimulated exocytosis. It was found that tolbutamide was unable to stimulate exocytosis in MIN6 cells pre-treated with anti-sense oligonucleotides against cAMP-GEF II or transfected with a dominant-negative mutant of the protein.

The most surprising finding in this study was the loss of the rapid PKA-independent component of capacitance increase in β-cells from $SUR1^{-/-}$ mice, suggesting a direct involvement of SUR1 in cAMP-activated granular priming. It has been reported that insulin secretion in islets from $SUR1^{-/-}$ mice has a lower sensitivity to cAMP (Nakazaki *et al.*, 2002; Shiota *et al.*, 2002). This was further verified by our measurements of insulin secretion in wildtype and $SUR1^{-/-}$ mice. Whereas insulin secretion was strongly enhanced by both GLP-1 and forskolin in the wild-type animals, these substances were much less effective in $SUR1^{-/-}$ islets. Wildtype β-cells contained prominent PKA-dependent and PKA-independent components of insulin secretion and only 55% was sensitive to Rp-cAMPS. In the $SUR1^{-/-}$ islets, the cAMP-increasing agents produced much smaller enhancement of the measured insulin release and the stimulatory effect was abolished by Rp-cAMPS. The failure of cAMP to induce the PKA-independent stimulatory mechanism was not due to loss of effector proteins and both cAMP-GEFII and the putative effector protein (Wang et al., 1997; Schoch et al., 2002) RIM2 were found to be expressed to equal extents in wildtype and $SUR1^{-/-}$ mice.

The direct effect of sulfonylureas on exocytosis have been suggested to involve ClC3 Cl^- channels, which possibly form an ion channel complex with a mdr-like 65kDa protein (the granular sulfonylurea-receptor, gSUR) and a V-type H^+-ATPas. The simultaneous operation of these proteins results in granular acidification and priming (Barg *et al.*, 1999; Barg *et al.*, 2001). Given that both effects of cAMP and sulfonylurea involve cAMP-GEFII, it is tempting to speculate

that they converge at the level of the ClC3-channel. Indeed, we were able to demonstrate that cAMP stimulates Cl^- influx in wildtype β-cells in a PKA-independent manner. The stimulatory action of Cl^- uptake by cAMP was totally absent in the $SUR1^{-/-}$ animals, suggesting that the ability of the granules to undergo ClC3 dependent granular priming is impaired in the $SUR1^{-/-}$.

To summarize these results, we argue that cAMP-dependent, PKA-independent rapid exocytosis is mediated by the cAMP-binding protein cAMP-GEFII, the plasma membrane sulfonylurea receptor SUR1, the granular sulfonylurea receptor gSUR and the granular ClC3-channel. A tentative model is presented in Fig. 10. Here, cAMP by binding to cAMP-GEFII, which is in close association with SUR1, facilitates the interaction between cAMP-GEFII and its effector protein RIM2. By interaction with gSUR and the ClC3 channel, cAMP-GEFII also promotes granular priming. In this model cAMP-GEFII plays a key role by linking the stimulating roles of cAMP and sulfonylurea. Likewise, the model demonstrate a dual role of SUR1 in both being part of the K_{ATP} channel important for initiation of electrical activity and being part of the protein complex regulating cAMP-stimulated and Ca^{2+}-dependent exocytosis. Thus, SUR1 emerges as a central player in both the triggering and amplifying pathways of glucose-stimulated insulin secretion.

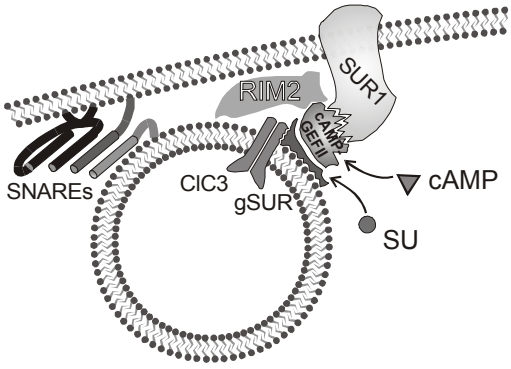

Figure 10. Hypothetical model for the associations among cAMP-GEF II, SUR1 and other regulatory proteins mediating granule acidification and priming. See text for details. Abbreviations used: SUR1: sulfonylurea receptor 1; gSUR: granular sulfonylurea receptor; ClC3: ClC3 chloride channel; RIM2: Rab-protein interacting molecule 2; SNARE: soluble NSF-attachment protein receptor.

4.4. Glucagon stimulates Ca^{2+}-dependent exocytosis in α cells (*IV*)

In pancreatic β-cells, insulin has been found to modulate its own release (Aspinwall *et al.*, 1999; Khan *et al.*, 2001). The possibility that glucagon exerts a similar autocrine action on Ca^{2+}-dependent exocytosis by binding to glucagon-receptors on the plasma membrane of the α-cells is discussed here.

4.4.1. cAMP-dependent potentiation of glucagon release

Capacitance measurements revealed that Ca^{2+}-dependent exocytosis was enhanced ~3-fold in the presence of 10 nM glucagon in both mouse (Fig. 11A) and rat α-cells. This was attributed to the elevated concentration of intracellular cAMP ($[cAMP]_i$) and it was found that $[cAMP]_i$ increased more than 3-fold in response to glucagon stimulation. As discussed in paper III, cAMP stimulates exocytosis of granules through both PKA-dependent (sensitive to Rp-cAMPS) and PKA-independent (resistant to Rp-cAMPS) mechanisms in insulin secreting β-cells. The action of glucagon on exocytosis in the α-cell was however totally PKA-dependent and was completely inhibited by the PKA-inhibitor Rp-Br-cAMPS. This does not necessarily mean that the PKA-independent pathway does not exist in the α-cell. Indeed, both PKA-dependent and PKA-independent effects on exocytosis was clearly detected in pancreatic α-cells when 0.1 mM cAMP was dialyzed directly into the cell interior and exocytosis was elicited by voltage-clamp depolarisations of increasing duration (5-850 ms).

The PKA-independent rapid enhancement of exocytosis involves, as in the β-cell (Paper III), the cAMP-binding protein cAMP-GEFII. The presence of this protein in α-cells was verified by immunostaining and RT-PCR. Further evidence for the involvements of cAMP-GEFII was achieved from capacitance measurements where the cAMP-GEFII agonist was able to enhance rapid exocytosis (<250 ms) in a PKA-independent manner. Experiments on α-cells from the $SUR1^{-/-}$ mice indicated that SUR1 also plays a major role for rapid exocytosis in α-cells, as is the case in β-cells (Paper III). The failure to detect the PKA-independent pathway upon elevation of $[cAMP]_i$ by glucagon might be due to the failure of glucagon to elevate cytoplasmic cAMP to sufficiently high concentrations in order to activate cAMP-GEFII. This assumption is based on the observations that 1) cAMP has a lower affinity for cAMP-GEFII than it has for PKA (Kashima *et al*., 2001), 2) activation of the PKA-independent pathway requires a 5-fold higher concentration of cAMP than the PKA-dependent mechanism (paper III), and 3) PKA-independent stimulation was prominent in presence of 10 μM forskolin when $[cAMP]_i$ was elevated more than 10-fold, whereas addition of glucagon only produced a < 4-fold increase of $[cAMP]_i$.

4.4.2. The stimulation by glucagon is mediated by glucagon receptors

In pancreatic β- and α-cells, regulation of the cAMP dependent signalling pathways has been found to depend on expression of specific receptors for GLP-1, GIP and glucagon (Huypens *et al.*, 2000). Receptors both recognizing GLP-1 and glucagon are found to exist in rodent and human pancreatic islets (Schuit & Pipeleers, 1985; Moens *et al.*, 1996; Huypens *et al.*, 2000). In β-cells, earlier data have demonstrated that glucagon is able to bind to the GLP-1 receptor (Thorens, 1992), which rises the possibility that glucagon exerts its effect by binding to the GLP-1 receptor (Kofod *et al.*, 1993). However, increasing evidence suggests that glucagon stimulates exocytosis of β-cells through its own receptors (Kawai *et al.*, 1995; Gromada *et al.*, 1997b; Huypens *et al.*, 2000). A similar situation is also the most likely explanation for the stimulatory action of glucagon in the pancreatic α-cells. Three pieces of evidence in rodent α-cells support this notion. First, the glucagon receptor gene was detected to be expressed in α-cells. Second, pharmacological inhibition of glucagon receptor activity using the potent glucagon-receptor antagonist des-His^{1}-[Glu^{9}]-glucagon-amide fully suppressed the stimulatory action of glucagon (Fig. 11B). No such effect was observed in presence of exendin-(9-39)-amide, a selective antagonist of the GLP-1 receptor. Third, the ability of glucagon to elevate $[cAMP]_i$ was completely counteracted by the glucagon receptor antagonist, not by exendin-(9-39)-amide. Thus, glucagon stimulates Ca^{2+}-dependent exocytosis in α-cells by binding to glucagon receptors in the plasma membrane.

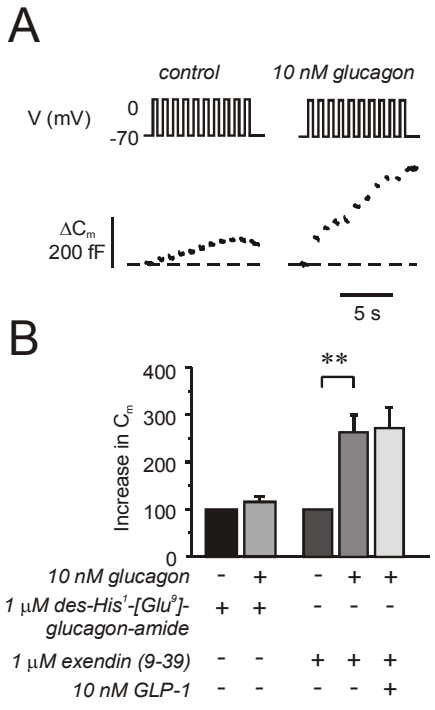

Figure 11. Glucagon stimulates exocytosis via binging to glucagon receptors in α-cells. **A.** A train of ten 500-ms depolarizations from -70 mV to 0 mV (top trace) elicited an increase in cell capacitance ($\Delta C_{m,}$ bottom trace) under control conditions (left) and 2 min after addition of 10 nM glucagon in the extracellular solution (right). **B.** Histogram summarizing the mean increase in cell capacitance (ΔC_m) under conditions indicated at the bottom.

4.4.3. The physiological role of an increased exocytotic capacity by glucagon

We demonstrate here that glucagon promotes Ca^{2+}-dependent exocytosis in the α-cell. However, we are unable to measure the effects of glucagon on glucagon secretion using traditional biochemical assays. Can the observed increased exocytotic responses be equated to enhanced glucagon secretion? Not necessarily. Substances that have the same effect on exocytosis can have opposite effects in assays measuring glucagon secretion. Such example is GLP-1 and forskolin. Both stimulates Ca^{2+}-dependent exocytosis in the α-cell, but GLP-1 inhibits (Gromada *et al.*, 1998) and forskolin stimulates glucagon secretion (Pipeleers *et al.*, 1985b). The explanation for this paradox lies in the complex interplay of several parallel processes in the α-cell performed to achieve glucagon secretion as the end result. Our data indicate that glucagon is more similar to GLP-1 than forskolin. This is mainly based on the measured cAMP-level, which is much higher in presence of forskolin compared to in the presence of either glucagon or GLP-1. Further evidence is provided from reported measurements on the glucagon receptor knock-out mouse (GcgR-/-; Gelling *et al.*, 2003). This mouse exhibits hyperglucagonemia and α-cell hyperplasia. Thus, it is most likely that the physiological response to glucagon receptor activation in the α-cell is reduced rather than stimulated glucagon secretion.

4.5. Significance to type-2 Diabetes

The development of type-2 diabetes is associated with β-cell dysfunction (Jensen *et al.*, 2002; Kahn, 2003) and a loss of the first phase of insulin secretion is an early feature of this disease (Cerasi, 1992). The appearance of secretory granules in different releasable pools and their possible relationship with first and second phase of insulin secretion highlight the necessity of elucidating the cellular mechanisms behind the secretory defect of type-2 diabetes. We and others have demonstrated that exocytosis of insulin-containing granules is initiated by Ca^{2+} influx and that there is a close association of primed granules and Ca^{2+}-channels, which is required for the first phase of insulin release. Thus, any interference with the coupling between the insulin granules and the Ca^{2+}-channel can be expected to impair rapid exocytosis and reduce the first phase. Exocytosis of insulin-containing granules in type-2 diabetes is reduced not only in response to glucose, but also to potentiators of insulin secretion such as hormones, incretins and sulfonylureas

(Fritsche *et al.*, 2000; Stumvoll *et al.*, 2002; Kjems *et al.*, 2003). Our experiments confirmed earlier observations that the second messenger cAMP, elevated by incretins and hormones, enlarges the pool of primed granules and accelerates recruitment of new granules (Renström *et al.*, 1997). This cellular mechanisms of cAMP account for enhanced first and second phase of insulin secretion. The traditional antidiabetic drugs sulfonylureas trigger insulin-secretion by binding to the plasma membrane sulfonylurea receptor, SUR1, followed by closure of the K_{ATP}-channel and depolarisation of the membrane. The finding that SUR1 is involved in cAMP-dependent granular priming makes SUR1 a bridge between the triggering (responsible for first phase) and the amplifying (responsible for second phase) pathways of insulin secretion. The new relationship between SUR1, cAMP and exocytosis potentially provides a new principle for the pharmacological treatment of type-2 diabetes.

5. Conclusions

1. Pancreatic β-cells contain fewer than 500 L-type Ca^{2+}-channels and they occur in triplets. Each triplet couples to individual secretory granules in the immediately releasable pool to form exocytotic complexes. This ensures immediate exposure of primed granules to high local $[Ca^{2+}]_i$ and efficient exocytosis.

2. A novel Ca^{2+}-channel antagonist Glacontryphan-M from the venom of *Conus marmoreus* selectively suppresses L-type Ca^{2+}-channel currents in a Ca^{2+}-dependent manner. Both the Gla-residues and the presence of extracellular Ca^{2+} are required for its function.

3. Cyclic AMP potentiates Ca^{2+} dependent exocytosis in β-cells through both PKA-dependent recruitment of granules from a reserve pool and PKA-independent granule priming. The latter is mediated by the interaction of the cAMP-binding protein cAMP-GEFII and the sulfonylurea receptor SUR1, normally a subunit of the plasma membrane K_{ATP}-channel responsible for controlling the membrane potential in the β-cell.

4. Glucagon increases Ca^{2+}-dependent exocytosis in rodent α-cells by binding to glucagon receptors. This stimulatory effect on exocytosis is secondary to the elevation of cAMP and activation of PKA.

6. Populärvetenskaplig sammanfattning på svenska

Glukos är den främsta energikällan för de flesta celler i kroppen. Det är av stor vikt att mängden glukos hålls på en jämn nivå, eftersom framförallt våra celler i hjärnan är mycket känsliga för stora variationer av glukoshalten i blodet. Regleringen av mängden glukos i blodet kontrolleras av hormonen insulin och glukagon. Insulin utsöndras från β-celler och glukagon från α-celler, båda belägna i bukspottkörtels Langerhanska öar. Insulin sänker glukoshalten i blodet efter en måltid medan glukagon höjer glukoshalten då kroppen behöver tillskott av energi såsom då vi motionerar eller är under stress. Patienter med åldersdiabetes eller typ-2 diabetes har ofta en försämrad utsöndring av insulin samtidigt med en ökad sekretion av glukagon. Det vill säga kontrollen av mängden glukos i blodet är satt ur funktion. Normalt sker insulinsekretion i två faser, en första snabb fas som pågår 5-10 min följt av en långsam mer kontinuerlig fas. Patienter med åldersdiabetes saknar den första snabba fasen och den andra fasen är något reducerad.

Insulin och glukagon befinner sig i små blåsor (granula) inne i repektive cell. Dessa blåsor smälter samman (fuserar) med cellens membran vid en given signal och innehållet kan komma ut i blodet. Signalen som initierar utsöndringen är ett ökat inflöde av kalcium-joner (Ca^{2+}) genom speciella spänningskänsliga, vattenfyllda kanaler (jonkanaler) i cellens membran. Utsöndringen av hormonen kan vidare påverkas av andra substanser, ett sådan substans är cykliskt AMP (cAMP). Ett nytt läkemedel mot typ-2 diabetes som är under prövning, GLP-1, verkar bl.a. genom att öka cAMP-koncentrationen inne i cellen. I denna avhandling har de cellulära mekanismer varmed cAMP och Ca^{2+} påverkar utsöndringen av insulin och glukagon undersökts. Fusionen av granula med plasmamembran och därmed utsödringen av insulin (eller glukagon) har studerats på enskilda celler med en speciell teknik som kallas kapacitansmätningar. Denna teknik bygger på principen att cellens membran elektriskt kan likställas med en plattkondensator. Vidare gäller att då ytan på en kondensator ökar så ökar dess kapacitans. Detta kan utnyttjas eftersom när ett granula smälter samman med cellens membran ökar dess yta och även dess kapacitans.

Det första delarbetet behandlar kopplingen mellan inflöde av Ca^{2+} och insulingranula. För att granula ska kunna fusera med cellmembranet krävs en hög koncentration av kalcium, mycket högre än medelkoncentrationen inuti cellen. Cellen

löser detta genom att Ca^{2+}-kanalen binder samman med insulingranula vid cellmembranet. På detta vis sker fusionen av granula snabbt samtidigt som energiåtgången för att återställa Ca^{2+}-koncentrationen till sin vilonivå inne i cellen minimeras.

I det andra delarbetet har en ny substans som utvunnits från en vattenlevande snigel, Conus Marmoreus, undersökts. Normalt är denna substans en del av snigelns gift, som används för att paralysera och fånga byten. Det visade sig att substansen blockerade de speciella Ca^{2+}-kanaler som finns i β-cellen. Substansen kan utnyttjas för att studera Ca^{2+}-kanalens betydelse i olika system inte bara vid insulinsekretion.

Vidare har betydelsen av cAMP för en ökad utsöndring av insulin studerats. Våra undersökningar visade på att cAMP stimulerar insulinsekretion både genom att aktivera ett protein som heter proteinkinas A (PKA) och genom att binda till ett annat protein, cAMP-GEFII. Proteinkinas A medverkar till att påskynda förflyttningen av insulingranula till cellens membran och påverkar framförallt den andra fasens insulinsekretion. Genom att binda till cAMP-GEFII stimulerar cAMP framförallt den snabba utsöndringen av insulin under den första fasen. Ett av de intressanta fynden i detta arbete berör den receptor, till vilken de antidiabetiska medicinerna sulfonylureas binder in på cellens membran. Genom att binda till denna receptor startas en kaskad som leder till utsöndring av insulin. Detta abete har visat att denna receptor också har en viktig funktion i ett av de avslutande stegen innan de insulininnehållande granula kan fusera med cellmembranet.

Avslutningsvis behandlas α-cellen som utsöndrar glukagon. Det visade sig att cAMP förmår påverka glukagonsekretion på ett liknade sätt som i β-cellen samt att glukagon har förmågan att stimulera sin egen sekretion åtminstone på enskilda celler.

De mekanismer som har undersökts i denna avhandling påverkar framförallt första fasens insulinsekretion som saknas hos patienter med typ-2 diabetes. Dessa undersökningar är därför av stor betydelse för att förstå uppkomsten av denna sjukdom samt för att finna nya och bättre läkemedel.

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