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## Regulation of glucagon secretion from pancreatic alpha cells

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Faculty of Medicine

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**Regulation of glucagon secretion  
from pancreatic alpha cells**

by

**Yang Zhang De Marinis**



**LUND UNIVERSITY**  
Faculty of Medicine

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**This thesis will be defended on January 22, 2010, at 13:15 in lecture theatre  
Medelhavet, Wallenberglaboratoriet, Malmö University Hospital, Malmö, Sweden**

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## I. List of Original Papers Included in the Thesis

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1. Patrick E. MacDonald, **Yang Z. De Marinis**, Reshma Ramracheya, Albert Salehi, Xiaosong Ma, Paul R. V. Johnson, Roger Cox, Lena Eliasson, Patrik Rorsman (2007) A  $K_{ATP}$  Channel-Dependent Pathway within  $\alpha$  Cells Regulates Glucagon Release from Both Rodent and Human Islets of Langerhans. *PLoS Biology* 2007 Jun; 5(6):e143
2. **Yang Z. De Marinis**, Caroline E Ward, Fernando Abdulkader, Martin Bengtsson, Reshma Ramracheya, Stefan Amisten, Abdella M Habib, Yuusuke Moritou, Enming Zhang, Quan Zhang, Frank Reimann, Anders Rosengren, Albert Salehi, Tadao Shibasaki, Fiona Gribble, Erik Renström, Susumu Seino, Lena Eliasson, and Patrik Rorsman (2010) PKA and Epac2 mediate the opposing effects of GLP-1 and adrenaline on glucagon release by modulation of N- and L-type  $Ca^{2+}$  channel-evoked exocytosis. (*under revision*)
3. **Yang Z. De Marinis**, Enming Zhang, Stefan Amisten, Jalal Taneera, Erik Renström, Patrik Rorsman, Lena Eliasson (2009) Enhancement of glucagon secretion in mouse and human pancreatic alpha cells by protein kinase C (PKC) involves intracellular trafficking of PKC $\alpha$  and PKC $\delta$ . (*published online Diabetologia*)  
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## **II. Introduction**

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Glucose is the main energy source of the body and the most important fuel of the brain. In order to let us move, eat, think and even rest, it is crucial for our body to maintain a steady glucose level in the blood circulation. This is achieved by two hormones secreted from the pancreas: glucagon and insulin.

### **The Importance of Glucagon in Blood Glucose Regulation**

Glucagon and insulin were discovered successively in 1920s [1]. Even by the time when glucagon was finally named in 1923 by Murlin et al. [2], it was considered to be a “contaminant” rather than a hormone of importance. For decades, glucagon has been overshadowed by the triumph of insulin in dramatically rescuing lives of diabetic patients. However, insulin alone, which solely decreases blood glucose, is not enough to maintain a steady glucose level; the glucose-elevating (hyperglycemic) hormone glucagon is also required. Glucagon increases blood glucose under hypoglycemic conditions. Together, glucagon and insulin keep the fluctuation of blood glucose in a very precise balance, by exerting coordinated but opposite effects.

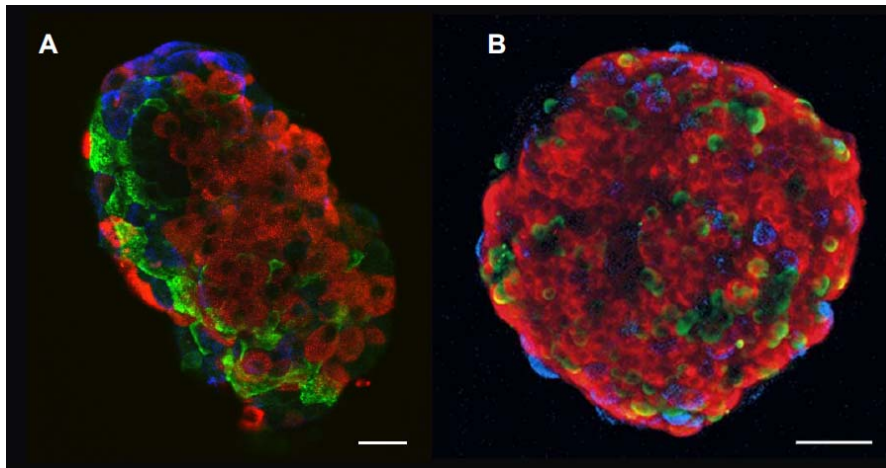
Glucagon is produced by pancreatic  $\alpha$ -cells in response to hypoglycemia [3, 4]. Once glucagon is released into the blood stream, it produces an increase in blood glucose by promoting glycogenolysis and gluconeogenesis in the liver. In addition to its physiological role in normal and extreme conditions, like stress and starvation, glucagon is also associated with pathology of diabetes mellitus. Diabetic patients do not solely have impaired insulin secretion and/or insulin action, but also increased plasma glucagon levels, which exacerbates the hyperglycemic effects of hypoinsulinemia [5, 6].

To date, the regulation of glucagon secretion from the pancreatic  $\alpha$ -cell is rather poorly understood. Studies on  $\alpha$ -cell physiology may lead to a better understanding of blood glucose control in the healthy individuals and diabetic patients.

### **The Pancreatic Islet Architecture**

“Pancreas” was first described by the Greek anatomist Herophilus (335-280 BC) and later on termed πᾶν (“all”, “whole”), and κρέας (“flesh”). It is an organ located between the stomach and the spine, and nestled in the curve of the small intestine. The pancreas is composed of both exocrine and endocrine units. The exocrine pancreas produces digestive enzymes and pancreatic juice, which are released into the duodenum. The endocrine pancreas is responsible for hormone production and secretion. In humans, the endocrine pancreas consists of up to one million islets of Langerhans, named after Paul Langerhans who discovered them in 1869. Each islet consists of about a thousand hormone-producing endocrine cells. These include the glucagon-producing  $\alpha$ -cells, the insulin-producing  $\beta$ -cells, the somatostatin-producing  $\delta$ -cells and the pancreatic polypeptide-producing (PP) cells (review: [7]).

In mouse islets (Fig. 1), the majority of the cells are  $\beta$ -cells (~70%) which are located in the core of the islet, while  $\alpha$ -cells (~20%),  $\delta$  cells (<10%) and PP cells (<5%) are located on the periphery of the islet [8]. Recent studies have shown that there is greater variability in the proportion of the different cell types in human islets [9]. There are more  $\alpha$ -cells (up to ~40%) and less  $\beta$ -cells (~50%) [9, 10]. Besides, the human pancreatic cells are not arranged in subdivisions like those of the mouse islet (with a core of  $\beta$ -cells and a mantle of non- $\beta$ -cells) but exhibits a more chaotic architecture [10]. These differences indicate that paracrine interactions (where a hormone released from one cell influences the function of the other endocrine cells) may be stronger in human islets than in rodent islets.



**Fig. 1 Confocal immunostaining of a mouse (A) and a human (B) pancreatic islet.** Pancreatic islet cells were labeled with antibodies against insulin (red), glucagon (blue) and somatostatin (green). Scale bar in A: 20  $\mu\text{m}$ ; scale bar in B: 50  $\mu\text{m}$ . Pictures are reconstructions of series of Z-stack confocal scanning images.

## Glucagon Secretion from Pancreatic $\alpha$ -cell

The pancreatic  $\alpha$ -cells are electrically excitable like other secretory cells and are equipped with different types of voltage-gated ion channels, which are responsible for generating action potentials and inducing  $\text{Ca}^{2+}$  influx which triggers glucagon release [11, 12]. In the  $\alpha$ -cell, secretion is under tight metabolic, paracrine, autocrine, neuronal and hormonal control. The details of this control will be considered below.

### Ion channels in the $\alpha$ -cell

#### ATP-dependent $\text{K}^+$ channel ( $\text{K}_{\text{ATP}}$ channel)

The role of the  $\text{K}_{\text{ATP}}$  channel has been exhaustively investigated in the  $\beta$ -cell, where it links the blood-glucose levels with insulin release [13, 14]. It accounts for the resting  $\text{K}^+$  conductance of the  $\beta$ -cell and maintains the membrane potential at negative values in the absence of glucose. Glucose-induced inhibition of the  $\text{K}_{\text{ATP}}$  channel underlies the



membrane depolarization and stimulation of insulin secretion seen at high glucose levels. Curiously, it is also present in mouse  $\alpha$ -cells [15] at a density rather similar to that of the  $\beta$ -cells ( $\sim 8,000/\text{cell}$ ) [16].

The  $K_{\text{ATP}}$  channel is composed of a transmembrane inwardly rectifying  $K^+$  channel subunit (Kir6.2) and high-affinity sulfonylurea receptor subunits (SUR1, SUR2A or SUR2B) [17, 18]. The assembly of Kir6.2 with different SUR subunits results in different  $K_{\text{ATP}}$  channels. In pancreatic islets, both SUR1/Kir6.2 and SUR2A/Kir6.2 channels have been detected [19]. The SUR2A/Kir6.2 channel has much less sensitivity to ATP than the SUR1/Kir6.2 channel and is present at only a moderate level in the islets [19]. The SUR1 subunit has 17 transmembrane segments and 2 nucleotide binding folds (NBF-1 and NBF-2), which sense the changes of intracellular ATP/ADP ratio [20, 21]. The regulation of the  $K_{\text{ATP}}$  channel by ATP and ADP is extremely complex. However, it appears that binding of ADP to SUR1 leads to activation of the channel thus relieving the inhibition induced by ATP binding to Kir6.2 [20]. In  $\alpha$ -cells, as in other cells, inhibition of  $K_{\text{ATP}}$  channels will result in depolarization that triggers opening of voltage-gated  $\text{Na}^+$ - and  $\text{Ca}^{2+}$ - channels [11, 15]. Intriguingly, this would be expected to stimulate rather than inhibit (which represent the physiological response) glucagon secretion. The role of  $K_{\text{ATP}}$  channels in  $\alpha$ -cells therefore represents an enigma.

### **Voltage-dependent $\text{Na}^+$ channel**

Voltage-gated  $\text{Na}^+$  channels are important for the upstroke of the action potential in the  $\alpha$ -cell [11]. It consists of a primary  $\alpha$ -subunit associated with one or more  $\beta$ -subunits. The  $\alpha$ -subunit is composed of four homologous domains (D1-D4) each containing six  $\alpha$ -helical transmembrane segments (S1-S6) [22]. In mouse  $\alpha$ -cells, the  $\text{Na}^+$  channel activates when the membrane potential exceeds -30 mV [11]. It subsequently undergoes spontaneous voltage-dependent inactivation. The inactivated state differs from the “ordinary” closed state in that the channel is refractory and cannot open again unless it is exposed to a brief period of repolarizing negative membrane potential. The  $\text{Na}^+$  channel in mouse  $\alpha$ -cells undergoes half-maximal inactivation ( $V_h$ ) at  $\sim -42$  mV. This can be used to identify  $\alpha$ -cells from  $\beta$ -cells ( $V_h \sim -90$  mV) and  $\delta$ -cells ( $V_h \sim -30$  mV) when performing patch-clamp experiments [11, 15]. In human pancreatic  $\beta$ -cells, the  $\text{Na}^+$  current contributes to the upstroke of action potentials, as in the  $\alpha$ -cell, and has a half-maximal inactivation at -42 mV [23].

### **Voltage-gated $\text{Ca}^{2+}$ channels**

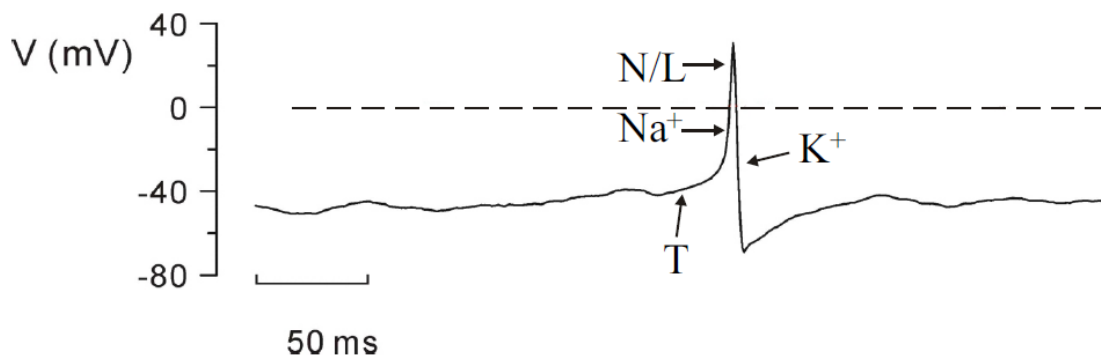
The voltage-gated  $\text{Ca}^{2+}$  channels detected in  $\alpha$ -cells include the low-voltage-activated T-type  $\text{Ca}^{2+}$  channel (opening at  $\sim -65$  mV) and high-voltage-activated N- and L-type  $\text{Ca}^{2+}$  channels (opening at  $\sim -40$  mV) [11, 15, 24, 25]. The influx of  $\text{Ca}^{2+}$  through  $\text{Ca}^{2+}$  channels into the  $\alpha$ -cell initiates exocytosis of glucagon-containing granules. The high-voltage-gated  $\text{Ca}^{2+}$  channels are composed of a pore-forming  $\alpha_1$ -subunit as well as additional auxiliary subunits ( $\alpha_2$ ,  $\delta$ ,  $\beta$  and  $\gamma$ ). There are 10 different  $\alpha_1$  subunits which give rise to pharmacologically distinct  $\text{Ca}^{2+}$  channels. Members of the  $\text{Ca}_v1$  family include the L-type  $\text{Ca}^{2+}$  channels,  $\text{Ca}_v2$  channels account for P/Q, N and R-type  $\text{Ca}^{2+}$  channels and  $\text{Ca}_v3$  produce T-type  $\text{Ca}^{2+}$  channels (see review [26]).

### Voltage-gated K<sup>+</sup> channels

There are at least two different types of voltage-gated K<sup>+</sup> channels in  $\alpha$ -cells: a delayed rectifying K<sup>+</sup> channel (K<sub>Dr</sub> channel) and a transient voltage-activated K<sup>+</sup> channel (A-channel) [27]. The function of voltage-gated K<sup>+</sup> channels is to repolarize the action potential. These channels consist of four  $\alpha$ -subunits, that form the actual conductance pore, together with interacting intracellular  $\beta$ -subunits (see review [28]). Based on sequence homology of the hydrophobic transmembrane cores, the  $\alpha$ -subunits of voltage-gated potassium channels have been grouped into 12 classes labeled K<sub>v</sub> $\alpha$ 1-12 [29].

### Stimulus-secretion coupling and exocytosis

The  $\alpha$ -cells are, like the neighbouring  $\beta$ - and  $\delta$ -cells [30], electrically excitable. They fire action potentials at low glucose concentrations or when the glucagon secretion is stimulated by amino acids like arginine [31]. These action potentials result from the opening of voltage-gated K<sup>+</sup>-, Na<sup>+</sup>- and Ca<sup>2+</sup> channels (Fig. 2) [15].



**Fig. 2 The pancreatic  $\alpha$ -cell action potential involves at least four types of voltage-gated current.** T: low voltage-activated T-type Ca<sup>2+</sup> current. Na<sup>+</sup>: Na<sup>+</sup> current. N/L: high voltage-activated N- and L-type Ca<sup>2+</sup> current. K<sup>+</sup>: voltage-dependent K<sup>+</sup>-current.

As discussed above, the role of K<sub>ATP</sub> channels in insulin secretion is well established [32], whereas the role of these channels in  $\alpha$ -cells is much less clear. If glucose closes these channels, then the  $\alpha$ -cells should depolarize which in turn should be expected to stimulate – and not inhibit – glucagon secretion.

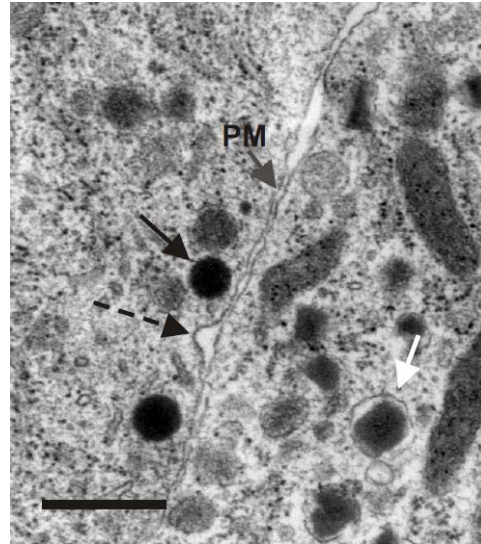
In this thesis we have explored the possible role of the K<sub>ATP</sub> channel in the control of glucagon secretion from rodent and human  $\alpha$ -cells (**Paper 1**). Under hypoglycemic conditions, glucose is transported into the  $\alpha$ -cells at a low rate, presumably involving the glucose transporter Glut1 [33]. Glucose metabolism is therefore not proceeding at maximum speed and the ATP/ADP ratio is not high enough (but higher than in  $\beta$ -cells) to completely block the K<sub>ATP</sub> channels. Therefore, the  $\alpha$ -cells membrane potential is maintained at a level sufficiently depolarized to allow action potential firing. During the action potentials, voltage-gated Ca<sup>2+</sup> channels open. The increase in intracellular Ca<sup>2+</sup> results in fusion of some of the glucagon-containing secretory granules with the plasma

membrane. In each  $\alpha$ -cell, there are  $\sim 8,000$  glucagon-containing granules [15]. The process by which glucagon is released from the interior of the granules to the external environment is referred to as exocytosis (Fig. 3).

An increase in glucose has been postulated to produce the closure of remaining active  $K_{ATP}$  channels, thereby leading to stronger membrane depolarization. This in turn causes voltage-dependent inactivation of the membrane conductance involved in action potential firing, culminating in inhibition of glucagon secretion and  $\alpha$ -cell exocytosis. This hypothesis will be discussed further in the *Result and Discussion* section.

Other studies have observed  $[Ca^{2+}]_i$  oscillations in the  $\alpha$ -cell under hypoglycemic conditions. The frequency of  $[Ca^{2+}]_i$  oscillations diminishes – but does not drop to zero – as the extracellular glucose concentration increases and hence glucagon release decreases [34]. Exactly how this decrease occurs is not clear but inactivation of an ion channel involved in the pacemaking of the  $\alpha$ -cell (via membrane depolarization induced by  $K_{ATP}$ -channel closure) is a possible mechanism.

According to an alternative hypothesis, high glucose levels lowers cytoplasmic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) and inhibits glucagon secretion by stimulating  $Ca^{2+}$  sequestration into the endoplasmic reticulum [35]. When the intracellular  $Ca^{2+}$  stores are filled, a depolarizing store-operated current is switched off and this would lead to membrane repolarization, cessation of action potential firing and inhibition of glucagon release [36].



**Fig. 3 Electron micrograph of human pancreatic islet.** The image highlights an  $\alpha$ -cell to the left and a  $\beta$ -cell to the right. Notice the exocytotic event (omega-shaped fusion; dotted arrow) in the  $\alpha$ -cell. Black arrow indicates a glucagon granule. White arrow indicates an insulin granule. PM: plasma membrane. Scale bar: 0.5  $\mu$ m.

## Regulation of glucagon secretion

### Autocrine and paracrine regulation

Glucagon exerts a positive feed-back autocrine effect on  $\alpha$ -cells [37] besides stimulating insulin and somatostatin secretion [38, 39]. The glucagon receptor is a G-protein coupled receptor [40] and by binding to the receptor on  $\alpha$  cells, glucagon stimulates exocytosis by elevating the intracellular cAMP level. Many hormones and neurotransmitters exert their effects via increases in the intracellular cAMP level. Cyclic AMP is synthesized from ATP by adenylyl cyclase situated close to the plasma membrane. Agents that increase intracellular cAMP (like GLP-1 and glucagon) stimulate insulin secretion from  $\beta$ -cell by activation of both protein kinase A (PKA)-dependent and -independent pathway. The

latter is through binding to downstream protein cAMP guanine nucleotide exchange factor II (cAMP-GEF II or Epac2) [41]. Cyclic AMP-dependent signaling pathways also play important role in the regulation of glucagon secretion but these are, in general, less well understood than in  $\beta$ -cells. In the **second paper** we describe that cAMP exert both inhibitory and stimulatory effects on the  $\alpha$ -cells via activation of PKA and Epac2, respectively.

Pancreatic  $\alpha$ -cells are surrounded by neighboring  $\beta$ -cells and  $\delta$ -cells. Thus, insulin and somatostatin are attractive candidates for paracrine regulation of glucagon secretion. Indeed, addition of exogenous insulin inhibits glucagon secretion but only at low glucose and not at high glucose [42, 43]. This inhibition may be attributed to a direct stimulatory effect of insulin on  $K_{ATP}$  channel activity. Such an effect has been reported in  $\beta$ -cells [44]. If a similar mechanism operates in  $\alpha$ -cells, it would produce membrane repolarization, reduce action potential firing, lower intracellular  $Ca^{2+}$  concentration and suppression of glucagon secretion. The  $\alpha$ -cell has also been proposed to be under paracrine control by  $Zn^{2+}$  [42, 45] as well as  $\gamma$ -Aminobutyric acid (GABA) [46] that are co-released with insulin.

Somatostatin secreted by  $\delta$ -cells have been shown to inhibit both glucagon and insulin secretion [47]. There are multiple somatostatin receptor (SSTR) subtypes but type 2 somatostatin receptor (SSTR2) is believed to be the functionally important subtype in  $\alpha$ -cells [48]. A previous study has demonstrated that transgenic mice lacking the expression of SSTR2 had increased arginine/ $K^+$ -stimulated glucagon secretion, but undisturbed glucagon secretion at 5.5 mM glucose [47].

Collectively, these findings argue that both insulin and somatostatin influence glucagon secretion. However, insulin and somatostatin release is not significant when glucose concentration is low [49, 50] and glucagon release can be inhibited without any concomitant changes in the release of the two other hormones. Clearly, further studies are needed to establish the relative importance of paracrine and intrinsic (i.e. within the  $\alpha$ -cell itself) control of glucagon secretion.

### **Hormonal regulation**

Glucagon secretion is under tight neuronal and hormonal regulation [51]. Important regulators include adrenaline, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). Adrenaline and GIP stimulates, while GLP-1 inhibits glucagon secretion [52, 53].

Adrenaline is secreted by chromaffin cells in the adrenal glands in response to stress, excitement or threat (like defending a thesis). It is also released locally within the pancreatic islets by adrenergic nerve endings. Adrenaline is a strong stimulus of glucagon secretion and simultaneously inhibits insulin secretion. These two effects likely account for the dramatic hyperglycemic action of adrenaline. It seems likely that adrenaline principally acts by binding to  $\beta_2$ -adrenoreceptors on the  $\alpha$ -cell and potentiate exocytosis by cAMP/PKA-dependent mechanisms [12, 54]. There is also evidence that adrenaline acts by  $\alpha_1$ -adrenoreceptors and this may account for a transient elevation of  $[Ca^{2+}]_i$  sometimes observed in response to application of adrenaline [54].

GLP-1 is released from the L-cells in the intestine in response to food intake. It is encoded by the proglucagon gene, which via alternative splicing also encodes glucagon and GLP-2 [55]. Previous investigations have shown that GLP-1 is capable of stimulating

glucose-induced insulin secretion from  $\beta$ -cells as well as inhibiting glucagon secretion from  $\alpha$ -cells [56]. This is an almost ideal profile of a compound to treat diabetes, a disease which involves both insufficient release of insulin as well as oversecretion of glucagon. Exenatide, a synthetic of GLP-1 analogue, as well as the dipeptidyl peptidase-4 (DPP-4) inhibitors vildagliptin and sitagliptin are already used in the treatment of type-2 diabetes [57, 58]. The exact mechanism by which GLP-1 inhibits glucagon release remains debated, however, and is still under intense investigation. A previous study from our laboratory has reported that GLP-1 increases cAMP production in rat  $\alpha$ -cells [37]. It has also been shown that GLP-1 abolishes spontaneous  $\text{Ca}^{2+}$  oscillations in mouse  $\alpha$ -cells [59]. During the course of this thesis work, we have examined in detail how GLP-1 may influence glucagon secretion by a direct effect. Such mechanisms may be unexpected given that most studies agree that  $\alpha$ -cells contain few (if any) GLP-1 receptors (**Paper 2**) [42, 60].

### **III. Aims of the Thesis**

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The overarching aim of this thesis was to explore the cellular regulation of glucagon secretion. We have examined the different types of ion channels present in  $\alpha$ -cells and intracellular second messengers with a view to elucidate how they cooperate in the control of  $\alpha$ -cell function.

#### **Specific aims**

1. Are  $K_{ATP}$  channels involved in the regulation of glucagon secretion from pancreatic  $\alpha$ -cells?
2. What are the roles of the different  $Ca^{2+}$  channels in the regulation of  $\alpha$ -cell exocytosis?
3. How can GLP-1 and adrenaline, which both increase intracellular cAMP, have opposite effects on glucagon secretion?
4. How does the activation of protein kinase C (PKC) contribute to the regulation of glucagon secretion?

## IV. Methodology

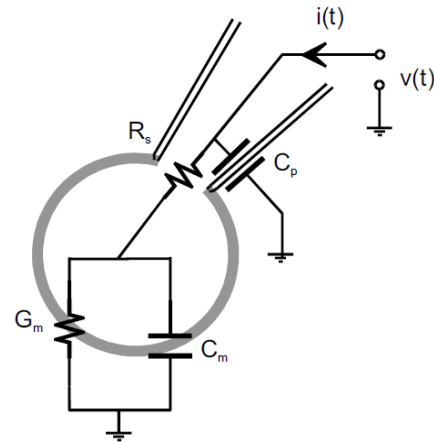
### Measurement of Pancreatic Hormone Release

We have studied hormone secretion from isolated structurally intact mouse, rat and human pancreatic islets. This allows secretion to be studied without interference from any processes in other organs. Hormone release was measured by radioimmunoassays (RIA). Freshly isolated islets were pre-incubated in Krebs-Ringer bicarbonate (KRB) buffer. Groups of twelve islets were then incubated for one hour in KRB buffer supplemented with various modulators of secretion. At the end of the test incubations, aliquots of the incubation medium was removed for determination of glucagon (and sometimes insulin and somatostatin) concentrations as previously described [61].

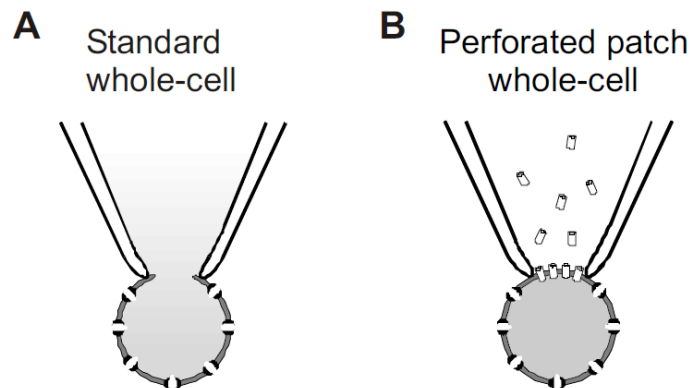
### The Patch-clamp Technique

The patch-clamp technique was developed by Erwin Neher and Bert Sakmann in the late 1970s [62]. It offers real-time high-resolution recordings of ion channel activities, exocytosis and membrane potential [63]. Capacitance measurements allow recording of exocytosis at the single-cell level and with millisecond resolution, something which cannot be accomplished using more standard (biochemical) approaches.

Patch-clamp recordings require the establishment of an electrical contact between the electrode and the cell interior needs (Fig. 4). The silver wire (that provides the electrical contact to the amplifier) is immersed in the pipette solution within a glass pipette. The glass pipette is fire-polished and the diameter of the open tip is  $\leq 1 \mu\text{m}$ . By applying gentle suction to the interior of the pipette, the cell membrane is brought in close contact with the glass pipette and this reproducibly results in a high-resistance seal (giga-seal). The seal is so tight that ions cannot pass between the glass and the membrane and the patch of membrane enclosed by the electrode is electrically isolated from the environment. It is also mechanically stable and thereby allows a number of further manipulations [64]. In this thesis we have employed either “standard whole-cell” or “perforated patch whole-cell” configurations (Fig. 5). These two recording configurations have both *pro and cons*, which are summarized below.



**Fig. 4 The equivalent electrical circuit of the standard whole-cell configuration.**  $R_s$ : series resistance.  $G_m$ : membrane conductance.  $C_m$ : membrane capacitance.  $C_p$ : pipette conductance.



**Fig. 5 Illustration of standard whole cell configuration and perforated patch whole-cell configuration.** In the whole-cell configuration (A), the ruptured plasma membrane results in exchange of the cytosol with the pipette solution. In the perforated patch whole-cell configuration (B), the plasma membrane remains intact. To establish electrical contact between the recording electrode and the cell interior the pore-forming antibiotic Amphotericin B is added to the pipette solution. Once inserted into the patch membrane, membrane currents and potentials can be recorded allowing both current- and voltage-clamp measurements.

## Standard whole-cell recording

The standard whole-cell configuration is achieved by rupturing the patch of membrane enclosed within the glass pipette after formation of a giga-seal. The exchange of intracellular pipette solution with cytosol allows the application of intracellular second messengers simply by including them into the pipette solution. The rate at which a molecule enters the cell depends on the diameter of the recording pipette, the width of the orifice in the membrane, the molecular weight of the substance to be applied (the larger the molecule, the slower the wash-in) as well as the size of the cell [65]. One limitation of the configuration is the progressive wash-out of the intracellular second messengers. This may explain the "run-down" of ion channel activities and exocytosis that often follows upon formation of the whole-cell configuration [66].

## Perforated patch whole-cell recording

In the perforated whole-cell configuration, intracellular metabolism is maintained. This is because the pores formed by amphotericin are only permeable to compounds with a molecular weight of <200 D. The perforated whole-cell configuration is technically more demanding than the standard whole-cell technique but it prevents the wash-out of intracellular component and allows measurements from metabolically intact cells. This is at the cost of not being able to control the composition of the cytosol [67] and this has to be accomplished by alternative less direct means (like applying membrane-permeable compounds, lipophilic [i.e. membrane-permeable] activators of intracellular enzymes etc.).



## **Study of exocytosis by capacitance measurement**

Exocytosis can be monitored as changes in membrane capacitance ( $\Delta C_m$ ) [68]. Capacitance refers to the ability to hold electrical charge. The lipid bilayer of the plasma membrane can be considered as a parallel-plate capacitor. Its capacitance is proportional to the surface area of the conductor plates. Accordingly, the membrane capacitance ( $C_m$ ) is proportional to the surface area ( $A_s$ ) of the plasma membrane. The specific capacitance ( $\epsilon$ ) of the cell membrane is  $9 \text{ fF}/\mu\text{m}^2$  [69]. During exocytosis, the insertion of the granule into the plasma membrane results in an increase in the membrane area that can be detected as an increase in membrane capacitance. An average glucagon-containing granule has a diameter of  $\sim 260 \text{ nm}$  [15, 30]. Assuming spherical geometry and using the specific capacitance quoted above, it can be estimated that a single glucagon granule contributes about  $2 \text{ fF}$  upon fusion with the cell membrane.

There are different ways to elicit exocytosis experimentally. In most of our experiments, we recorded the capacitance increases that were evoked by 500-ms depolarizations from  $-70 \text{ mV}$  to membrane potentials between  $-50$  to  $+20 \text{ mV}$  or by trains of five or ten 500-ms depolarizations from  $-70$  to  $0 \text{ mV}$  applied at  $1 \text{ Hz}$ .

## **Intracellular Calcium Measurement**

An imaging range of fluorescent  $\text{Ca}^{2+}$ -indicators have been developed [70] based on the pioneering work of Tsien and colleagues [71]. We have used both a combination of fluo-4 and fura red (for ratiometric measurements; **Paper 1**) and fluo-4 alone (**Paper 2**). Upon binding of  $\text{Ca}^{2+}$ , the fluorescence of fluo-4 increases dramatically, whereas that of fura red is decreased. All indicators were excited by a  $488\text{-nm}$  argon laser, and emitted fluorescence was collected through  $500\text{-}550 \text{ nm}$  and  $650\text{-}710\text{-nm}$  band-pass filters for the fluo-4 and fura red signals, respectively.

## **Immunocytochemistry**

We have employed immunocytochemistry to establish cell identity and to investigate the distribution of certain proteins within the cell. Single mouse or human cells were cultured overnight to allow adhesion to the glass cover-slip. The cells were fixed with 4% paraformaldehyde in  $\text{Ca}^{2+}$ -free PBS for 1 hour and permeabilised with 5% Triton X-100 overnight. Unspecific binding was blocked with 5% donkey serum before incubating with different primary antibodies overnight. After washing with PBS, the cells were exposed to different secondary antibodies. The labeled cells were visualized by confocal microscope using the  $488 \text{ nm}$  (Cy2),  $550 \text{ nm}$  (Cy3) and  $633 \text{ nm}$  (Cy5) lasers for excitation. Unspecific binding of the secondary antibodies could be excluded by control experiments performed in the absence of the primary antibodies.

In **Paper 3** the fixation of the cells were preceded by stimulation in the presence of different drugs. In short, the culture media was replaced by KRB buffer ( $1 \text{ mmol/l}$

glucose) during 30 minutes of pre-incubation. The cells were then incubated for 1 h in KRB buffer supplemented with or without the different stimuli before being fixed as described above.

## **Flow Cytometry**

To obtain pure  $\alpha$ -cells from mice expressing YFP under pro-glucagon promoter [72], we performed Flow Cytometry on dispersed islets using a Dakocytomation MoFlo cell sorter. The excitation was set at 488 nm and emission monitored at 530 nm and 580 nm [72]. The first 200 Venus-positive cells were collected into HBSS and inspected immediately by fluorescence microscopy, to confirm that the percentage of fluorescent cells was at least 90%. Subsequent cells were sorted into 1 ml of RNAlater (Ambion). Based on the mRNA-content, the Venus-positive fraction consisted exclusively of  $\alpha$ -cells.

## **Gene Expression**

In **Paper 2**, Venus-positive purified cell total RNA was prepared using RNeasy Mini kit (Qiagen) followed by DNase digestion (RQ1 DNase, Promega) and reverse transcription (SuperScript III, Invitrogen), according to the manufacturers' instructions. The resulting cDNA was subjected to 40 cycles of quantitative real-time PCR (RotorGene 2000, Corbett Research) in 10  $\mu$ l reactions containing 1X JumpStart PCR Master Mix (Sigma) and 0.2  $\mu$ M of the respective primers. In **Paper 3**, human and mouse pancreatic islets were dissolved in TRIzol (Invitrogen, CA, USA) and stored at -80°C. Total islet RNA was extracted according to a modified TRIzol protocol and reverse transcribed into cDNA using TaqMan Reverse transcription (Applied Biosystems, CA, USA) as described elsewhere [73]. Relative quantitative real-time PCR (qPCR) of human and mouse protein kinase C (PKC) genes was performed using the QuantiFast SYBR Green PCR Kit and QuantiTect Primer Assays (all from Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Pancreatic islet PKC gene expression was calculated relative to the housekeeping gene GAPDH using the  $\Delta\Delta$ Ct method [74].

## V. Results and Discussion

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### PAPER 1

#### Direct effect of glucose on glucagon secretion

Glucagon secretion is inhibited by hyperglycemia and stimulated by hypoglycemia. Many investigations have investigated the neuronal and paracrine regulation of glucagon secretion [27]. It remains disputed, however, whether glucose exerts a direct influence on the  $\alpha$ -cell. We investigated insulin and glucagon release together with  $\text{Ca}^{2+}$  measurements from  $\alpha$ - and  $\beta$ -cells in intact rodent and human pancreatic islets. Glucagon release was maximally suppressed at glucose concentrations (7- and 8.3 mM), just above the threshold concentration for stimulation of insulin secretion in mouse and rat islet. This argues that glucagon secretion must, at least in part, be regulated by mechanism not mediated by insulin released from the  $\beta$ -cell.

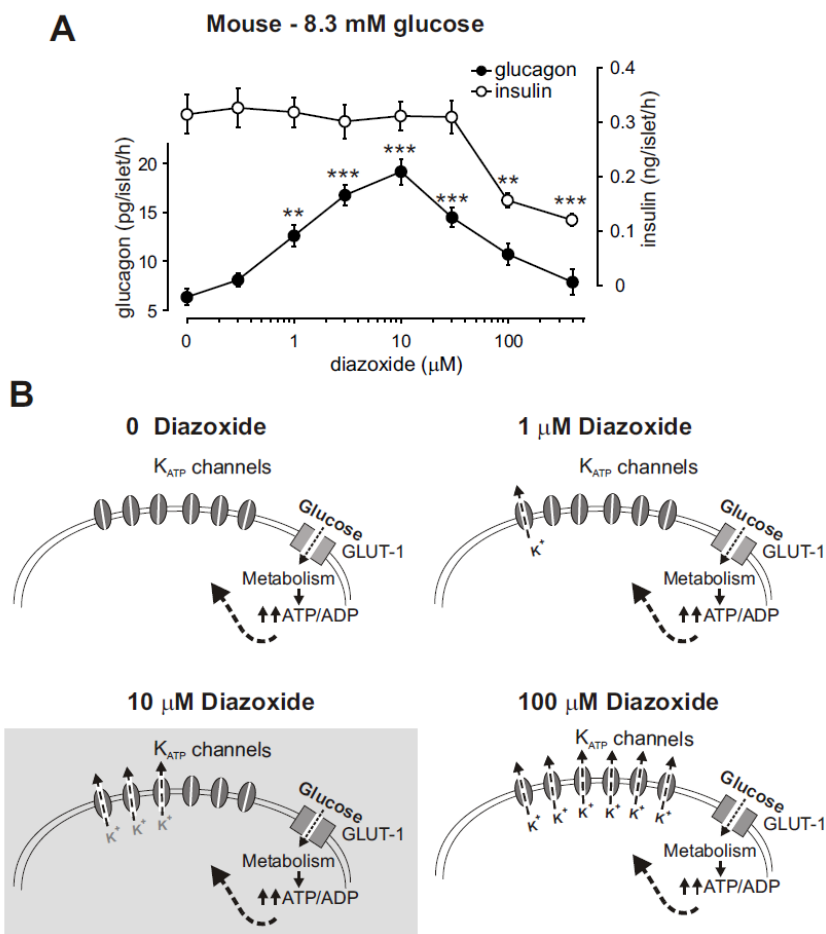
We also investigated other possible paracrine modulators of glucagon such as GABA and  $\text{Zn}^{2+}$ . We measured glucagon secretion in single mouse and rat islets at 1 and 7 mM glucose in the presence of the GABA<sub>A</sub> antagonist SR-95531 and the  $\text{Zn}^{2+}$  chelator  $\text{Ca}^{2+}$ -EDTA. Importantly, glucose remained capable of suppressing glucagon secretion even when  $\text{Zn}^{2+}$ - and GABA-mediated signaling was prevented. It is notable, however, that the inhibitory effect was somewhat reduced in the presence of the GABA receptor antagonist. Thus, it appears that part of the effect of glucose may be mediated by GABA released from the  $\beta$ -cells. Recent data suggest that this is also the case in human islets (Braun et al., submitted). Nevertheless, it seems reasonable to conclude that the ability of glucose to inhibit glucagon secretion to a major extent reflects a direct (intrinsic) effect on the  $\alpha$ -cell. So, what is the nature of this regulation?

#### $\text{K}_{\text{ATP}}$ -dependent regulation of glucagon release

One possibility is that the  $\text{K}_{\text{ATP}}$  channel constitutes the link between glucose metabolism and electrical activity in the  $\alpha$ -cell [11]. In order to study the role of the  $\text{K}_{\text{ATP}}$  channel in the regulation of glucagon release, we examined  $\alpha$ -cell  $[\text{Ca}^{2+}]_i$  and glucagon release in the presence of increasing concentrations of  $\text{K}_{\text{ATP}}$  channel activator diazoxide and the  $\text{K}_{\text{ATP}}$  channel inhibitor tolbutamide.

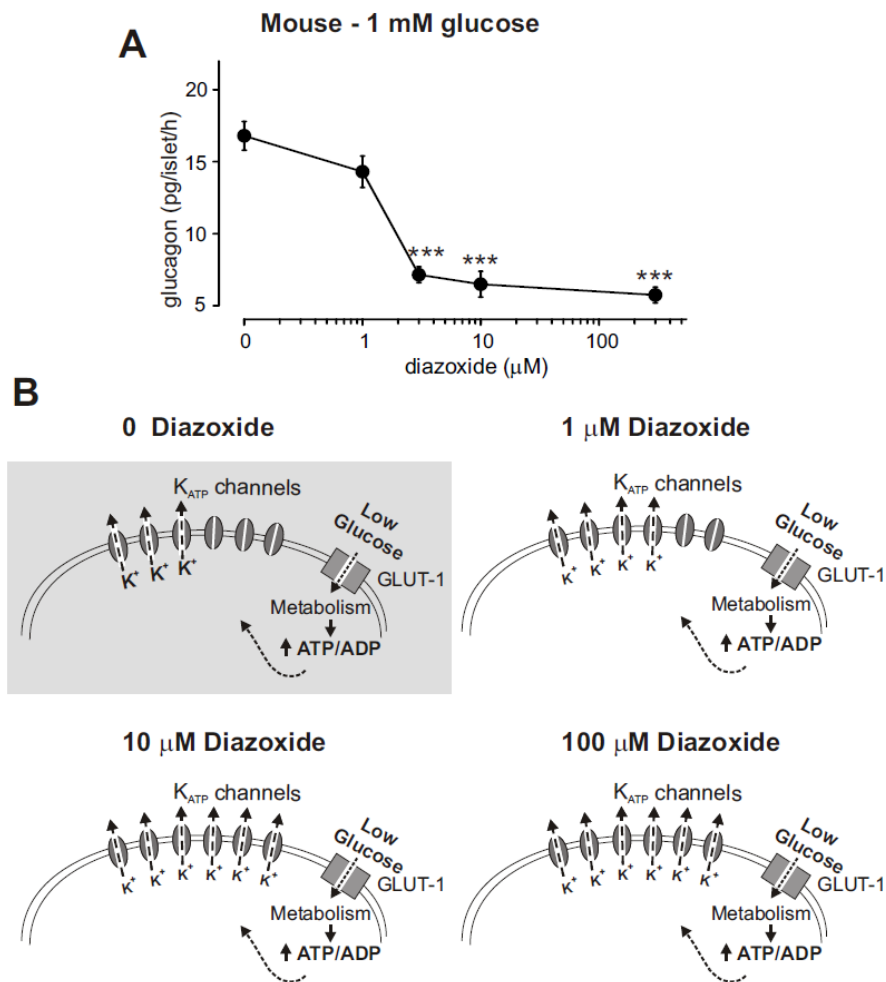
Glucagon secretion was first inhibited in the presence of 8.3 mM glucose (Fig. 6A). By analogy to the situation in the  $\beta$ -cell [13], we propose that the  $\alpha$ -cell  $\text{K}_{\text{ATP}}$  channels are fully inhibited under these conditions (Fig. 6B). The inhibitory effect of glucose could be counteracted in a dose-dependent fashion by increasing concentrations of diazoxide up to 10  $\mu\text{M}$  (Fig. 6A). This we attribute to the opening of  $\text{K}_{\text{ATP}}$  channels (Fig. 6B). The effect is not secondary to inhibition of insulin secretion since insulin secretion is stable at diazoxide concentrations  $\leq 30 \mu\text{M}$ . Higher concentrations of diazoxide inhibited glucagon secretion in parallel with an inhibition of insulin release (Fig. 6A). This is likely to result

from strong activation of the  $K_{ATP}$  channels with resultant hyperpolarization of the  $\alpha$ -cell and suppression of electrical activity (Fig. 6B).



**Fig. 6 Glucagon release from isolated and intact mouse islets.** (A) Glucagon (filled circles) and insulin (open circles) secretion measured in the presence of 8.3 mM glucose at increasing concentrations of diazoxide. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared with zero diazoxide. (B) Simplified schematic of  $K_{ATP}$  activity in the presence of different concentrations of diazoxide.

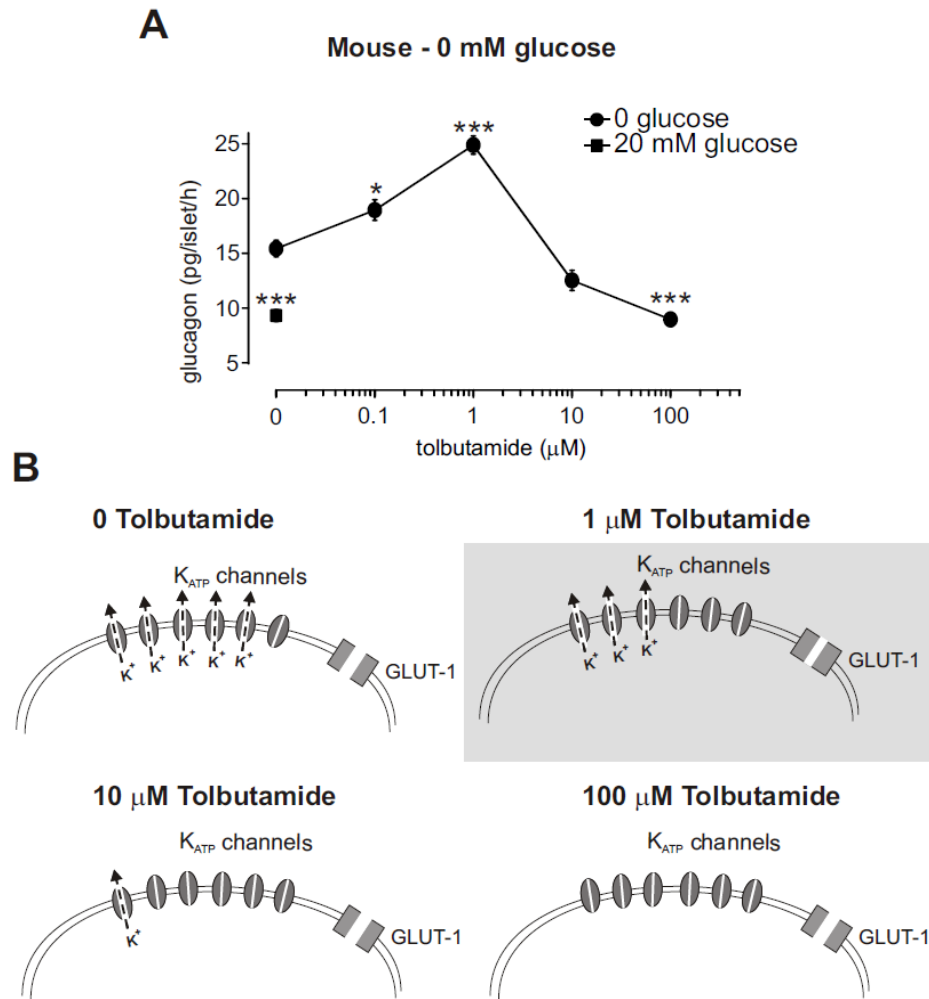
The same experiments were performed in the presence of 1 mM glucose (Fig. 7). In the absence of diazoxide glucagon release is stimulated at this glucose concentration. We believe that this is due to a partial opening of the  $K_{ATP}$  channels resulting in an intermediate depolarization of the plasma membrane (Fig. 7B). Increasing concentrations of diazoxide (0-100  $\mu$ M) produced a monotonic inhibition of glucagon secretion (Fig. 7A). We postulate that this is because any activation of  $K_{ATP}$  channels will hyperpolarize the  $\alpha$ -cell and that this will produce a concentration-dependent inhibition of  $\alpha$ -cell electrical activity (Fig. 7B).



**Fig. 7 Glucagon release from isolated and intact mouse islets.** (A) Glucagon secretion measured in the presence of 1 mM glucose at increasing concentrations of diazoxide. (B) Simplified illustrations of  $K_{ATP}$  activity in the presence of different concentrations of diazoxide. \*\*\*  $p < 0.001$  compared with zero diazoxide (A).

We compared the effects of diazoxide with those of tolbutamide, a blocker of the  $K_{ATP}$  channel. When tolbutamide is applied in the absence of glucose, increasing concentrations initially (up to 1  $\mu\text{M}$ ) produce a slight stimulation of glucagon secretion but then results in progressive inhibition of release (Fig. 8A). In the absence of both tolbutamide and glucose, the  $K_{ATP}$  channels are as active as they can be in the  $\alpha$ -cell (Fig. 8B; but note that  $K_{ATP}$  channel activity is not so great that electrical activity is prevented). The ability of a low concentration of tolbutamide to stimulate glucagon secretion indicates that  $K_{ATP}$  channel activity at 0 mM glucose is somewhat higher than that associated with maximum action potential firing. Closure of the  $K_{ATP}$  channels will then (by analogy to the situation in the  $\beta$ -cell) increase action potential firing and stimulate glucagon secretion (Fig. 8B). Further inhibition of  $K_{ATP}$  channels leads to stronger depolarizations which in turn may inhibit electrical activity and secretion by inactivation

of the channels involved in action potential firing. This is supported by the finding that when different concentrations of diazoxide and tolbutamide were tested in  $[Ca^{2+}]_i$  imaging experiments, the  $Ca^{2+}$  oscillations could be evoked in a way that echoed the secretion responses. Importantly, similar effects of diazoxide and tolbutamide on glucagon secretion and  $[Ca^{2+}]_i$  were also observed in human islets.



**Fig. 8 Glucagon release from isolated and intact mouse islets.** (A) Glucagon secretion measured in the absence of glucose at increasing concentrations of tolbutamide. Glucagon secretion in response to 20 mM glucose is indicated by the filled square. (B) Simplified illustrations of  $K_{ATP}$  activity in the absence of glucose at different concentrations of tolbutamide. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$  compared with zero tolbutamide (A).

Taken together, these data suggest that glucagon secretion from the pancreatic  $\alpha$ -cell is similar to insulin secretion from the  $\beta$ -cell. It involves closure of the  $K_{ATP}$  channels, and maximal secretion is possible within an optimal “window” (shaded panels in Fig. 6-8) of intermediate  $K_{ATP}$  channel activity.

## **Role of Na<sup>+</sup> channels in glucose-regulated glucagon secretion**

It is implicit from the above argument that the K<sub>ATP</sub> channels must be almost fully closed even in the absence of glucose. This is in agreement with measurements of the ATP/ADP ratio in  $\alpha$ -cell fractions obtained by flow cytometry [75]. These measurements suggest that the ATP/ADP ratio in  $\alpha$ -cells exposed to 1 mM glucose is similar to that of  $\beta$ -cells exposed to 5 mM. Measurements of K<sub>ATP</sub> channel activity in  $\beta$ -cells indicate that the K<sub>ATP</sub> channels are blocked by >90% at this glucose concentration, which is close to the threshold for initiation of insulin secretion and action potential firing in the  $\beta$ -cell. It is possible that because the inward current of  $\alpha$ -cells is much larger than in  $\beta$ -cells (~500 pA vs. ~100 pA) that they are capable of firing action potentials even before the K<sub>ATP</sub> channels are completely closed. This difference is a consequence of the  $\alpha$ -cell Na<sup>+</sup> channel being active in the physiological range of membrane potentials [15]. A central role of Na<sup>+</sup> channels in action potential firing is also suggested by the fact that glucagon release was completely inhibited by Na<sup>+</sup> channel antagonist TTX. In the presence of TTX, glucose exerts no further inhibitory action. It is important to point out that this does not necessarily mean that glucose acts by inhibiting the Na<sup>+</sup> channels. The only thing that we can conclude from these experiments is that inhibition of Na<sup>+</sup> channels and glucose both produce the same downstream effect.

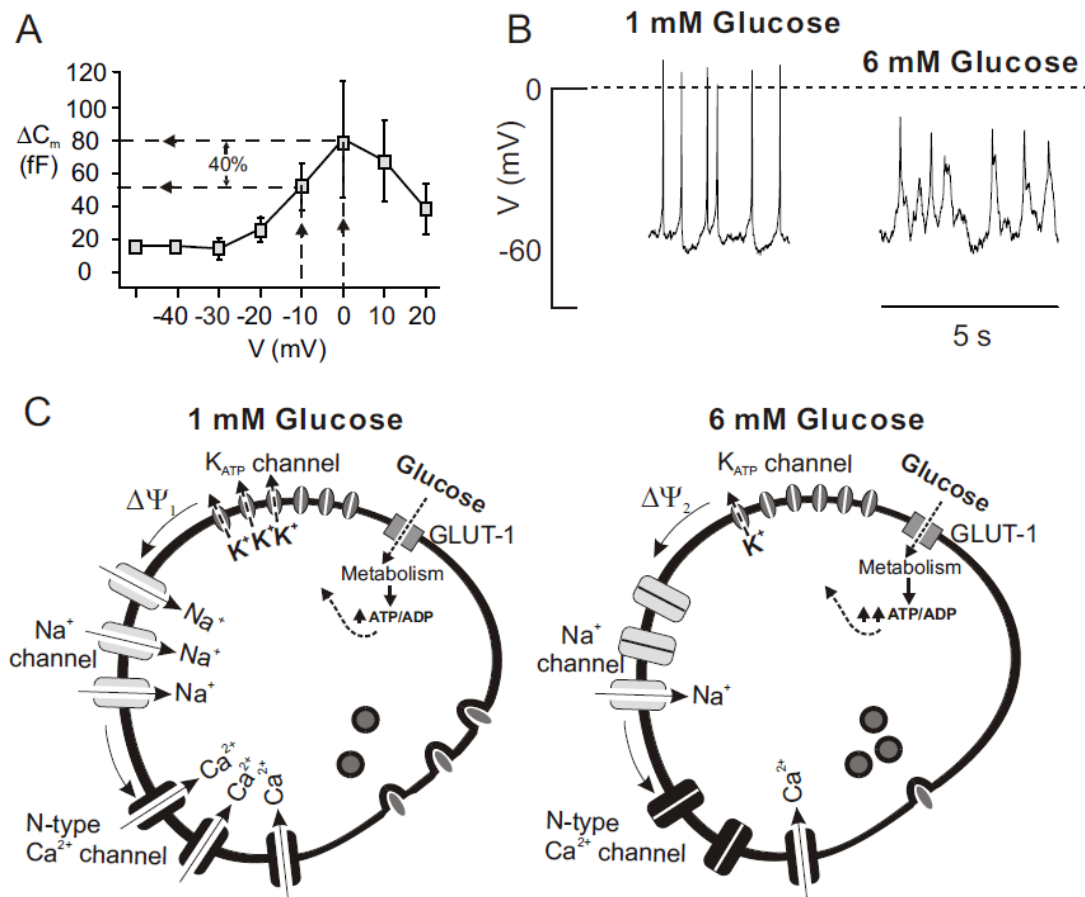
## **Role of N-type Ca<sup>2+</sup> channels in glucagon secretion**

As discussed above, the voltage-dependent Na<sup>+</sup> channel is important for the upstroke of the  $\alpha$ -cell action potential. The significance of the  $\alpha$ -cell action potential is that it takes the  $\alpha$ -cell membrane potential into a range of voltages where high voltage-activated (HVA) Ca<sup>2+</sup> channels (N- and L-type Ca<sup>2+</sup> channels) activate. The opening of these channels will result in further depolarization. Importantly, it will also result in the Ca<sup>2+</sup>-influx that initiates exocytosis of the glucagon granules. Opening of N- and L-type Ca<sup>2+</sup> channels account for 30% and 60% of the total Ca<sup>2+</sup> current in mouse  $\alpha$ -cell, respectively [15].

The N-type Ca<sup>2+</sup>-currents undergoes voltage-dependent inactivation [76]. Using perforated patch-whole-cell recordings we could observe that the  $\omega$ -conotoxin sensitive N-type Ca<sup>2+</sup> channel component decreased when the membrane potential exceeded -50 mV and exhibited half-maximal inactivation at ~-30 mV. This may explain why the magnitude of the exocytotic responses decreases when the  $\alpha$ -cell membrane potential is held at more positive voltages. Thus, exocytosis elicited by a voltage-clamp depolarization to 0 mV from -30 mV was only 25% compared to that elicited from -70 mV.

## Correlation between action potential amplitude and exocytosis

In  $\alpha$ -cells, exocytosis is maximal at voltages around zero mV (Fig. 9A). Any reduction of the action potential amplitude would have drastic effects on exocytosis under these conditions. For example, a reduction of the peak of the action potential by as little as 10 mV would be expected to decrease the exocytotic response by ~40%. This is interesting because glucose has been found to produce a small membrane depolarization (only a few mV) and reduce the peak voltage of the action potentials by >10 mV [77]. This is quite difficult to analyze as the action potential amplitude is very variable. However, the most recent analysis (Braha, Zhang and Rorsman, in preparation) indicates that in  $\alpha$ -cells exposed to 6 mM glucose, the distribution of action potential amplitudes is shifted toward lower values compared to those observed at 1 mM glucose (Fig. 9B). Importantly, a subgroup (10-15%) of overshooting action potentials (i.e. those going beyond zero mV) almost disappeared at the higher glucose concentrations. Given the steepness of the relationship between membrane voltage and exocytosis in  $\alpha$ -cells in intact islets [30], this



**Fig. 9 Connection between action potential amplitude and exocytosis.** (A) Voltage dependence of exocytosis in mouse  $\alpha$ -cells. (B) Electrical activity recording from an individual  $\alpha$ -cell exposed to 1 and 6 mM glucose. (C) Illustrations of ion channel activity and exocytosis at 1 mM and 6 mM glucose. See main text for details



can be predicted to have a significant effect on  $\alpha$ -cell exocytosis and glucagon secretion. This reduction of the action potential amplitude could also be explained by partial inactivation of the  $\text{Na}^+$ -current as a result of the glucose-induced membrane depolarization. The difference between 1 mM and 6 mM glucose is illustrated in Fig. 9C. At 1 mM glucose the  $\text{K}_{\text{ATP}}$ -channels are partially opened leading to a depolarization  $\Delta\Psi_1$  of the membrane. This depolarization is enough to activate voltage-dependent  $\text{Na}^+$  channels and downstream N-type  $\text{Ca}^{2+}$ -channels. The influx of  $\text{Ca}^{2+}$  will trigger exocytosis. In the presence of 6 mM glucose, remaining active  $\text{K}_{\text{ATP}}$ -channels close, resulting in a stronger depolarization ( $\Delta\Psi_2 > \Delta\Psi_1$ ). This will inactivate some of the downstream  $\text{Na}^+$ -channels resulting in action potentials of lower amplitude, less activation of N-type  $\text{Ca}^{2+}$  channels, reduced  $\text{Ca}^{2+}$  influx and suppression of exocytosis.

## PAPER 2

### **Role of L-type Ca<sup>2+</sup> channels in glucagon secretion**

As discussed above, mouse  $\alpha$ -cells are equipped with both N- and L-type Ca<sup>2+</sup> channels. Although the L-type Ca<sup>2+</sup>-current is much larger than the N-type Ca<sup>2+</sup>-current, it was (somewhat paradoxically) only N-type Ca<sup>2+</sup> channels that appeared important for  $\alpha$ -cell exocytosis (**Paper 1**). However, work on rat  $\alpha$ -cells have indicated that the situation changes under conditions associated with elevation of intracellular cAMP (such as adrenaline or forskolin). Under these conditions, both  $\alpha$ -cell exocytosis and glucagon secretion depend on Ca<sup>2+</sup>-influx via L-type Ca<sup>2+</sup> channels and are highly sensitive to nifedipine or isradipine but resistant to  $\omega$ -conotoxin [78]. As now shown in **Paper 2**, this also seems to apply to mouse  $\alpha$ -cells. We have examined the functional significance of the switch from N- to L-type Ca<sup>2+</sup> channels in response to cAMP. It turns out that this is highly significant not only to the mechanism by which adrenaline stimulates glucagon secretion but also the processes that explain the ability of GLP-1 to inhibit glucagon secretion.

### **Expression of GLP-1 and Adrenaline Receptors on $\alpha$ -cell**

Despite previous reports on the inhibitory effect of GLP-1 on glucagon secretion at low glucose [79], the expression of the GLP-1 receptor on  $\alpha$ -cells is still rather controversial. Earlier reports have shown the absence or very low levels of the GLP-1 receptor (GLP-1R) in human, rat and mouse  $\alpha$ -cells [60]. Another study proposed that GLP-1 receptors are distributed among a subpopulation (20%) of rat  $\alpha$ -cells [80]. We detected by PCR a very low expression level of GLP-1 receptor (Glp1r) in mouse  $\alpha$ -cells, only 1-2% of that of  $\beta$ -adrenergic receptors (Adrb1 and Adrb2), which was confirmed by immunohistochemistry. By contrast, mouse  $\beta$ -cells expressed Glp1r at very high levels.

### **GLP-1 modulates glucagon secretion by direct effect on $\alpha$ -cell?**

Despite the low GLP-1 receptor density in  $\alpha$ -cells, GLP-1 seemed capable of inhibiting glucagon secretion without any concomitant stimulation of insulin or somatostatin secretion. This makes it unlikely that the effect of GLP-1 on glucagon secretion is mediated by paracrine modulation and instead it favours a direct effect of the incretin on the  $\alpha$ -cell. The inhibitory action of GLP-1 on glucagon secretion was not due to binding of GLP-1 to other receptors, as it was completely abolished by the GLP-1 receptor antagonist exendin (9-39).

Activation of the classical GLP-1 receptor is associated with stimulated production of cAMP. We speculated that the difference in GLP-1 and adrenaline receptor density in  $\alpha$ -cells may lead to different capacity in producing cAMP. We tested this by exposing cells to increasing concentrations of the adenylate cyclase activator forskolin (1 nM-10  $\mu$ M).

We found that whereas  $\sim 10$  nM forskolin mimicked the inhibitory effect of GLP-1, a 100- to 1000-fold higher concentration was required to exert an adrenaline-like (stimulatory) effect. Thus, the effects of GLP-1 and adrenaline in terms of “forskolin equivalents” (10 nM vs. 1-10  $\mu$ M) correlate well with the relative receptor abundance (100-fold higher for adrenaline compared to GLP-1). We speculate that the number of receptors in the  $\alpha$ -cells sets an upper limit to how much cAMP can be generated in response to the agonists

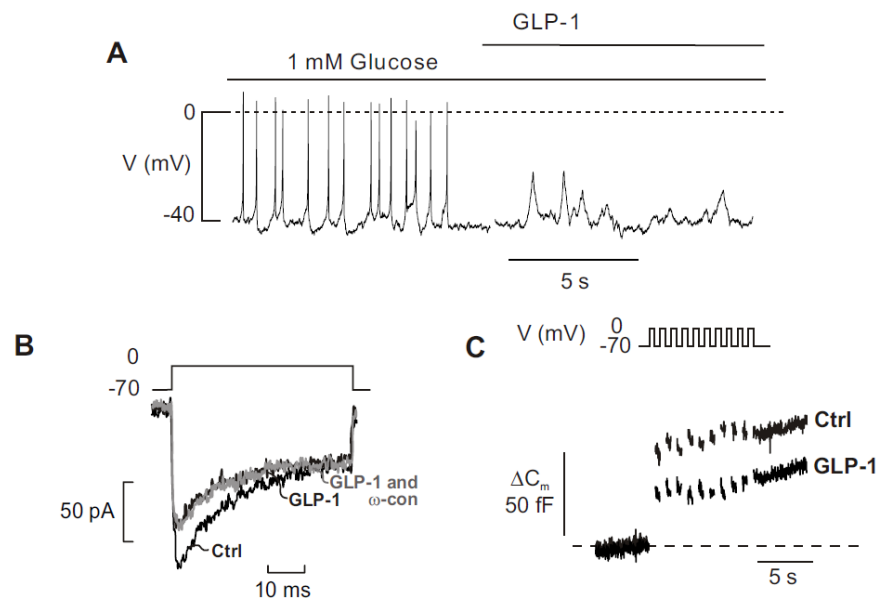
### **Effects of GLP-1 are mediated by a small increase in intracellular cAMP that activates PKA and *inhibits* N-type $\text{Ca}^{2+}$ channels**

We next asked the question; which cellular mechanisms are involved in the inhibitory action of GLP-1 on glucagon secretion? Interestingly, the inhibitory effect of GLP-1 on glucagon secretion was prevented in the presence of Rp-cAMPS, suggesting the involvement of protein kinase A (PKA).

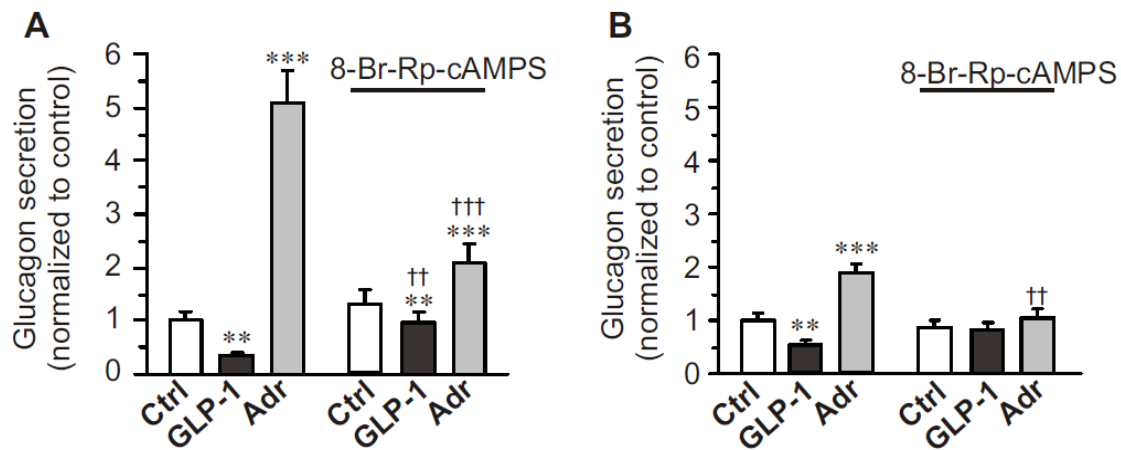
The inhibition of glucagon secretion by GLP-1 was associated with a reduction in the action potential amplitude without changing the interspike membrane potential (Fig. 10A). This demonstrates that GLP-1 does not interfere with the regulation of the  $\text{K}_{\text{ATP}}$  channel activity. We also observed that GLP-1 had no further inhibitory effect on glucagon release in the presence of the N-type  $\text{Ca}^{2+}$  channel blocker  $\omega$ -conotoxin. Therefore we investigated the possibility that GLP-1 suppresses action potential amplitude through its inhibition of the N-type  $\text{Ca}^{2+}$  channel activity. Indeed, patch-clamp measurement revealed that GLP-1 reduced the voltage-dependent  $\text{Ca}^{2+}$  current in single  $\alpha$ -cells, which could not be further inhibited by  $\omega$ -conotoxin (Fig. 10B).

Exocytosis in  $\alpha$ -cells could also be inhibited by GLP-1 (Fig. 10C), which resembles the inhibitory effect of  $\omega$ -conotoxin on  $\alpha$ -cell exocytosis (**Paper 1**). It is worth to note that the inhibitory effect of GLP-1 on exocytosis was observed at 1 mM glucose, since the effect was different from our previous study performed at 5 mM glucose, where we could observe a stimulation of exocytosis [37]. It is therefore pertinent to speculate that the GLP-1 action is glucose concentration dependent. This finding also raises the interesting possibility that glucose-induced inhibition of N-type  $\text{Ca}^{2+}$ -channels may contribute to the inhibitory action of glucose on glucagon secretion.

Both adrenaline and GLP-1 stimulate cAMP production but adrenaline produces a larger effect [37]. An increase in cAMP signals exerts its biological actions by both PKA-dependent and PKA-independent pathways. The latter is likely to be mediated by Epac2 [81]. We tested the involvement of Epac2 in the control of glucagon secretion by GLP-1 using Epac2 null mice. In Epac2 knockout islets, GLP-1 remained inhibitory but was prevented by the PKA inhibitor 8-Br-Rp-cAMPS (Fig. 11). In summary, our data suggest that the inhibitory effect of GLP-1 in  $\alpha$ -cells is mediated through PKA-dependent inhibition of N-type  $\text{Ca}^{2+}$ -channels and reduction of  $\text{Ca}^{2+}$  dependent exocytosis.



**Fig. 10 Effects of GLP-1 on  $\alpha$ -cell action potential firing and N-type  $\text{Ca}^{2+}$ -channel activity.** (A) Electrical activity recorded from an individual  $\alpha$ -cell exposed to 1 mM glucose (left), and 5 min after addition of 100 nM GLP-1 (right). (B) Whole-cell  $\text{Ca}^{2+}$ -currents evoked by membrane depolarization from -70 mV to 0 mV under control conditions (Ctrl; 5 mM glucose), 5 min after addition of GLP-1 (10 nM) and 5 min after addition of 100 nM  $\omega$ -conotoxin in the continued presence of GLP-1 (GLP-1 and  $\omega$ -con; grey). (C) Changes in membrane capacitance ( $\Delta C_m$ ) elicited by ten voltage-clamp depolarizations from -70 mV to 0 mV under control conditions (1 mM glucose) and 4 min after the addition of 10 nM GLP-1.



**Fig. 11 Glucagon secretion from wildtype (A) and Epac2 null (B) mice.** Glucagon secretion under control conditions (Ctrl; 1 mM glucose), in the presence of 100 nM GLP-1 or 5  $\mu$ M adrenaline (Adr) in the absence and presence of 10  $\mu$ M 8-B4-Rp-cAMPS. \*\* $p$ <0.01 and \*\*\* $p$ <0.001 vs. Ctrl in the absence or presence of 8-Br-Rp-cAMPS; †† $p$ <0.01 and ††† $p$ <0.001 vs. corresponding value in the absence of 8-Br-Rp-cAMPS.

## **Adrenaline stimulates L-type $\text{Ca}^{2+}$ channels and exocytosis via a large increase in cAMP, which activates Epac2 and PKA**

Because there are many more  $\beta$ -adrenoreceptors in the  $\alpha$ -cells, it is likely that the binding of adrenaline to these receptors leads to a much larger increase in cAMP, and stimulation of glucagon secretion by both PKA-dependent and PKA-independent mechanisms. Whereas PKA has a high affinity to cAMP and responds to submicromolar levels of cAMP, Epac2 requires 10- to 20-fold higher concentrations of cAMP [81]. To confirm this, we compared glucagon secretion from wildtype and Epac2 knockout mice and the effects of GLP-1 and adrenaline. As already discussed, GLP-1 retained its inhibitory action in Epac2 knockout islets whereas the stimulatory effect of adrenaline was much reduced.

We were surprised that adrenaline remained stimulatory in Epac2 knockout islets. Subsequent experiments revealed that the stimulatory component that remained was sensitive to 8-Br-Rp-cAMPS. Taken together with the PKA-dependent inhibitory effect of GLP-1, this raised the interesting possibility that there are several PKA isoforms: one that responds to low cAMP levels and mediate the effect of GLP-1 and one that requires higher cAMP levels and that *contributes* to the stimulatory effect of adrenaline (see below).

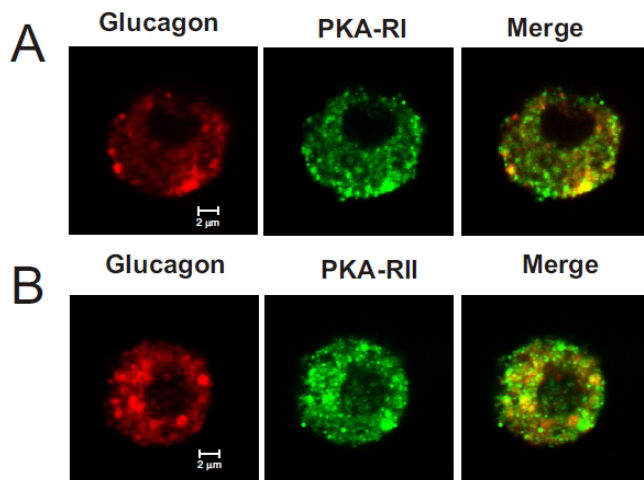
The stimulatory effect of adrenaline and high forskolin was also studied by capacitance measurements. Significant exocytotic responses were observed in  $\alpha$ -cells exposed to both reagents. Importantly, exocytosis was also observed at voltages more negative than those required to evoke exocytosis under control conditions (-30 to -20 mV instead of -10 to zero mV). This raises the interesting possibility that in the presence of high cAMP levels, exocytosis in the  $\alpha$ -cell is less sensitive to a reduction of the action potential amplitude.

In the  $\alpha$ -cell, a large increase in  $[\text{cAMP}]_i$  amplifies the exocytotic response through the activation of both PKA-dependent and PKA-independent pathways. Indeed, the effect of intracellular cAMP was mimicked by intracellular addition of the Epac2-selective agonist 8CPT-2Me-cAMPS. Interestingly, in the presence of the Epac2-selective agonist, exocytosis was partially isradipine-resistant. This we attribute to 8CPT-2Me-cAMP not activating PKA. Accordingly, N-type  $\text{Ca}^{2+}$  channels remain active and evoke exocytosis. In addition, we have preliminary data suggesting that the Epac2 agonist may stimulate L-type  $\text{Ca}^{2+}$ -channel activity.

## **The Presence of Different PKA Isoforms in Mouse $\alpha$ -cell**

The PKA family includes four regulatory R subunits ( $\text{RI}\alpha$ ,  $\text{RI}\beta$ ,  $\text{RII}\alpha$ ,  $\text{RII}\beta$ ) and two catalytic C subunits ( $\text{C}\alpha$  and  $\text{C}\beta$ ). The PKA holoenzyme is composed of two R subunits and two C subunits. Based on the difference in R subunits, the PKAs are categorized into type I ( $\text{RI}\alpha_2\text{C}_2$  and  $\text{RI}\beta_2\text{C}_2$ ) and type II ( $\text{RII}\alpha_2\text{C}_2$  and  $\text{RII}\beta_2\text{C}_2$ ) [82]. We were able to detect the presence of both PKA type I (PKAI) and type II (PKAII) in mouse  $\alpha$ -cells (Fig. 12). PKAI has a more cytoplasmic distribution, while PKAII is distributed close to the vicinity of the plasma membrane. It has been shown previously that specific PKA

isoforms can be activated upon generation of distinct cAMP pools, and different PKA isoenzymes play distinct roles [83]. PKAI is more sensitive to cAMP than PKAII, and RI subunits disassociate from C subunits in response to a lower cAMP concentration [84]. We therefore speculate that the opposite effects of PKA on glucagon secretion in the presence of adrenaline and GLP-1 are due to selective activation of PKA isoenzymes by different cAMP levels. According to this scenario, GLP-1 activates PKAI, resulting in inhibition of N-type  $\text{Ca}^{2+}$  channels leading to reduced exocytosis and glucagon release. Adrenaline also activates PKAI, as well as PKAII and Epac2, which both require higher cAMP-concentrations. Due to its localization it is likely that PKAII stimulates refilling of granules whereas Epac2 has a stimulatory effect on exocytosis at the release site close to the L-type  $\text{Ca}^{2+}$  channels. Several PKA subunit knockout mice exist. Studies on islets from these mice would allow a more detailed investigation of the specific roles of the PKA isoenzymes in the regulation of glucagon secretion.



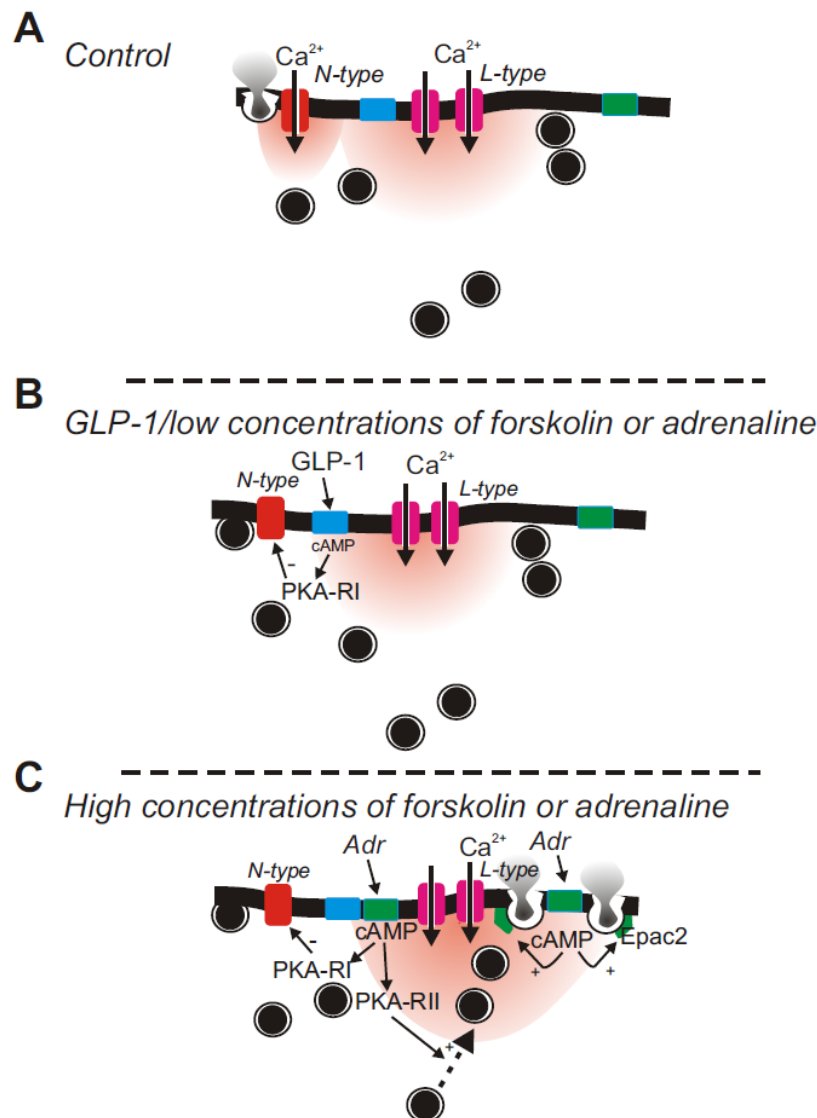
**Fig. 12 Confocal immunostaining of cells dispersed from single mouse islets.** Cells were labeled with antibodies against glucagon (red) and PKA-RI (green, A) or PKA-RII (green, B). Right panel shows the superimposed images (merge)

### A model for the opposite effects of GLP-1 and adrenaline on glucagon secretion

Based on these observations we propose the following model for the regulation of glucagon secretion (Fig. 13). Under control conditions (hypoglycemia alone), the  $\alpha$ -cell is electrically active. Entry of  $\text{Ca}^{2+}$  via the N-type  $\text{Ca}^{2+}$ -channels during the action potentials triggers exocytosis of glucagon-containing secretory vesicles. This accounts for the high basal glucagon secretion observed (Fig. 13A). Although the N-type  $\text{Ca}^{2+}$ -channels only account for 20% of the whole-cell  $\text{Ca}^{2+}$ -current (**Paper 1**), glucagon secretion evoked by low glucose alone appears almost exclusively dependent on  $\text{Ca}^{2+}$ -influx via these channels.

Postprandial release of GLP-1 inhibits glucagon secretion. Through binding to GLP-1Rs, expressed at low levels, GLP-1 causes a small increase in intracellular cAMP concentration that is sufficient to activate PKAI. PKA-dependent phosphorylation of N-type  $\text{Ca}^{2+}$ -channels leads to strong inhibition of their activity and reduced exocytosis (Fig. 13B).

In the presence of adrenaline, many (100- to 1000-fold) more  $G_s$ -coupled receptors are activated and resulting in larger increases in intracellular cAMP levels (Fig. 13C). This leads to activation of PKAI and, in common with GLP-1 as discussed above, this inhibits the N-type  $Ca^{2+}$ -channels. However, the large increase in cAMP also activates the low-affinity cAMP sensor Epac2. This increases  $Ca^{2+}$ -influx via the L-type  $Ca^{2+}$ -channels, which need not be localized to the glucagon granules. This results in a dramatic rise in the release probability of the glucagon granules and increased glucagon secretion. In addition to the activation of Epac2 and PKAI, adrenaline also activates PKAII, which requires higher cAMP levels than PKAI. We speculate that this promotes mobilization of secretory granules to release sites.



**Fig. 13 Model of the control of glucagon secretion from  $\alpha$ -cells.** (A) Under control conditions (hypoglycemia). (B) In the presence of GLP-1 or low concentrations of forskolin or adrenaline. (C) In the presence of high forskolin or adrenaline concentrations.

## PAPER 3

### Expression of PKC in Mouse and Human Pancreatic Islet and Its Effect on Glucagon Release upon Activation

In addition to PKA-dependent stimulation of glucagon release described in **Paper 2**, protein kinase C (PKC) has also been demonstrated to modulate secretion from the  $\alpha$ -cells [85, 86]. Genetic screening has unveiled 10 distinct PKC isotype genes [87]. The PKC superfamily has been subdivided based on their properties: conventional/classical PKCs (cPKCs;  $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ), novel PKCs (nPKCs;  $\eta$ ,  $\epsilon$ ,  $\delta$  and  $\theta$ ) and atypical PKCs (aPKCs;  $\iota/\lambda$  and  $\zeta$ ).

We have investigated the gene expression pattern of different PKC isoforms in mouse and human pancreatic islets. We found that the classical PKC isoforms (PKC $\alpha$ , PKC $\beta$ 1) were the most highly expressed in mouse, whereas the novel (PKC $\delta$ , PKC $\epsilon$ , PKC $\eta$ , PKC $\theta$ ) and atypical PKC (PKC $\iota$ , PKC $\zeta$ ) isoforms were more abundant in human islets. This illustrates the potential pitfalls of extrapolating observations made in rodent systems to the situation in man.

Activation of PKC by exogenous diacylglycerol (DAG) [88, 89] or by the phorbol ester and DAG analogue PMA augments insulin [90] and glucagon secretion in rat [85]. In our study, PMA strongly stimulated glucagon secretion at 1 mM glucose in both mouse and human islets. The effect was inhibited by the PKC inhibitor bisindolylmaleimide (BIM). Membrane capacitance measurements on single mouse islets revealed that the stimulatory effect of PMA is due to an enhanced exocytotic response, which increased > 4-fold in the presence of PMA. The increase in membrane capacitance was not accompanied with a change in the voltage-dependent Ca<sup>2+</sup>-current. This suggests that PKC stimulates exocytosis at a late stage at the level of fusion with the plasma membrane. Interestingly, BIM alone reduced the increase in membrane capacitance by ~60% in the majority of the cells investigated. This argues that tonic background PKC activity is required to maintain the exocytotic capacity of the  $\alpha$ -cells. The reduction in membrane capacitance in the presence of BIM was associated with a ~30% reduction in the Ca<sup>2+</sup> current. Thus, it is likely that the reduced exocytotic response in presence of BIM is a combination of the inhibition of exocytosis *per se* and the reduced Ca<sup>2+</sup> influx.

A possible downstream target for PKC phosphorylation is Munc-18, which is one of the central proteins involved at several levels in the exocytotic process [91]. It has been shown that Munc-18 phosphorylation by PKC can modulate the kinetics of exocytosis [92]. Indeed, our data in  $\alpha$ -cells are consistent with a scenario in which PKC-dependent phosphorylation of munc-18 promotes fusion, priming and docking of granules.

### Intracellular Translocation of PKC $\alpha$ and PKC $\delta$ upon Activation

It has been shown that PKC increases its membrane affinity upon stimulation. Activation of PKC by DAG or PMA induces translocation of PKC from the cytoplasm to the plasma

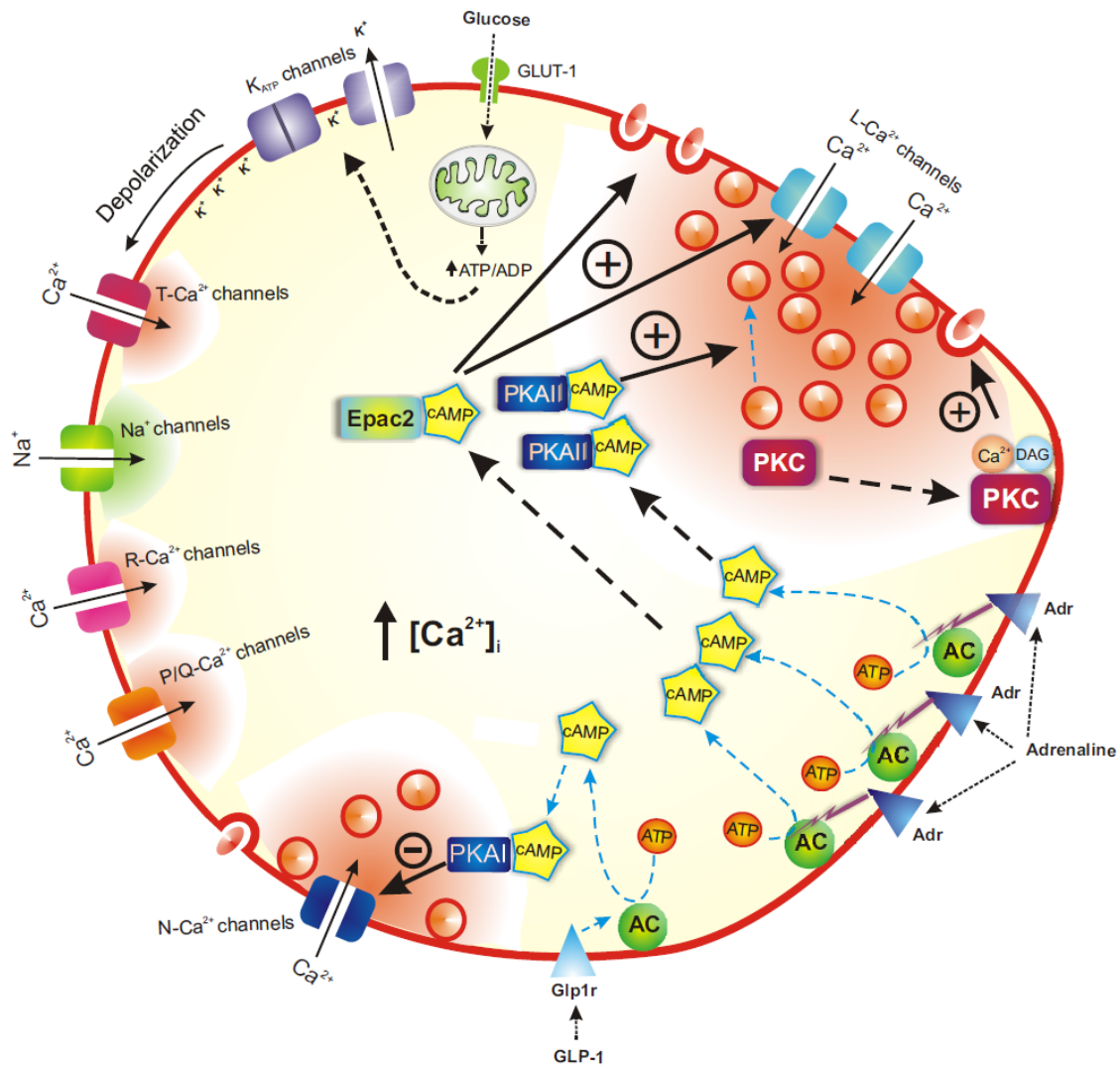


membrane [93]. Due to the extremely low gene expression of PKC $\beta$ 1 (*PRKCB*) in human islets, we selected PKC $\alpha$  for further analysis of activation and translocation of classical PKC isoforms in  $\alpha$ -cells. Among the novel PKC isoforms, PKC $\delta$  and PKC $\epsilon$  are highly expressed in both species and have been described to be involved in exocytosis [94, 95]. We opted for PKC $\delta$  in our further study since PKC $\epsilon$  showed a very weak expression by confocal immunocytochemistry in  $\alpha$ -cells.

We demonstrated that PKC $\delta$  Ca<sup>2+</sup>-independently translocated from cytosol to cell periphery upon PMA stimulation in both mouse and human  $\alpha$ -cells. This re-distribution places PKC $\delta$  in close proximity to any downstream substrates involved in exocytosis. However, we observed differences in the translocation pattern of PKC $\alpha$  between mouse and human  $\alpha$ -cells. In mouse  $\alpha$ -cells, PKC $\alpha$  is preferentially located in the vicinity of the plasma membrane already at hypoglycemic condition (1 mM glucose), while activation by PMA was necessary for the translocation of PKC $\alpha$  to the cell membrane in human  $\alpha$ -cells. PKC $\alpha$  is Ca<sup>2+</sup>-sensitive [96] and it is possible that in human  $\alpha$ -cells, the intracellular Ca<sup>2+</sup>-concentration is lower than in mouse  $\alpha$ -cells [34, 97]. This will result in a Ca<sup>2+</sup>-dependent translocation of PKC $\alpha$  to the plasma membrane in mouse  $\alpha$ -cells, whereas in human  $\alpha$ -cells this process is less Ca<sup>2+</sup>-dependent. Interestingly, the Ca<sup>2+</sup>-dependent PKC $\alpha$  translocation at 1 mM glucose in mouse islets was totally inhibited in presence of 400  $\mu$ M of the K<sub>ATP</sub> channel opener diazoxide or 2  $\mu$ M of the L-type Ca<sup>2+</sup> channel blocker isradipine. We also know (as discussed above) that exocytosis in the absence of cAMP is N-type channel dependent. Further studies are required to elucidate the roles of the different Ca<sup>2+</sup> channels in the  $\alpha$ -cells. We suggest based on our observation that L- and N-type Ca<sup>2+</sup> channels fulfil different functions in the  $\alpha$ -cell in the absence of cAMP, that Ca<sup>2+</sup>-influx through L-type Ca<sup>2+</sup> channel is mediating PKC $\alpha$  translocation, whereas exocytosis is N-type Ca<sup>2+</sup> channel dependent.

## VI. General Conclusions: A Pancreatic $\alpha$ -cell Model

Fig. 14 integrates the findings reported in this thesis with previous knowledge about  $\alpha$ -cell function to provide a model for the regulation of glucagon secretion by glucose, hormones and neurotransmitters,



**Fig. 14 Model of the control of glucagon secretion from  $\alpha$ -cells.** See text for detail.

In  $\alpha$ -cells,  $K_{ATP}$  channel activity regulates the membrane potential of the  $\alpha$ -cell. Glucagon secretion only occurs within a narrow window of  $K_{ATP}$  channel activity. Excursions beyond this “window” lead to inhibition of glucagon secretion regardless of whether channel activity is increased (as with diazoxide) or reduced (as with tolbutamide and perhaps glucose).

Under hypoglycemic conditions, the  $K_{ATP}$  channels are already strongly inhibited. The remaining  $K_{ATP}$  channel activity maintains the  $\alpha$ -cell membrane potential sufficiently

polarized to prevent channel inactivation and yet allows regenerative electrical activity. This electrical activity depends on voltage-gated T-type  $\text{Ca}^{2+}$  channels, TTX-sensitive  $\text{Na}^+$  channels and voltage-gated  $\text{K}^+$  channels (A-type). The significance of electrical activity is that it leads to the opening of high-voltage activated N- and L-type  $\text{Ca}^{2+}$ -channel. We postulate that glucose inhibits glucagon secretion by inhibiting the few  $\text{K}_{\text{ATP}}$  channels that are active in the  $\alpha$ -cell membrane in the absence of glucose. This leads to stronger depolarization and (partial) inactivation of the  $\text{Na}^+$  channels with resultant decrease in the amplitude of action potential height. As a result, N-type  $\text{Ca}^{2+}$  channels become less activated and this in turn leads to inhibition of glucagon secretion.

GLP-1 also inhibits glucagon secretion. However, our data suggest it acts by a mechanism slightly different from that used by glucose. Rather than acting via closure of  $\text{K}_{\text{ATP}}$ -channels, GLP-1 leads to direct inhibition of the N-type  $\text{Ca}^{2+}$  channels. This effect is mediated by PKA and results from the small increase in intracellular cAMP that follows upon the activation of the few GLP-1 receptors present in the  $\alpha$ -cell. It is possible that PKA-dependent inhibition of N-type  $\text{Ca}^{2+}$  channels also contributes to the inhibitory action of glucose. This is suggested by the finding (unpublished observations) that the effect of glucose can be antagonized by inhibition of PKA by 8-Br-Rp-cAMPS.

Adrenaline, on the other hand, stimulates glucagon secretion. Beta-adrenoreceptors are highly expressed in  $\alpha$ -cells and activation of these receptors will result in a significant increase in intracellular cAMP levels. This results from a combination of several effects: 1) inhibition of N-type  $\text{Ca}^{2+}$  channels by the mechanisms discussed above for GLP-1 by activation of PKAI; 2) stimulation of PKAII (which requires higher cAMP concentrations than PKAI) and stimulated mobilization of new glucagon granules to the release sites; and 3) activation of the low-affinity cAMP sensor Epac2. The latter effect leads to a strong enhancement of exocytosis already at negative voltages. This may be significant as our membrane potential recordings (unpublished) indicate that adrenaline induces high-frequency action potential firing but that their amplitude is reduced compared to control conditions (low glucose alone). The shift in voltage dependence therefore allows glucagon secretion in response to these low-amplitude action potentials. In addition, it appears that activation of Epac2 activates the L-type  $\text{Ca}^{2+}$  channels.

Activation of PKC stimulates glucagon secretion without affecting the  $\text{Ca}^{2+}$  currents. Classical PKCs are activated by binding to  $\text{Ca}^{2+}$  and/or diacylglycerol. Novel PKCs are sensitive only to diacylglycerol. Upon activation, PKC translocate to the vicinity of plasma membrane and stimulate  $\alpha$ -cell exocytosis, possibly by phosphorylating the membrane protein Munc-18. In mouse, the intracellular translocation of PKC $\alpha$  (which belongs to classical PKC) in hypoglycemia is dependent on  $\text{Ca}^{2+}$  influx through the L-type  $\text{Ca}^{2+}$  channel.

## VII. Populärvetenskaplig Sammanfattning

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Glukos är den viktigaste energikällan i vår kropp. För att vi ska kunna röra oss, äta, dricka, ja till och med vila, måste kroppen upprätthålla en stabil blodglukosnivå. Mängden glukos i blodet regleras av hormonerna insulin och glukagon. Insulin utsöndras från  $\beta$ -celler och glukagon från  $\alpha$ -celler, båda belägna i bukspottkörtelns Langerhanska öar. Insulin frisätts efter en måltid och sänker blodsockernivån genom att signalera till muskler, lever och fettvävnad att ta upp och lagra överskottet av glukos. Mellan måltider utsöndras istället glukagon som frigör glukoslageren då vi behöver tillskott av energi. Patienter med diabetes har inte bara ofullständig insulinsekretion och/eller insulinresistens utan också förhöjda nivåer av glukagon vilket förstärker det förhöjda blodglukosnivåerna. Med hänsyn till glukagonets betydelse vid regleringen av blodglukos är kunskaperna om hur utsöndringen regleras från  $\alpha$ -cellerna relativt okänd. I den här avhandlingen har jag undersökt regleringen av glukagonsekretion genom att utnyttja ett flertal olika tekniker, däribland patch-clamptechniken. Med hjälp av denna teknik kan vi på enskilda celler mäta strömmar genom något som kallas jonkanaler. Dessa finns i cellens membran och möjliggör utbyte av joner mellan insidan och utsidan av cellen under vissa förutsättningar. Vi kan också mäta förändringar i spänning över cellmembranet. Glukagon befinner sig inne i  $\alpha$ -cellen i små blåsor som kallas granula. När dessa granula sammansmälter med cellens membran så kommer glukagon att utsöndras ut i blodbanan. Denna process benämns exocytos och kan mätas som en ökning av cellens membrankapacitans. Det sistnämnda är möjligt då cellmembranet är elektriskt kan liknas vid en plattkondensator.

De pankreatiska  $\alpha$ -cellerna är elektriskt aktiva, liksom de insulinproducerade  $\beta$ -cellerna och kroppens nervceller. Den elektriska aktiviteten regleras av strömmar genom jonkanaler i cellens membran. I mitt avhandlingsarbete har jag påvisat hur glukos reglerar utsöndringen av glukagon genom en mekanism som omfattar ett flertal olika jonkanaler genomsläppliga för  $K^+$ ,  $Na^+$  och  $Ca^{2+}$ -joner.

GLP-1 är ett hormon som utsöndras i tarmarna. Detta hormon har visat sig stimulera insulinsekretion samtidigt som det inhiberar glukagonsekretion. Analoger till detta hormon är därför utmärkta läkemedel för behandling av diabetes. Jag har undersökt de cellulära mekanismer varmed GLP-1 inhiberar glukagonsekretion. GLP-1 binder till en receptor på  $\alpha$ -cellens membran, vilket medför att en intracellulär molekyl som benämns cAMP bildas. Normalt brukar en ökad mängd cAMP stimulera utsöndring, och det är också det som sker i den insulinproducerade  $\beta$ -cellen. Vi har kunnat visa på att GLP-1 inhiberar utsöndringen av glukagon, eftersom  $\alpha$ -cellen har mycket få GLP-1-receptorer. Detta leder till att en mycket liten mängd cAMP bildas inne i  $\alpha$ -cellen, vilket istället stimulerar en signalväg som inhiberar den elektriska aktiviteten och utsöndringen av glukagon.

Jag har också undersökt hur adrenalin påverkar utsöndringen av glukagon. Adrenalin är ett hormon som utsöndras då vi utsätts för press eller känner oss hotade, och därför behöver höja vårt blodsocker. Detta hormon ökar också mängden cAMP inne i  $\alpha$ -cellen, men till betydligt högre nivåer än GLP-1. Våra undersökningar har visat på att adrenalin därmed kommer att påverka andra signalvägar, som leder till ökad utsöndring av glukagon.

Slutligen har jag undersökt ytterligare en signalväg inne i  $\alpha$ -cellen. Denna involverar ett proteinkinase som heter proteinkinase C (PKC). Stimulering av PKC stimulerar exocytos i  $\alpha$ -cellen och därmed utsöndringen av glukagon. I denna undersökning fann vi vissa skillnader mellan regleringen av  $\alpha$ -celler från möss och  $\alpha$ -celler från människa. Det är därför viktigt att validera att samma mekanismer gäller i människa som i mus.

Sammanfattningsvis har mina undersökningar utökat kunskapen om hur utsöndringen av glukagon från bukspottkörtelns  $\alpha$ -celler regleras. Detta medför en ökad förståelse av  $\alpha$ -cellens betydelse vid uppkomst av diabetes samt ökade möjligheter att finna nya läkemedel för behandling av denna sjukdom.

## VIII. Acknowledgements

---

The story of this book began when I was to start my PhD education in the Department of Physiological Science in Lund University. I visited Professor **Patrik Rorsman** who later became my co-supervisor and whom I have already heard about before: a genius scientist with great new ideas all the time. Besides his scientific ideas and knowledge, his optimism and enthusiasm have never ceased to impress me. He does have the power to affect you with his passion for science, as well as his infectious loud laughter at various parties. To Patrik, I am very grateful, for guiding me into the wonderland of electrophysiology, for all the patient discussions, and for the guidance, encouragements and great support!

I was then introduced to Dr. **Lena Eliasson** who became my main supervisor along with Patrik over my PhD education. She is a great scientist and always supportive and at the mean time, leaves great space and freedom for me to develop. Lena isn't one of those 'watching over your shoulders' type of supervisor, nor did she assist by my side. She helps me to figure out where problem lies and guides me to solve them as independently as possible. With her, we share the most interesting and exciting discussions over the manuscripts, as well as joy of family and life. I appreciate very much the freedom she gave to me in research, as well as her trust in my capacity which she has helped me to develop even greater.

Since I started my PhD education, I have been very lucky to meet many great people. First of all, I would like to thank **Britt-Marie**, you are so professional and generous. And above all, you are a great friend! I enjoyed many lovely conversations with you about the lab, motorcycle rides and football stars. Our trip to Lausanne was so much fun! But next time we should not forget to bring our ski equipments instead of bags of test tubes!

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## Yang De Marinis

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