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Cell coupling and exocytosis measured in intact mouse pancreatic islets Control of {delta}-cell secretion

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Cell coupling and exocytosis measured in intact pancreatic islets

Control of δ -cell somatostatin secretion

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Doctoral thesis

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The cover shows a confocal image of a mouse pancreatic islet in which the δ-cells have been labelled.

Contents

1. List of Original Papers

I. Sven Göpel*, **Quan Zhang** [*], Lena Eliasson, Xiao-song Ma, Juris Galvanovskis, Takahiro Kanno, Albert Salehi and Patrik Rorsman (2004). Capacitance measurement of exocytosis in mouse pancreatic α-, β- and δ- cells within intact islets of Langerhans *Journal of Physiology* 556:711-726

II. **Quan Zhang**, Martin Bengtsson, Chris Partridge, Albert Salehi, Matthias Braun, Roger Cox, Lena Eliasson, Paul RV Johnson, Erik Renström, Toni Schneider, Per-Olof Berggren, Sven Göpel, Frances M Ashcroft & Patrik Rorsman (2007). R-type Ca^{2+}-channel-evoked CICR regulates glucose-induced somatostatin secretion *Nature Cell Biology* 9: 453-460.

III. **Quan Zhang**, Albert Salehi, Martin Bengtsson, Stephan Collins, Juris Galvanovskis, Fiona Gribble, Frank Reimann and Patrik Rorsman.(2008) Glucose regulation of somatostatin release by cAMP/PKA-dependent modulation of CICR in mouse pancreatic δ-cells. *Manuscript*

IV. **Quan Zhang**, Juris Galvanovskis, Fernando Abdulkader, Chris Patridge, Sven Göpel and Patrik Rorsman. (2008)Cell coupling in mouse pancreatic islet cells measured in intact islets of Langerhans. *Manuscript.*

They will be referred herein by their Roman numerals

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[*] Equal contribution to the study

2. Introduction

2.1 *Pancreatic islets of Langerhans and diabetes mellitus*

Pancreatic islets of Langerhans are clusters of endocrine cells embedded in the exocrine parenchyma of the pancreas. They are named after the German pathologist Paul Langerhans who made the discovery of this micro-organ and his very first description of them---'*islands of clear cells* '. Although Paul Langerhans did not define the function of these 'islets', they were later identified to bear the important function of regulating blood glucose homeostasis by secreting several hormones with immediate impact on glucose disposition and mobilization. Between 500,000 to several million oval or spheroid shaped islets are localized near the capillary system in the human pancreas. This location of the islets enables 20% of the pancreatic blood to perfuse the islets (that themselves compose only 1~2% of the entire pancreas mass with the mass of 1~3 g).

The major hormones released from pancreatic islets into the blood are insulin (the body's only glucose-lowing or *hypoglycaemic* hormone), glucagon (one of the body's most important glucose-elevating or *hyperglycaemic* hormones)

β-cell (65-80%)

α-cell (15-20%)

δ-cell (5-15%)

50 µm

Figure 1. Pancreatic islet contains α-,β- and δ-cells

Immunostaining of a human islet. α-, β- and δ-cells are labelled as red, green and blue, respectively.

Picture is a reconstruction of a series of Z-stack confocal scanning images
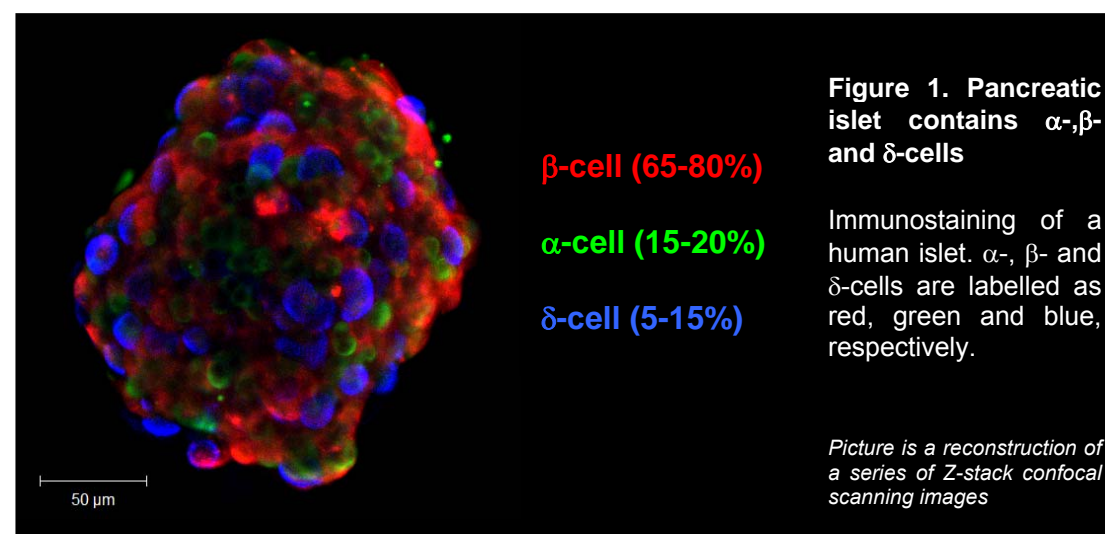
The islet cells are categorized according to the hormones they release into three major types (see a human islet in *Figure1*): insulin-secreting β-cells (which form 65-80% of the islet), glucagon-producing α-cells (comprising 15-

20% of the islet) and somatostatin-releasing δ-cells (the remaining 5-15%). The three major cell types are all electrically active and use electrical signals to couple variations of the blood glucose concentration (and other stimuli) to regulate hormone output. Insulin and glucagon together efficiently regulate plasma glucose and ensure that is maintained at concentrations around 4.5 mM. Large deviations from this range are dangerous. For example, when the blood glucose falls below 2 mM (hypoglycaemia), severe damage in the brain may result due to lack of the fuel for normal function. Chronic hyperglycaemia, which is a typical condition observed in diabetes mellitus, induces complications such as retinopathy, neuropathy, nephropathy, coronary artery disease and peripheral vascular disease (Adler, Boyko et al. 1997; Stratton, Adler et al. 2000; Stratton, Kohner et al. 2001; Caramori and Mauer 2003).

As insulin is the only glucose-lowering hormone and only secreted from the pancreatic β-cells, it is not hard to understand that the function of the islet is tightly related to the pathogeny of diabetes mellitus. It is now recognized that type-2 diabetes results from failure of the islets to release adequate amounts of insulin (O'Rahilly, Nugent et al. 1986). However, the inability of the insulin-sensitive tissues to lower the glucose level in response to insulin (insulin resistance) also contributes (Martin, Warram et al. 1992). Frequently, diabetes involves a combination of insufficient insulin secretion and insulin resistance (Lillioja, Mott et al. 1993).

Diabetes mellitus can be categorized into several types. Among them, insulin-dependent diabetes mellitus (IDDM)/type-1 diabetes (T1DM) and non-insulin-dependent diabetes mellitus (NIDDM)/type-2 diabetes (T2DM) are the most common ones. T1DM is a T-cell-mediated autoimmune disease, in which the immune system produces one or several β-cell-specific antibodies which cause(s) the destruction of the insulin-secreting cells. In type-2 diabetes, lowered capacity of the β-cell to secrete enough insulin (in relative or absolute terms) in relation to the requirements (insulin resistance) is the key feature. Furthermore, the imbalance between insulin and glucagon causes decreased glycogen synthesis, increased gluconeogenesis and impaired glucose

disposition. Collectively these defects result in the development of overt hyperglycaemia.

Although impaired insulin secretion is the most apparent cause of diabetes, the two other hormones appear to have abnormal secretion patterns in the diabetic complications. The *bihormonal concept* (Lefebvre and Luyckx 1979) postulates that elevated glucagon secretion in the diabetes in combination with insufficient insulin lead to diabetes. Indeed, diabetes may well be a *multihormonal disorder* as the release of somatostatin is perturbed secretion in spontaneously diabetic mouse models (*db/db* and *ob/ob* mice) (Patel, Cameron et al. 1977).

In conclusion, the pancreatic islets play a central role in the maintenance of the plasma glucose concentrations and abnormalities of the release of the hormones produced and secreted by them is a key element of the aetiology of clinical diabetes. Against this background, it is clear that it is important to understand the regulation of the different islet cells and the control of islet hormone secretion. Ultimately, this should be done in human islets but it may be assumed that similar processes operate in rodent islets and such studies can therefore guide subsequent work using human tissue.

2.2 *Electrical activity and exocytosis of the pancreatic islet cells*

All islet cells are electrically excitable and their electrical activities are regulated by glucose concentration/metabolism (Kanno, Gopel et al. 2002). At elevated glucose levels, the β-cells start firing action potentials (Henquin and Meissner 1984). Conversely, the α-cells generate action potentials at low glucose concentrations and electrical activity is suppressed when glucose is elevated (Rorsman and Hellman 1988). Like β-cells, δ-cells are electrically active in response to glucose but their threshold is lower than in the β-cell (3 mM vs. 7 mM glucose in mouse δ-cells vs. β-cells) (Nadal, Quesada et al. 1999; Gopel, Kanno et al. 2000).

Electrical activity is tightly linked to the exocytosis of the hormone-containing secretory granules in pancreatic islet cells. Exocytosis is the process in which

granules fuse with the plasma membrane in order to release their cargo (*Figure2*).

Figure 2. Schematic of the Ca^{2+}-dependent exocytosis

Ca^{2+}-dependent exocytosis is triggered by electrical activity and leads to fusion of the secretory granules and emptying of their cargo.
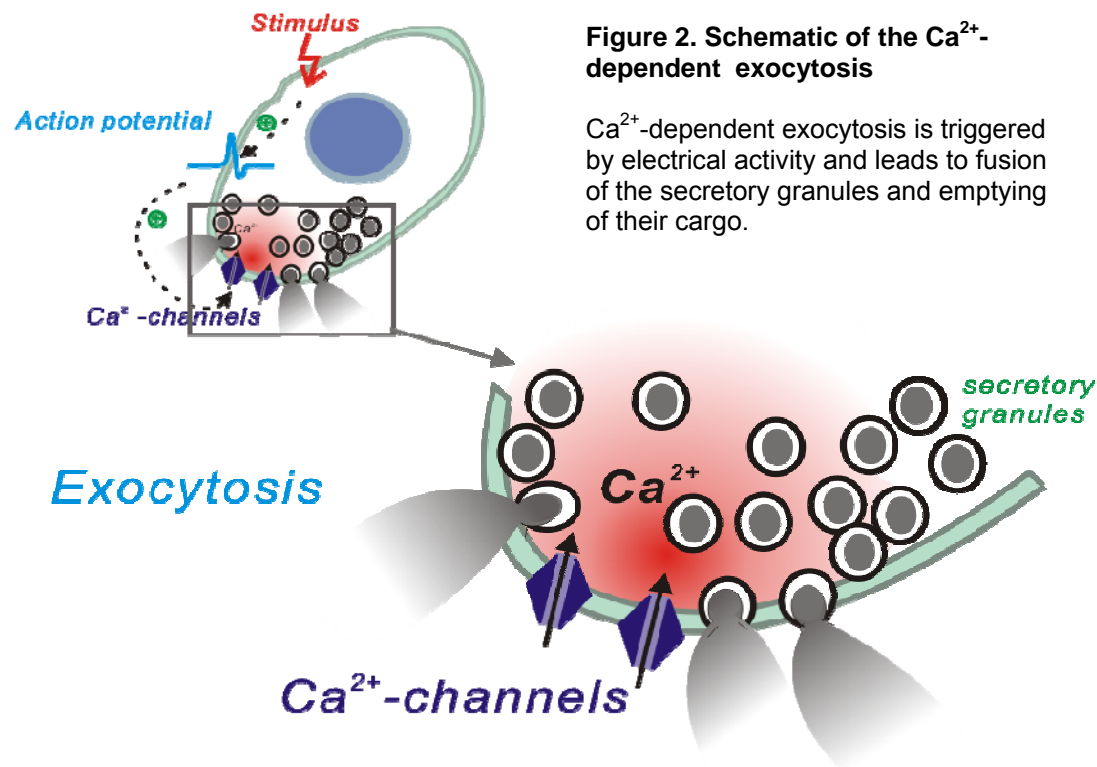
Exocytosis in pancreatic endocrine cells is Ca^{2+} dependent and responds to the increase in cytosolic Ca^{2+} that is normally secondary to opening of the voltage-gated Ca^{2+}-channels that occurs during electrical activity. Thus, the electrical excitability of islet cells enables glucose to regulate the hormone secretion (*stimulus-secretion coupling*) and fine-tune pancreatic hormone release to the functional demands during hypo- and hyperglycaemia.

At the single-cell level, exocytosis in both α- and β-cells monitored by capacitance measurements (see *Methods*, *Section 4.3*) is biphasic and exhibits a rapid/early phase and a slow/late phase (Barg, Ma et al. 2001). This phenomenon has been attributed to the release of granules belonging to different functional pools (Rorsman and Renstrom 2003). The rapid phase represents the release of the granules from a readily releasable pool (RRP) of granules that are believed to be docked at the cell membrane. Measurements in our own (Olofsson, Gopel et al. 2002) and other (Wan, Dong et al. 2004) laboratories indicate that RRP contains 1-5 % of all the granules in the insulin

secreting β-cells. The remaining >95% of granules form a reserve pool which must undergo a number of priming reactions to attain release competence. It is possible that the latter reactions are initiated with a slight delay with respect to 1st phase secretion and have a limited capacity and that these features explain the biphasic nature of secretion. *Figure 3* summarizes a current view on the relationship between granule pools and the different phases of insulin secretion (for review: (Rorsman and Renstrom 2003)). Importantly, type-2 diabetes is associated with a complete loss of 1st phase insulin secretion and a dramatic reduction of 2nd phase release (Rorsman, Eliasson et al. 2000). By analogy, it is likely that δ- and α-cells also contain distinct granule pools and that secretion from these cells exhibits discrete components. However, this is much less studied. Indeed, very little is known about the exocytotic properties of these cells.
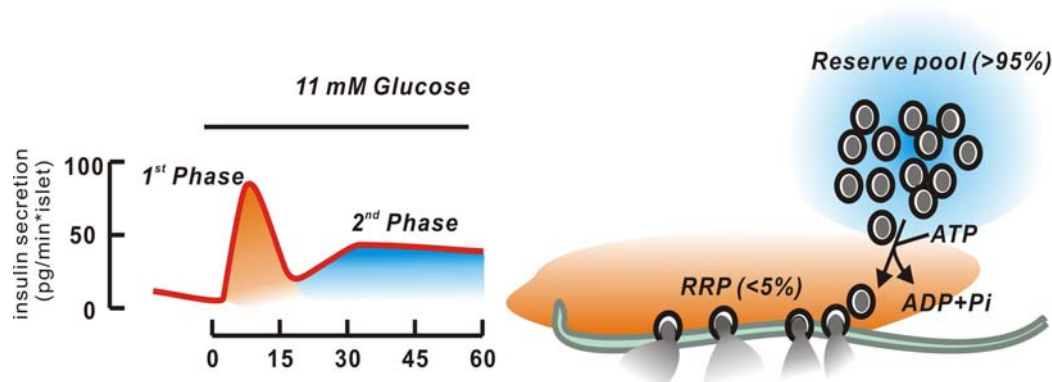
Figure 3. **Schematic of biphasic glucose-induced insulin secretion (left) and the functional pools of granules in a β-cell (right).**
Brown indicates 1st phase of secretion and the RRP; blue marks 2nd phase of secretion and the reserve pool. The arrows indicate the process of ATP-dependent refilling of RRP by the mobilization of granules from reserve pool.

2.2.1 *β-cell stimulus-secretion coupling*

The β-cells are equipped with ATP-sensitive K^+-channels (K_{ATP} channels). During hyperglycaemia, glucose is transported into the cell via glucose transporter (Glut-2, in mouse (Schuit, Huypens et al. 2001)and Glut-1 in man (De Vos, Heimberg et al. 1995)) and metabolized to produce ATP. The enzyme glucokinase plays a central role and in many ways can be regarded as the "*glucosensor*" (Matschinsky 2002). Thus, inhibition of glucokinase with

mannoheptulose leads to inhibition of insulin secretion(Johnson, Shepherd et al. 2007). The increase in the intracellular ATP/ADP ratio resulted from the acceleration of glucose metabolism leads to inhibition of the K_{ATP} channels. The reduced K^+-conductance causes membrane depolarization which, when the threshold potential is exceeded, leads to opening of L-type Ca^{2+} channels. This produces further depolarization and accounts for the upstroke of the β-cell action potential. Typically, electrical activity in mouse β-cells consists of bursts of action potentials superimposed onto depolarized plateaus (lasting 5-10 s) that are separated by repolarized intervals (Kanno, Rorsman et al. 2002). The rhythmic firing of action potentials explains the pulsatile nature of insulin secretion (Rosario, Atwater et al. 1986). In addition to the high-frequency oscillations (1-4 oscillations per min) observed in electrophysiological measurements and $[Ca^{2+}]_i$-recordings from intact islets, there is also evidence for a much slower rhythm (frequency: 1 oscillations every 10 min) in isolated cells (Dryselius, Grapengiesser et al. 1999). The relationship between these two types of oscillations seen in vitro and the oscillations in plasma insulin seen systemically in man (Hellman, Gylfe et al. 1994)remains unclear.

When the extracellular glucose is restored to the normal range (following the uptake of glucose into insulin-sensitive tissues), glucose uptake into the β-cell is reduced, and the ATP/ADP-ratio is thus lowered. Consequently, K_{ATP}-channels re-open to lead to membrane repolarization and the cessation of action potential firing and inhibition of insulin secretion. Thus, a mechanism for feedback control of plasma glucose via insulin secretion from the β-cells is established.

Interestingly, β-cells in the mouse islets β-cells are electrically coupled to each other allowing the 1000 β-cells within in an islet to synchronize the electrical and secretory activities (i.e. the islet cells function as an electrical and secretory syncytium). Exactly how this synchronization is achieved is not fully established but it seems likely that both electrical (via gap junctions) (MacDonald and Rorsman 2006)and paracrine (exerted by hormones secreted by neighbouring cells) are involved (Zarkovic and Henquin 2004).

2.2.2 Stimulus-secretion coupling in the α-cell

Glucagon is secreted in response to hypoglycaemia and inhibited by hyperglycaemia. Thus, one would expect electrical activity in α-cells to be the opposite to that of the β-cell. Indeed, mouse and rat α-cells fire action potentials during hypoglycaemic conditions. These originate from a negative voltage (<-50 mV) and peak at voltages as positive as +20 mV (i.e. an amplitude of 70 mV) (Gopel, Kanno et al. 2000). A low-voltage-activated (LVA) Ca^{2+} channel (T-type Ca^{2+} channel) is believed to function as a pacemaker of α-cell electrical activity by producing the small depolarization. This in turn leads to the opening of voltage-gated Na^+-channels that underlie the upstroke of action potential in mouse α-cells. During the action potential, the membrane potential attains values positive enough to open high-voltage-activated Ca^{2+} channels (HVA Ca^{2+} channels)(Leung, Ahmed et al. 2006). Our measurements indicate that ω-conotoxin-sensitive N-type Ca^{2+}-channels are particularly important for glucagon secretion evoked by hypoglycaemia alone (MacDonald, De Marinis et al. 2007) and opening of these channels is tightly linked to exocytosis of the glucagon-containing granules (Barg, Galvanovskis et al. 2000).

Surprisingly, the α-cells are equipped with K_{ATP} channels of exactly the same type as those found in the β-cells. In the β-cells, these channels represent a key component of their glucose-sensing. How can the presence of such channels in α-cells be reconciled with the fact that secretion in these cells is inhibited by high glucose and their ability to respond with reduced action potential firing as well as lowered cytoplasmic $[Ca^{2+}]_i$? Several hypotheses have been proposed (see Gromada et al. 2007 for a review). Our group has championed the idea that glucose-induced closure of the K_{ATP}-channels, by analogy with the situation in β-cells, leads to membrane depolarization. However, because α-cells express a different complement of voltage-gated ion channels than the β-cell, membrane depolarization leads to *reduced* rather than increased electrical excitability (Gopel, Kanno et al. 2000). For example, the N-type Ca^{2+}-channels that undergo voltage-dependent inactivation (i.e.

enter a functionally inhibited state(Hille 2001)); when the membrane potential becomes more positive than -60 to -50 mV (MacDonald, De Marinis et al. 2007).

2.2.3 *Stimulus-secretion coupling in the δ-cell*

Compared to the wealth of information about the function of the β-cell and the increasing understanding of the control of the α-cells, the regulation of the δ-cells remains a largely unexplored 'territory'. The low number of δ-cells in the islets and the difficulty of isolating them (which in turn may reflect their complicated architecture; see *Cover*) have slowed progress. However, the fact that somatostatin is a potent inhibitor of both insulin and glucagon secretion (Schuit, Derde et al. 1989) suggests it plays a highly significant role within the islet. Thus, efforts to explore the regulation of δ-cell somatostatin secretion should have a high priority.

Like the β-cells, δ-cell electrical activity is triggered by elevation of glucose (Gopel, Kanno et al. 2000). This effect has been attributed to an increase in the intracellular ATP/ADP ratio which leads to closure of K_{ATP} channels. The depolarization of membrane opens voltage-dependent Na^+-channels as well as voltage-dependent Ca^{2+}-channels which form the upstroke of action potential (Gopel, Kanno et al. 2000). The resultant increase in intracellular $[Ca^{2+}]_i$ is then assumed to trigger exocytosis of somatostatin-containing vesicles. This scenario is consistent with the observation that sulphonylureas stimulate somatostatin secretion (Efendic, Grill et al. 1982). Thus, in many ways the δ-cell is similar to the β-cell but this conclusion rests on very limited data.

2.3 *Cross-talk between islet cells*

Systemic glucose homeostasis is likely to be achieved by the co-operation of the different endocrine cells in the islets. There are several means suggested for their interacting with each other. Electrical coupling has been suggested as ultrastructural studies showed that gap-junctions exist in most cells and recorded by patch clamping experiments (Eddlestone, Goncalves et al. 1984;

Perez-Armendariz, Roy et al. 1991; Speier, Gjinovci et al. 2007). With regard to electrical coupling, accurate measurement of the gap-junctional conductance and information about its magnitude relative the plasma membrane conductances in intact islets are lacking. Such information is essential to establish whether the gap-junctional coupling is sufficient to explain the synchronization of insulin secretion. As well, intracellular dye injection showed a coupling between β-cells and between β- and non-β- cells (Meda, Santos et al. 1986) but the significance of this remains unclear. Electrical coupling is believed to underlie synchronization of electrical activity across the entire islet.

In addition to electrical coupling, the close physical proximity of the different islet cells to one another provides the structural basis for a paracrine regulation via factors released into the islet interstitium. *Figure.4* schematically summarizes the paracrine cross-talk postulated to exist within a pancreatic islet. In β-cells, insulin have been reported to have both inhibitory (mediated by activation of K_{ATP} channels) (Khan, Goforth et al. 2001) and stimulatory effects (via intracellular Ca^{2+} mobilization) (Aspinwall, Lakey et al. 1999; Barker, Leibiger et al. 2002). There is also evidence that it acts on the α-cell to inhibit glucagon secretion (Gromada, Franklin et al. 2007). In addition to these effects mediated by insulin itself, other factors released from the β-cell like GABA (Braun, Wendt et al. 2004) and Zn^{2+} have also been reported to modulate glucagon release (Franklin, Gromada et al. 2005; Gromada, Franklin et al. 2007). Whereas glucagon enhances both insulin and somatostatin secretion, most likely via elevation of cAMP (Funk 2006), somatostatin inhibits the release different somatostatin receptors (SSTRs)
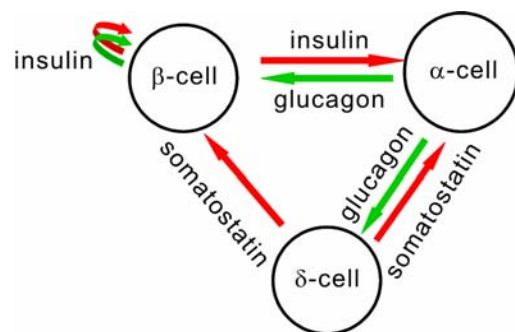
Figure 4. Schematic of paracrine/autocrine interactions among islet cells.

Inhibitory or stimulatory effects of hormones are indicated by red or green arrows, respectively.

which have different affinities to sst-14 (locally released somatostatin) and 28 (somatostatin secreted from the gastrointestinal tract) (Singh, Brendel et al. 2007).

Collectively, the interactions between the different islet cells illustrate how important it is to consider the islet cells as one organ. Whereas hormone measurements are typically carried out using intact islets, most electrophysiological experiments on islet cells have been performed on isolated cells. This is obviously far from ideal. Not only are the interactions described above disrupted. It is also possible that the mechanical/enzymatic dissociation of the islets as well as subsequent cell culture as such change the electrophysiological properties of the islet cells leading to erroneous results. Thus, the electrophysiological characterization should be carried out on a preparation as close to the intact tissue as possible and with a minimal delay after removal from the body. Methods have been developed to allow measurements in pancreatic slices (Speier, Yang et al. 2005) or isolated islets (Gopel, Kanno et al. 1999; Gopel, Kanno et al. 2000; Gopel, Kanno et al. 2000; Kanno, Rorsman et al. 2002). Experiments using these preparations have revealed important differences between the islet cell properties *in situ* and after cell isolation. For example, most studies indicate that the characteristic bursting pattern of electrical activity regarded as hallmark of the β-cell is not seen in isolated cells(Smith, Ashcroft et al. 1990); an effect that may be due to the loss of a K^+-conductance activating gradually during electrical activity (Gopel, Kanno et al. 1999)and that represents a mosaic of K_{ATP}-channels and (probably) a Ca^{2+}-activated K^+-channel (Kanno, Rorsman et al. 2002; Zhang, Houamed et al. 2005)

3. Aims of Study

The overall aim of this study is to increase our understanding of the electrophysiological and secretory properties of the intact islet micro-organ. The specific aims were to:

1. document the exocytotic properties of pancreatic β-cells (and to a lesser extent those of the α-cell) in intact islets and to correlate exocytosis detected by capacitance measurements with biochemical assays of insulin (and glucagon) release;

2. examine the glucose regulation of the somatostatin-releasing δ-cells and the involvement of processes distal to the closure of the K_{ATP}-channels;

3. characterize the control of δ-cell exocytosis by Ca^{2+}-induced Ca^{2+}-release and identify some of the signal transduction pathways involved; *and*

4. assess β-cell electrical coupling in intact islets and assess whether it is sufficient to account for the synchronization of all β-cells in the entire islet.

4. Methods

The various techniques that were employed during the course of this thesis project are briefly summarized below,

4.1 *The patch-clamp technique*

Developed by Neher and Sakmann in the late 1970s (Neher, Sakmann et al. 1978), patch-clamping offers a powerful technique to study cell electrophysiology. A key element is the establishment of a tight seal between the glass of the recording electrode and the lipid of the plasma membrane. This seal has a very high electrical resistance, typically above 1 GΩ, and is commonly referred to as the *giga seal*.

Once the giga-seal has been formed, there are two ways of establishing electrical contact with the cell interior: either the membrane enclosed within the orifice of the electrode is ruptured by a pulse of negative pressure <u>or</u> pore-forming compounds (such as amphotericin B or nystatin) are used to perforate the membrane patch. The two techniques are referred to as "standard" and "perforated patch" whole-cell techniques, respectively. In the latter technique, intracellular metabolism is maintained intact, whereas the former approach involves the replacement of the cytosol with an artificial buffer. The standard whole-cell approach thus provides an efficient means of introducing both low-

and high-molecular weight compounds into the cytosol simply by adding them to the electrode-filling solution. Thus, Ca^{2+}-indicators can be applied selectively into the cell from which the electrophysiological measurements are made whereas more conventional loading protocols (using the AM esters) will label all cells.

The electrophysiological measurements were made with a software-controlled amplifier (EPC9, HEKA electronics, Lambrecht/Pfalz, Germany) that is connected to the cell via the recording probe. Thus, the cell membrane potential and current injection can be manipulated via a computer interface. In this study, stimulation protocols and analysis were performed using the software packlages Pulse and Pulsefit (both from HEKA). In electrophysiological experiments, two types of measurements can be performed. Basically, the experiments are carried out either in the voltage- or current-clamp modes to measure membrane currents and potentials, respectively.

4.2 *Capacitance measurement and its application in intact islets*

Capacitance measurements have revolutionized the study of exocytosis. In electrical terms, the lipid bilayer of cell membrane corresponds to a capacitor. The capacitance of the membrane can be measured fairly easily at high temporal resolution. Importantly, the capacitance (*C*) of the membrane is proportionally related to the membrane area (*A*; i.e. $C \propto A$). The increase in cell surface area that results from fusion of secretory granules with the plasma membrane can therefore be monitored as an increase in cell capacitance (Neher and Marty 1982). Biological membranes have a specific capacitance of 10 fF/µm^2. Based on ultrastructural data and approximating the granules to spheres/spheroids, it can be estimated that fusion of a secretory granule will contribute approximately 1 fF, 2 fF and 3 fF in δ-, α- and β-cells, respectively. In the case of the β-cell, capacitance steps of ~3 fF have been observed experimentally (MacDonald, Obermuller et al. 2005).

All the capacitance measurements in the studies included in this thesis were carried out in intact islets (see *Figure 5* for an experimental setup) using a 1.25 kHz sine wave. The selection of this frequency, which is higher than that used for single-cell measurements, was made to minimize pick-up of capacitance from neighbouring electrically coupled cells. In most experiments, the cells were stimulated using brief (10-850 ms) voltage pulses. Because of the increase in membrane conductance that reflects the opening of voltage-gated ion channels during the stimulus, it is difficult to reliably monitor the capacitance increase during the depolarizations and the measurements were therefore interrupted during the stimuli. It is emphasized that prior to the studies reported here, capacitance measurement of islet cells had only been performed on individual cells maintained in tissue culture. Although very valuable data was obtained from single-cell studies, the high rate of exocytosis suggested by these measurements (several orders of magnitude higher than that suggested by more traditional biochemical approaches) caused some disquiet. The approach of applying capacitance measurements
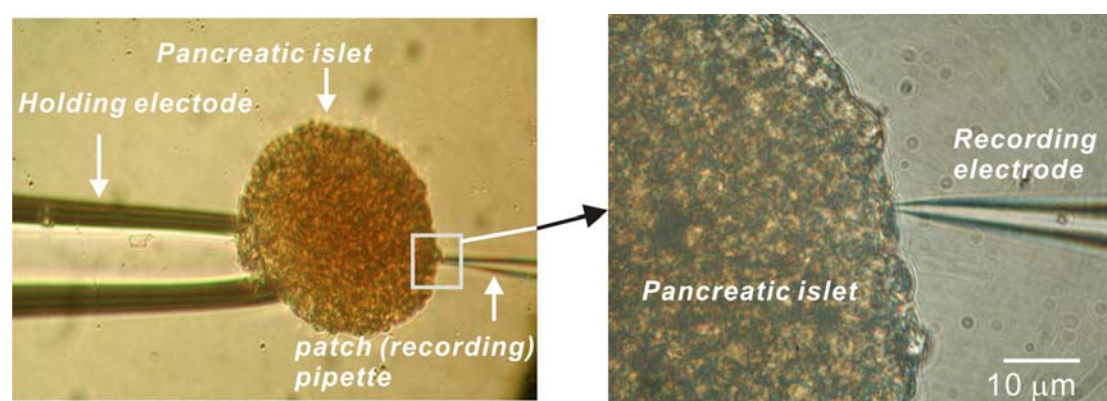
Figure 5. Whole-islet patch-clamping.

Left panel indicate the islet ommobilized with a holding electrode and a patch-electrode has been placed on the islet for recording. The right panel shows a magnification of the pipette position on the surface of a peripheral cell.

to cells within the intact islets should allow more direct comparison of biophysical (capacitance measurements) and biochemical (e.g. RIA) methods to monitor secretion.

4.3 _Calcium Measurement and Imaging_

Cytosolic calcium ($[Ca^{2+}]_i$), functions as an important intracellular 2nd messenger in the process of exocytosis. Its role in hormone secretion was recognized ~40 years ago by the work of Douglas (Douglas and Rubin 1961), which in turn was inspired by the studies of Bernard Katz on synaptic transmission (Katz and Miledi 1967). The study of Ca^{2+}-dependent processes was greatly facilitated by the introduction of fluorescent Ca^{2+}-indicators that can be loaded into intact cells (Tsien, Pozzan et al. 1982). In this study, indo-1 and fluo-4 have been used. Indo-1 is a single-excitation (350 nm) dual-emission (485 and 405 nm) dye. Upon an increase in $[Ca^{2+}]_i$, light emitted at 485 nm (F_{485}) and 405 nm (F_{405}) will increase and decrease, respectively. The increase of $[Ca^{2+}]_i$ is then reported as F_{485}/F_{405} ratio. The advantage of a ratiometric dye is that it eliminates artefacts caused by bleaching of the fluorescent indicator, changes in autofluorescence etc. A further advantage is that the signal will be independent of the thickness of the cell. Fluo-4 is a single-excitation single-emission wavelength indicator. The excitation wavelength is 488 nm and the reporting emission is about 500 nm. Importantly, its fluorescence increases dramatically (>100-fold) upon Ca^{2+}-bidning. Although being a single-excitation and -emission dye, fluo-4 has several advantages that render it attractive for cell physiological studies. For example, in not requiring UV light for its excitation, fluo-4 can be used in experiments involving photorelease of caged compounds (Rapp 1998), the liberation of which is effected by UV-light.

The combination of Ca^{2+}-imaging with standard whole-cell patch-clamp recordings is a valuable addition to the experimental battery of techniques. In this type of measurements, the indicators are infused into the cytosol via the patch electrode. Thus the precise $[Ca^{2+}]_i$ change from a _single_ cell within the intact islet can be obtained (_Figure 6_), whereas conventional loading protocols will label all cells within the tissue. The combination of whole-cell patch-clamping and $[Ca^{2+}]_i$-imaging therefore allows different processes to be correlated at the single-cell level.

For the experiments reported in papers II-III, a Photon Technology International (PTI, Birmingham, NJ) RM-2/2005 fluorescence microscopy system or a BioRad Radiance 2100 scanning laser confocal system mounted on Nikon Eclipse E600FN upright microscope were used. The whole-islet $[Ca^{2+}]_i$ imaging described in *Paper IV* were performed using a Zeiss 510META system.

For off-line signal processing, the Felix software (PTI) was used for the indo-1 experiments, whereas the confocal images were analyzed using Zeiss Laser Scanning Microscope LSM 510 software.
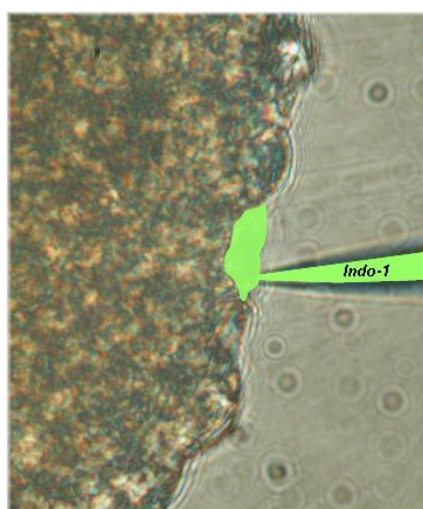
Figure 6. Single-cell labelling of a cell with indo-1 via a patch- clamp electrode.

The indicator (green for display purposes) is added to the pipette-filling solution and diffuses into the patch-clamped cell following the establishment of the standard whole-cell configuration.

4.4 *Photoliberation of Caged InsP$_3$*

In standard whole-cell recordings, the electrode solutions are dialysed into the cytosol. The exchange of the cytosol with the electrode solution is fairly slow and may require several minutes. This does not represent a major problem when the cell is stimulated with voltage pulses in which the stimulus can be applied once exchange has been complete. However, when testing the effects of second messengers that may trigger exocytosis by themselves, the mixing of the electrode solution and cytosol represents a real problem and (at least for potent compounds) the effect may be over even before the electrophysiological measurements have commenced! It is against this background that the usefulness of caged compounds should be considered. Using these compounds, the compound of interest is loaded into the cell as a biologically inert caged precursor (a "cage" has been added around the

molecule). Once complete exchange of the cytosol with the electrode solution has been achieved, the precursor can be converted into its active form by a brief (ms) flash of UV-light producing instant and uniform liberation of the substance throughout the cell. In this thesis, a caged form of $InsP_3$ (50 μM) was infused into the cell together with Ca^{2+} indicator fluo-4 (25 μM) via the recording electrode. The liberation of $InsP_3$ was effected by a UV pulse (JML-C2, Rapp Optoelectronic, Hamburg, Germany).

4.5 *Hormone release measurements*

Insulin, glucagon and somatostatin secretion from intact islets was determined by radioimmunoassay. Briefly, groups of 10 islets were preincubated for 30 minutes at 37°C in a Krebs-Ringer bicarbonate buffer (pH 7.4) consisting of 120 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1 mM glucose, and 10 mM HEPES (pH 7.4) and then incubated for another 60 min in the same medium supplemented with different combinations of test substances. The medium was gassed with 95% O_2 and 5% CO_2 to obtain constant pH and oxygenation. Immediately after incubation, an aliquot of the medium was removed for determination of hormone content as reported before (Salehi, Panagiotidis et al. 1995).

4.6 *Immunocytochemistry and cell type confirmation.*

As mentioned above, patch clamping and capacitance measurement was applied to intact islets in this study. *Figure 5* shows the experimental set-up. The islet is held in place within the experimental chamber by a wide-bore holding electrode. Patch-clamp electrodes are then positioned on the opposite pole of the islet. Methods for electrophysiological identification of the different islet cells have been proposed (Gopel, Kanno et al. 1999; Gopel, Kanno et al. 2000). We have tested the validity of the electrophysiological identification by establishing a biocytin-streptavidin tracing protocol. The polar tracer biocytin is included in the electrode solution dialyzing the cell interior following establishment of the whole-cell configuration at a concentration of 1 mg/ml. Following the electrophysiological measurements (during which biocytin labels the cell), the islet is fixed in phosphate buffer solution (PBS) containing 4%

paraformaldehyde. Islets are then permeabilized with 0.1% Triton X-100 in PBS containing 5% goat serum for 1 h to block non-specific binding. This was followed by incubation in a mixture of primary antibodies (guinea pig anti-insulin, mouse anti-glucagon and rabbit anti-somatostatin) for 2 h at room temperature. After washing in PBS, the islets were incubated in goat serum containing a mixture of secondary antibodies (goat anti-guinea pig Alexa-633, goat anti-mouse Alexa-405 and goat anti-rabbit Alexa-488) for 1 h at room temperature. After another rinse in PBS, the islet was incubated with PBS containing rhodamine-conjugated streptavidin for 30 min to visualize biocytin. The identity of the cell was then confirmed by confocal microscopy. *Figure. 7* shows membrane currents evoked by voltage-clamp pulses from -70 mV to zero mV recorded from a β-cell (A) and a δ-cell (B) identified as outlined above. As can be seen, the β-cell only exhibits a sustained inward current (reflecting opening of voltage-gated Ca^{2+}-channels), whereas the response in the δ-cell is biphasic; reflecting the presence of Na^+ (spiky part) and Ca^{2+} currents (sustained part).
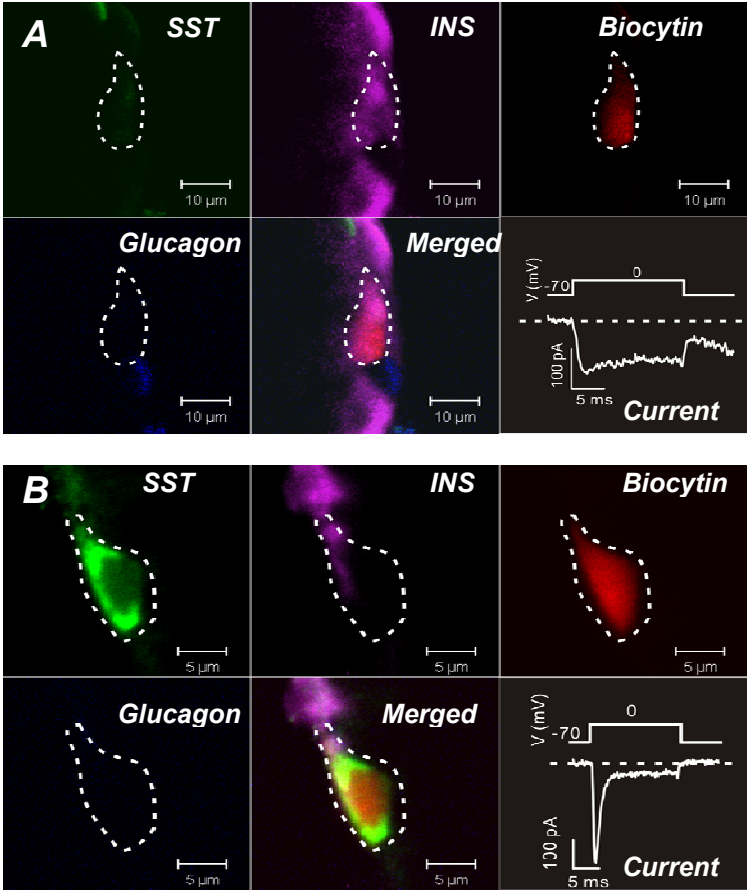
Figure 7. Cell type confirmation using biocytin tracing

A. Biocytin (red) is marked in an insulin positive (*INS*, purple) cell in an intact islet. The transmembrane current[*] triggered by a depolarization from -70 mV to 0 mV recorded from the same cell is shown in lower right.

B. As in *A*, but biocytin labelled a somatostatin positive (*SST*, green) cell. Transmembrane current recorded from the same cell is displayed in the lower right panel.

*: The failure of the current to return to the zero level is due to an action potential being fired in a neighbouring cell (see section 5.4).

5. Results

5.1 _Capacitance measurements of islet cells in the intact islets (Paper I)_

As discussed above, islet cells are electrically excitable and utilize electrical signals to couple changes in the blood glucose concentration to stimulation or inhibition of islet hormone release (Ashcroft & Rorsman, 1995). Hormone release has traditionally been studied by biochemical methods such as radioimmunoassay (RIA). With the latter technique, insulin can be detected at the level of ~100 amol (500 fg) of insulin (Westerlund and Bergsten 2001). For comparison, an insulin granule contains 8 fg of insulin (Huang, Shen et al. 1995; Rorsman and Renstrom 2003).

Understanding of the fundamental processes involved in hormone secretion requires means to study exocytosis in single cells with high temporal resolution. The technique of capacitance measurements provides a powerful means to study secretion down to the level of the single granule(Neher 1998) and it has been successfully applied to pancreatic islet cells (Ammala, Eliasson et al. 1993). However, except for a single study (Rose, Efendic et al. 2007), all capacitance measurements in islet cells to date have been carried out on individual cells (Gillis and Misler 1992; Ammala, Eliasson et al. 1993; Gromada, Bokvist et al. 1997; Mariot, Gilon et al. 1998) maintained in tissue culture, whereas hormone release measurements are typically performed on intact islets. Ideally, capacitance measurements should be applied to islet cells in the intact tissue to allow direct comparison of the secretion data obtained with the two techniques. In _Paper I_, we have characterized the exocytotic properties of three different types of islet cells in intact islets. Here I will concentrate on the data dealing with the β-cells and only in passing consider the α-cells. The properties of the δ-cells are described extensively in sections 6.2 and 6.3 and will therefore not be discussed here.
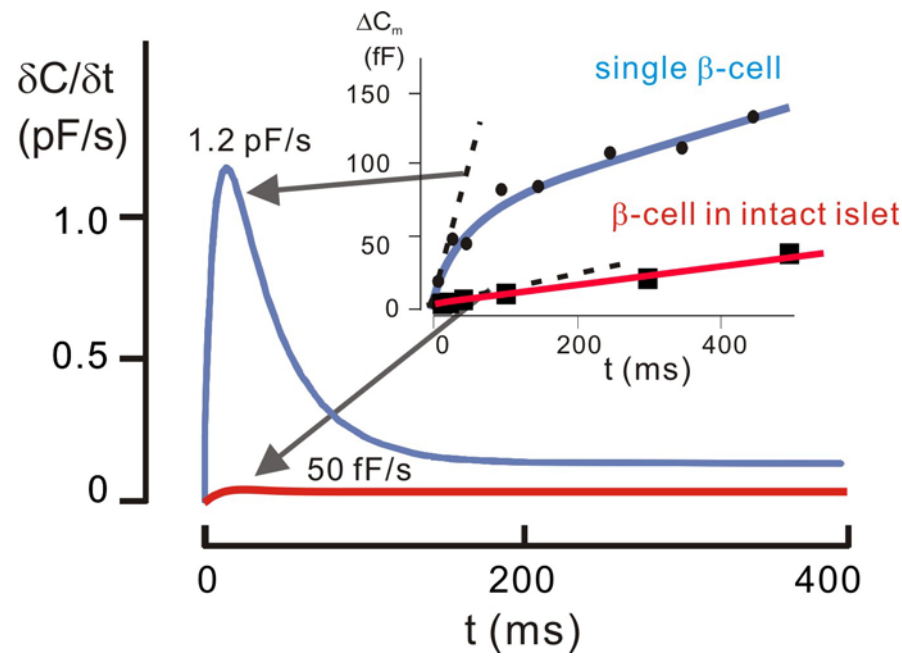
Figure 8. Exocytotic rate of β-cells within intact islets (red) and dispersed single cells (blue) derived from the reconstruction of capacitance increase triggered by progressively longer depolarizations from -70 mV to 0 mV (inset). Blue traces illustrate the data represented in a previous single-cell study(Barg, Galvanovskis et al. 2000) . Red traces are derived from *Paper I.* Arrows indicate the peak exocytotic rate (dashed lines in *Inset*)

Different from the single-cell data, exocytosis in intact isles evoked by progressively longer (from 10-850 ms) depolarizations exhibits monophasic kinetics. As shown in *Figure 8*, exocytosis proceeds at a steady rate of ~50 fF/s with little sign of the rapid initial phase observed in single β-cells. It seems likely that the exocytotic properties change as a consequence of the mechanical dissociation of the islet and/or loss of paracrine signalling. A further difference between isolated β-cells and β-cells in intact islets was observed during repetitive stimulation (ten 500-ms depolarization from -70 mV to 0 mV). In isolated cells, the first pulse typically evoked the largest response with subsequent pulses producing progressively smaller increases. This behaviour (similar to depression in neurones (Katz 1966)) was interpreted in

terms of the β-cell having a limited pool of readily releasable granules. By contrast, depression was much less evident in intact islets. However, when exocytosis was elicited by an action potential-like train (100 50-ms depolarization at a frequency of 4 Hz), exocytosis exhibited two distinct phases with a peak rate of ~40 fF/s obtained at 7 s after onset of stimulation. The total capacitance increase recorded in this type of experiments amounted to 1100 fF. Interestingly enough, the latter value is considerably higher than that observed from the low-frequency repetitive stimulation experiments although the duration of the onset of stimuli is the same (5 s). Thus, although β-cells *in situ* clearly exhibit phase exocytosis, it is different from that seen in isolated cells and the correlation between 1st phase insulin secretion and RRP granules suggested by single-cell measurements is perhaps not as strong as the single-cell data led us to believe.

5. 1.2 *Voltage-dependent exocytosis in β-cells*

As mentioned earlier (see *section 2.2*), β-cell exocytosis is a Ca^{2+}-dependent process. The entry of extracellular Ca^{2+} into the cytosol is mediated by voltage-dependent Ca^{2+} channels (VDCC). Understanding the correlation between β-cell action potential firing and insulin secretion requires detailed information about the voltage dependence of exocytosis. We studied this by applying 500-ms depolarization from -70 mV to membrane potentials between -40 mV and +40 mV. The exocytotic responses elicited by these depolarizations exhibit a bell-shaped voltage dependence which superimposes that of the Ca^{2+} current measured simultaneously. The peak response was observed during depolarizations to +20 mV and averaged 35 fF. Of note, a depolarization to -20 mV (which corresponds to the peak of β-cell action potential) evoked a capacitance increases of <5 fF. However, it should be noted that the action potential is as short as 50 ms whereas the pulse length was 500 ms. The capacitance increases can be converted into granules released using a conversion factor of ~3 fF/granules. Taken together, these data suggest that every action potential may lead to the exocytosis of 0.1-0.2 granules (i.e. 5 fF/[3 fF/granule * 500 ms/50 ms]).

5.1.3 *β-cell exocytosis was blocked by nifedipine and increased by pertussis toxin.*

Ca^{2+} entry via VDCCs provides the Ca^{2+} triggering exocytosis. β-cells express at least three different types of voltage-gated Ca^{2+}-channel: L-, R- and P/Q-type (Schulla, Renstrom et al. 2003). Exocytosis in isolated β-cells is strongly dependent on opening of L-type Ca^{2+} channels. We tested whether these channels are also important for exocytosis in intact islets using the L-type-selective antagonist nifedipine (25 μM). It blocked the Ca^{2+}-current by ~50%, exocytosis by 60% and glucose-induced insulin secretion by 80%. The fact that the effect on glucose-induced insulin secretion is stronger than the inhibition of insulin secretion we attribute to the fact that nifedipine not only affects exocytosis but also electrical activity (Rosario, Barbosa et al. 1993). Collectively these results confirm that the L-type Ca^{2+} channel is essential for the insulin secretory process.

We hypothesized that the fact that the peak rate of β-cell exocytosis *in situ* is only <5% of that in isolated cells reflects paracrine inhibition via factors released by neighbouring cells. Many of these effects (like the effects of somatostatin (Renstrom, Ding et al. 1996) and autocrine inhibition by GABA$_B$ receptors (Braun, Wendt et al. 2004) are mediated by activation of inhibitory G-proteins. We tested this by pretreating the islets with pertussis toxin (100 ng/ml for >4h). However, this increased exocytosis by only 40%. We therefore conclude that the low rate of exocytosis in intact islets is unlikely to result from paracrine suppression and that it instead reflects a more fundamental functional change.

In addition, we obtained some data from cells taken to be α-cells (expressing Na^+-currents as well as a transient outward K^+-current; see (Gopel, Kanno et al. 2000). We were thereby able to confirm that that exocytosis and glucagon secretion depend on Ca^{2+}-influx via N-type Ca^{2+}-channels. Moreover, α-cells exhibited marked depression during repetitive stimulation, suggesting they are specialized for brief bouts of secretion rather than sustained activity.

5.2 R-type Ca^{2+}-channel-evoked Ca^{2+}-induced Ca^{2+} release regulates glucose-induced somatostatin secretion (Paper II)

Earlier experiments have identified an islet cell type that is electrically silent in the absence of glucose and starts generating action potentials in the presence of 10 mM glucose (Gopel, Kanno et al. 2000). Unlike what was observed in β-cells, these action potentials were not grouped into bursts. Whole-cell recording on such cells revealed that they differed from β-cells in displaying a prominent Na^{+}-current that could be activated from physiological membrane potentials (i.e. -70 mV and more positive). Taken together this indicates that they may represent δ-cells which secrete somatostatin in response to glucose. Biocytin tracing in combination with patch-clamp recordings and subsequent immunocytochemistry have confirmed this conclusion (see Methods; Section 4.6).
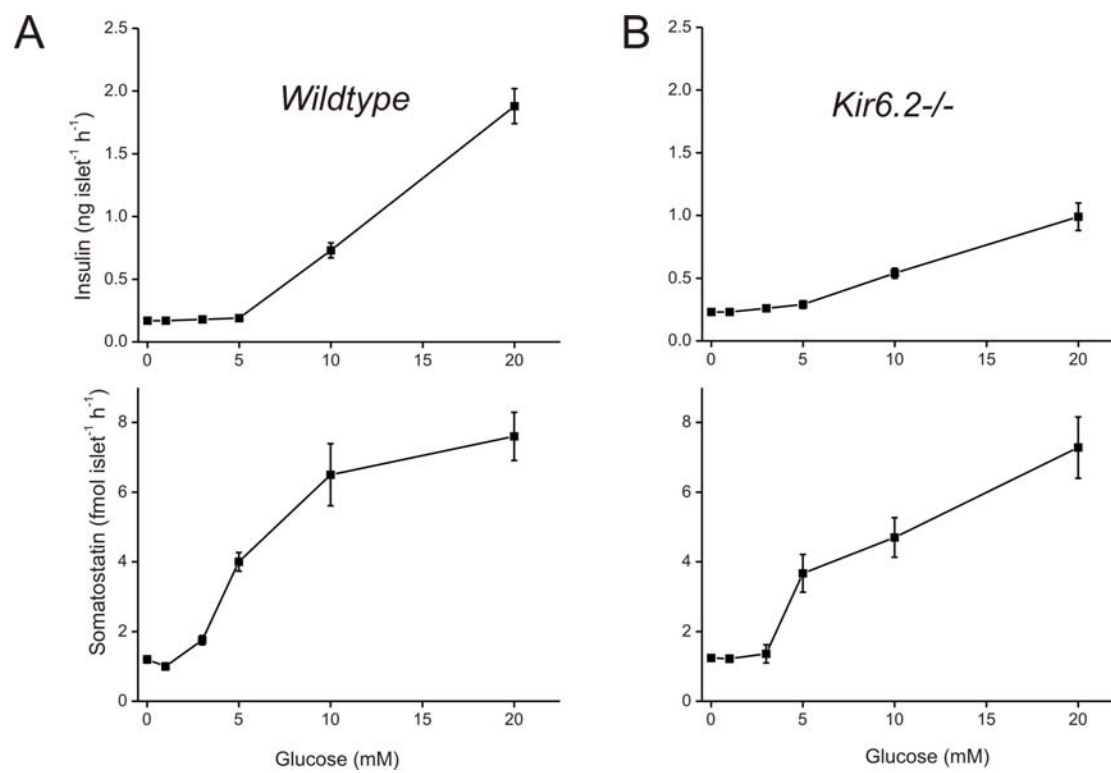
Figure 9. Glucose-induced somatostatin and insulin secretion in wildtype and Kir6.2-deficient islets. Hormone secretion was measured in static incubations in the presence of increasing glucose concentrations. Note stimulation of somatostatin secretion at 3 mM glucose whereas an elevation beyond 5 mM is required to evoke insulin secretion.

5.2.1 *Stimulus-secretion coupling of the δ-cell: involvement of K_{ATP}-channels*

Previous work had revealed that the δ-cells are equipped with K_{ATP}-channels and that these channels are active in the absence of glucose. Inhibition of the K_{ATP}-channels resulted in membrane depolarization and the appearance of action potentials. The magnitude of the tolbutamide-sensitive K^+-conductance was only 2.7 nS (30% of that in β-cells (Gopel, Kanno et al. 2000)) , a finding that might explain why the somatostatin is stimulated by glucose concentrations as low as 3 mM whereas 6-7 mM is required for insulin secretion (*Figure 9*). It was proposed that the stimulus-secretion coupling in the δ-cell is similar to that of the β-cell and that they respond to an increase in plasma glucose via acceleration of glucose metabolism, elevation of the intracellular ATP/ADP ratio, membrane depolarisation, action potential firing, Ca^{2+}-influx and Ca^{2+}-dependent exocytosis of somatostatin-containing secretory granules.

Figure 10. Effects of CICR inhibitors on glucose-regulated insulin (A), glucagon (B) and somatostatin secretion (C).

Data from *Paper II*

* p<0.001 vs. 10 mM glucose alone

It was therefore surprising that tolbutamide is a weak stimulus of somatostatin release and that glucose-induced somatostatin secretion was unaffected in islets isolated from SUR1- or Kir6.2-deficient mice whereas the release of insulin more strongly perturbed (*Figure. 9*).

5.2.2 Regulation of somatostatin release beyond electrical activity

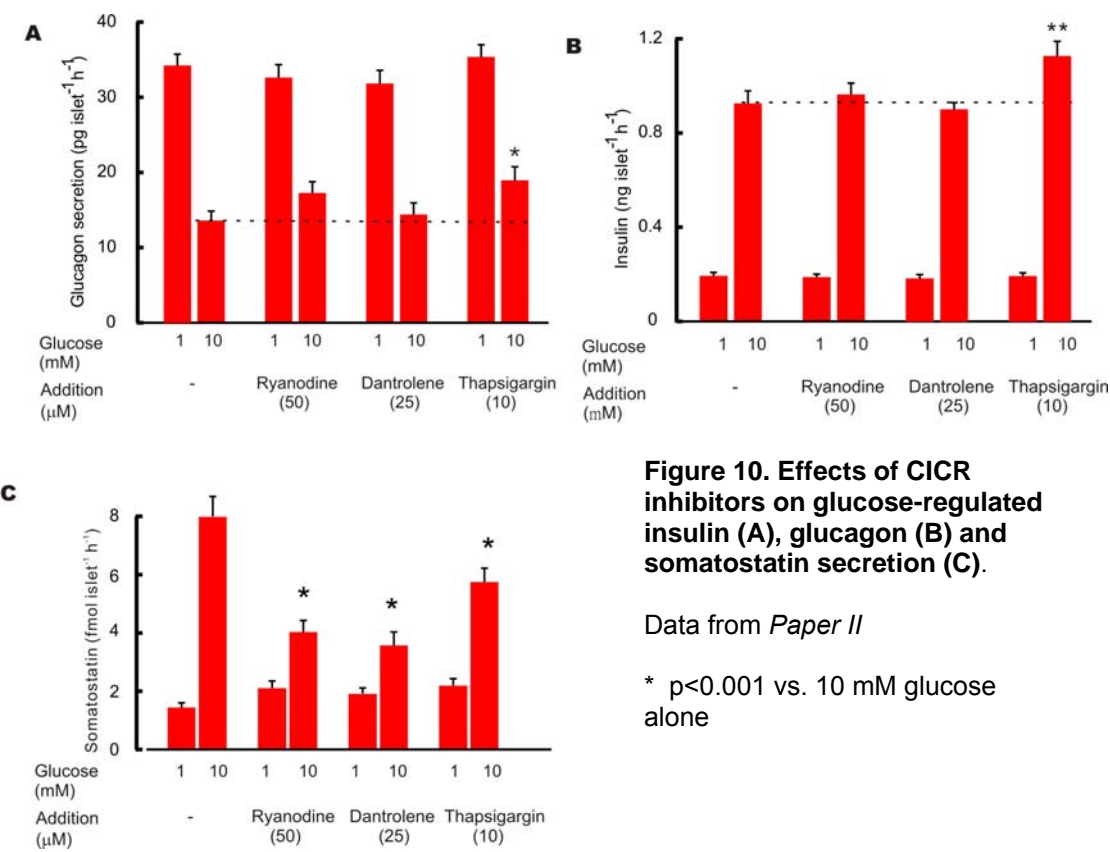In *Paper I* it was noted that in some δ-cells a large part of the exocytotic response occurred after the depolarization. By contrast, exocytosis in β-cells was typically restricted to the stimulation period. It was proposed that "*exocytosis in δ-cells depends on processes other than just Ca^{2+} influx through plasmalemmal Ca^{2+} channels*". In *Paper II* this aspect was investigated in detail. This work has led to the proposal that glucose regulates somatostatin secretion by triggering Ca^{2+}-induced Ca^{2+} release (CICR). It was found that in mouse islets, somatostatin secretion is uniquely sensitive to antagonists of CICR such as ryanodine and dantrolene (*Figure 10*). Parallel recordings of cell capacitance and cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in δ-cells in intact islets[†] reveal that CICR gives rises to a long-lasting elevation of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$)

Figure11. Depolarization-triggered capacitance increase (middle trace) and $[Ca^{2+}]_i$ increase (lower trace) in voltage-clamped δ-cell in intact islet.

Recording was obtained from a δ-cell at 20 mM glucose. $[Ca^{2+}]_i$ was measured using indo-1. Shadowed area highlights ongoing exocytosis during $[Ca^{2+}]_i$ –oscillations. Dashed lines indicate prestimulatory levels.
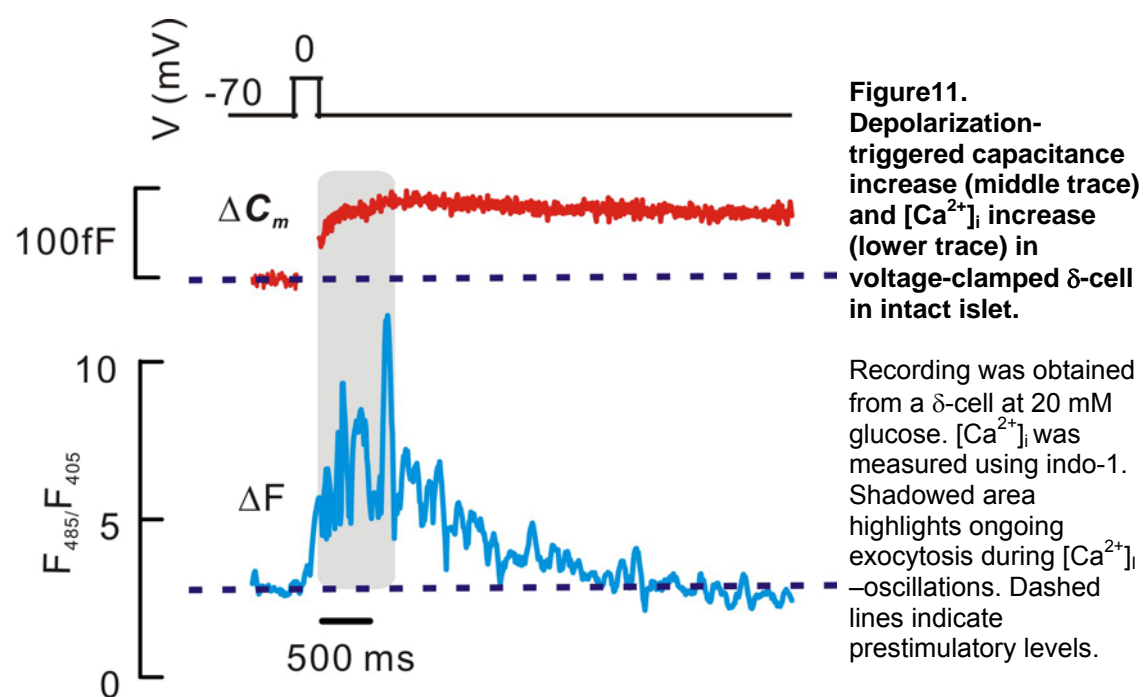
[†] Note that when combining whole-cell patch-clamp measurements with $[Ca^{2+}]_i$ imaging, a single cell within the islet is loaded with the Ca^{2+} dye via the recording electrode, thus ensuring that the Ca^{2+} signal is derived exclusively from the cell connected to the recording electrode and the one used for the capacitance measurements (see Methods)

that is associated with a slow component of capacitance increase (*Figure 11*); both of which were blocked by ryanodine. Indeed, in the presence of ryanodine, the $[Ca^{2+}]_i$ transients in δ-cells became similar to those observed in β-cells. Exocytosis in δ-cell was also reduced by dantrolene and following depletion of the intracellular Ca^{2+} stores by the SERCA (sarco-endoplasmic reticulum Ca^{2+}-ATPase) inhibitor thapsigargin. Overall, there was an excellent agreement between capacitance measurements and biochemical hormone secretion measurements. These data argue that intracellular Ca^{2+} stores play an important role in somatostatin secretion. It should be noted, however, that membrane depolarization, electrical activity Ca^{2+}-influx through the voltage-gated Ca^{2+}-channels is still required to initiate secretion but that the exact magnitude of the secretory response is chiefly determined by CICR. As will be shown below, 80% of the secretory response results from events distal to membrane depolarization.

5.2.3 *Somatostatin secretion depends on R-type Ca^{2+}-channels*

The initial electrophysiological characterization of the δ-cells revealed that they were equipped with high voltage-activated (HVA) Ca^{2+}-channels but their molecular identity was not established (Berts, Ball et al. 1996; Liu, Hellman et al. 1999; Nadal, Quesada et al. 1999). Pharmacological evidence presented in Paper II suggests that in mouse δ-cells, the Ca^{2+}-current flows through R- (blocked by SNX482) and L-type (blocked by isradipine) Ca^{2+}-channels. It is of interest, however, that although the latter type of Ca^{2+}-channels account for 60% of the whole-cell current, isradipine is almost of no effect on glucose-induced somatostatin secretion. By contrast, SNX482 exerted a very strong inhibitory effect. Moreover, in experiments on islet isolated from R-type Ca^{2+}-channel knockout mice ($Ca_v2.3^{-/-}$), glucose-induced somatostatin secretion was also strongly inhibited and reduced to the level observed in wild type islets exposed to SNX482.

Confocal $[Ca^{2+}]_i$ imaging under control conditions in the absence and presence of R- and L-type Ca^{2+}-channel blockers revealed that although the two types of Ca^{2+}-channels may be situated within 1-2 μm of each other, it is only the Ca^{2+}-influx through R-type Ca^{2+}-channels that triggers CICR. Thus,

the evidence suggests that R-type Ca^{2+}-channels must be tightly associated with the ryanodine receptors (which mediate CICR). This conclusion is also underpinned by the finding that the short latency between membrane depolarization and CICR which is <10 ms (*Figure. 12B*). This is almost as rapid as observed in skeletal muscle (Escobar, Monck et al. 1994). However, unlike what is seen in skeletal muscle, depolarization alone is not sufficient to trigger CICR and at voltages at or beyond the reversal potential for Ca^{2+}-entry, CICR in the δ-cell is negligible (*Figure. 12C*). This leads us to conclude that although the association between the R-type Ca^{2+}-channels and the RyRs is tight, they do not actually assemble into a complex (*Figure. 12A*).
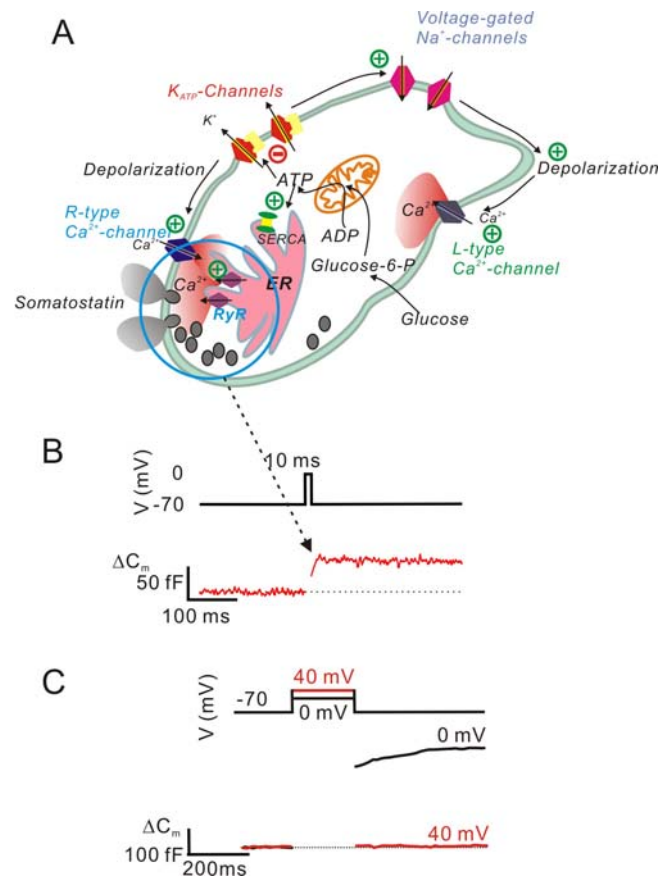
Figure 12. RyR3-mediated CICR is in tight association with R-type Ca^{2+}-channel and depends on Ca^{2+}-influx.

A. Schematic of δ-cell stimulus-secretion coupling. Solid arrows indicate ion/molecule movements and depolarization. The symbols ⊕/⊖ indicate stimulatory and inhibitory effect, respectively. **B.** 10 ms-Depolarization-evoked capacitance increase in a δ-cell. Slow component of exocytosis (arrow) reflects the tight coupling between R-type Ca^{2+} channel and RyR (highlight the tight coupling in **A** by the blue circle) . **C.** δ-cells exocytosis triggered by depolarizations to 0 mV (black trace) and 40 mV (red trace).

5.2.4 *Studies of somatostatin secretion in depolarized islets*

We also studied somatostatin secretion from islets depolarized with 70 mM K^+. This bypasses effects of glucose and other modulators of secretion on membrane potential and will allow us to study the late effects in isolation. Depolarization alone stimulated somatostatin secretion ~4-fold over that seen under standard control conditions (normal extracellular K^+). Increasing

glucose concentrations applied in the presence of high-K$^+$ resulted in a concentration-dependent stimulation of somatostatin secretion that was half-maximal at 10 mM and plateaued at concentrations 20 mM where release was enhanced >4-fold over that seen with high-K$^+$ alone.

Secretion evoked by high-K$^+$ in combination with high glucose was strongly inhibited by mannoheptulose, ryanodine and SNX482 but largely unaffected by isradipine and xestospongin C. All these compounds had little effect on secretion evoked by high-K$^+$ alone. Taken together, these data confirm the involvement of CICR, ryanodine receptors (RyRs) and R-type Ca^{2+}-channels in somatostatin secretion, whereas any major role of L-type Ca^{2+}-channels and InsP$_3$-signalling can be excluded. They also show that glucose stimulates regulates CICR and somatostatin secretion via its metabolism although the identity of the second messenger mediating the effect remained to be established.

However, it seems likely that glucose-induced sequestration of Ca^{2+} into the sER (sarco-endoplasmic reticulum) is involved. There is a precedence for this mechanism in the β-cell where glucose promotes the uptake of Ca^{2+} into intracellular stores (Tengholm, Hellman et al. 2001).

5.3 Glucose triggers CICR in δ-cells by a PKA-dependent mechanism? (Paper III)

There remained a number of important questions after the initial studies reported in *Paper II*. A particularly important question to resolve was the identity of the second messenger mediating the effect of glucose on CICR also remained obscure.

Membrane potential recordings revealed that δ-cell generate electrical activity already at 6 mM glucose, a concentration that was not sufficient to evoke action potential firing in the β-cell. It was also found that the action potentials in δ-cells, unlike what is the case in the β-cell, are dependent on activation of TTX-sensitive voltage-gated Na$^+$-channels. In accordance with this observation, TTX reduced glucose-induced somatostatin secretion by ~30%.

To address the mechanisms by which glucose evokes CICR and somatostatin secretion, we applied perforated patch whole-cell recordings in conjunction with capacitance measurements to δ-cells in intact pancreatic islets. Exocytosis was recorded at low and high glucose concentrations. In the initial experiments, 2 µM forskolin was included in the extracellular medium to enhance exocytosis. Contrary to our expectation, the exocytotic responses recorded in δ-cells under these experimental conditions were fairly large already in the absence of glucose and were not affected by glucose.

We reasoned that exocytosis being already enhanced by forskolin may deplete the readily releasable pool of granules, thus obscuring any additional effect exerted by glucose. Indeed, when tested in the absence of forskolin, glucose stimulated exocytosis ~3.5-fold, an effect that was counteracted by mannoheptulose.

A low concentration of forskolin (2 µM) was likewise found to stimulate exocytosis to the same extent as produced by glucose. However, when tested in the presence of glucose, this concentration of forskolin was ineffective and the adenylate cyclase activator was only able to stimulate exocytosis in the presence of glucose when tested at a 5-fold higher concentration (10 µM). These results are suggestive of an interesting cross-talk between cAMP and glucose in the control of somatostatin secretion.

We tested this aspect in hormone release measurements. If glucose acts by increasing intracellular cAMP, then the effects on secretion might be mediated by activation of protein kinase A. If this is the case, then glucose-stimulated somatostatin secretion should be sensitive to inhibitors of PKA. Indeed, glucose-induced somatostatin secretion was reduced by ~50% by the PKA-inhibitor 8-Br-Rp-cAMPS.

We tested the effects of elevating intracellular cAMP using the high-K^+ protocol described in *Section 5.2.4*. Forskolin (2 µM and 10 µM) increased exocytosis >2-fold in the absence of glucose. However, when tested in the

presence of 20 mM glucose, forskolin failed to exert any major additional stimulatory effect. The amplifying action of glucose under these conditions was also partially antagonized by Rp-cAMPS. Importantly, there was no effect of 8-Br-Rp-cAMPS on somatostatin secretion evoked by high-K^+ alone. Taken together the latter data indicate that glucose increases cAMP and activates protein kinase A by a direct effect exerted distally to membrane depolarization. Unfortunately, it is not trivial to measure the effects of glucose on cAMP in δ-cells in the intact islets so it has not yet been possible to verify this assumption by direct measurements but work is in progress to address this important aspect.

It seems probable that cAMP/PKA enhances CICR by phosphorylation of the RyRs. With regard to the latter, the identity of these could be more firmly established. Using islet cell fractions enriched for δ-cells, it could be demonstrated that pancreatic islets only express RyR3 at appreciable levels and that it appears exclusively expressed in the δ-cells.

In addition to the effect of cAMP that is mediated by PKA, there also appears to be an effect evoked by high cAMP-levels (likely to result from exposure to 10 µM forskolin) that is resistant to PKA-inhibition. In α- and β-cells, such PKA-independent effects are mediated by the low-affinity cAMP-sensing protein cAMP-GEFII (Kashima, Miki et al. 2001) and it is now important to determine whether this protein is also expressed in δ-cells and what effects activation of this protein has on CICR and somatostatin secretion.

In addition to the effect of glucose mediated by activation of PKA, glucose was also found to promote intracellular Ca^{2+} sequestration as proposed in *Paper II*. This was evaluated by photoreleasing $InsP_3$ in δ-cells incubated in the absence or presence of 20 mM glucose. In *Paper II* we demonstrated that the δ-cells possess RyR and $InsP_3$ receptors and that they act on a common the Ca^{2+}-store. We therefore reasoned that the size of the intracellular Ca^{2+}-stores can be estimated by $InsP_3$ release. It should be noted that the data obtained with xestospongin C indicate that glucose-evoked CICR does not

involve InsP$_3$ receptors. By photorelease of InsP$_3$, we could demonstrate that glucose increased the intracellular Ca^{2+} stores ~5-fold.

We conclude that glucose stimulates somatostatin secretion by the combination of three effects: it evokes electrical activity, it elevates intracellular cAMP and thereby sensitizes the RyR3s and it increases CICR by stimulation of intracellular Ca^{2+} sequestration. The relative contribution of these effects to the stimulatory action of glucose is considered below (Section 6.3).

5.4 Electrical coupling between β-cells in intact islets.(Paper IV)

Islet β-cells are responsible for rapid and precise regulation of the blood glucose concentration. Of the ~1000 cells in an islet, 80% are insulin-secreting β-cells. In addition to chemical communication (paracrine and autocrine), the β-cells communicate electrically with each other. Ultrastructural studies have provided evidence for gap junctions between islet cells (Orci, Malaisse-Lagae et al. 1975). There is also electrophysiological evidence, obtained by sharp intracellular electrodes (Meissner 1976; Eddlestone, Goncalves et al. 1984; Mears, Sheppard et al. 1995) as well as patch-clamp measurements (Perez-Armendariz, Roy et al. 1991) for the existence of electrical coupling within the islet. Connexin-36, a protein giving rise to gap junctions, is expressed in islets and its ablation leads to loss of electrical coupling (Serre-Beinier, Le Gurun et al. 2000; Willecke, Eiberger et al. 2002).

Gap junctions enable the diffusion of ions and molecules between cells. The electrical current associated with the flux of ions can be envisaged to alter the membrane potential of the neighbouring cells. This provides a means of propagating and synchronizing electrical activity. The advantage of electrical coupling is that it can be expected to be much faster than paracrine/autocrine signalling involving diffusion of molecules in the islet interstitium. In *Paper IV* we have made use of the whole-islet patch-clamp method to provide the first

high-resolution measurements of the gap-junctional conductance in intact pancreatic islets.

5.4.1 *Gap junction conductance estimated from patch clamping data obtained from intact islets*

In perforated patch whole-cell recordings, switching between the current-clamp mode and voltage-clamp modes revealed an oscillating holding current that correlated with bursting electrical activity. Indeed, the waveforms recorded under these conditions resemble upside-down bursts of action potentials and we attribute them to electrical activity in electrically coupled neighbouring cells. A total gap junctional conductance of 1.2 nS between β-cell in intact islet was derived by analysis of these data. This value represents the gap-junctional conductance provided by all the cells coupled to the voltage-clamped cell. We next investigated the gap junction between a pair of coupled β-cells by applying a depolarization from -70 mV to >+10 mV. Under these conditions, current waveforms (likely to correspond to an action potential in - at least - one of the neighbours) were observed. By comparing the threshold potential from which action potentials are evoked under these experimental conditions (-43 mV) with the amplitude of the first voltage-clamp depolarization that triggered an action potential waveform, we estimate that the best coupled neighbour experiences ~30% of the voltage applied via the patch electrode to the voltage-clamped cell. From this we were able to estimate that the gap-junctional conductance between two adjacent β-cells to be 0.17 nS. When compared with the total conductance of 1.2 nS, this value indicates that every β-cell may be coupled to as many as ~7 other β-cells.

5.4.2 *Correction of K_{ATP} conductance measurement*

The K_{ATP} channels provide the glucose-sensitive resting conductance of the β-cell. As discussed above, closure of these channels results in membrane depolarization and electrical activity. The relationship between the glucose concentration and K_{ATP}-channel activity has previously been determined in isolated cells (Ashcroft, Harrison et al. 1984). It is clear, however, that estimates of the K_{ATP}-channel activity in β-cells in intact islets and how it is

affected by glucose would be very valuable. Unfortunately, measurements of the K_{ATP}-channel conductance in intact islets using the standard ±20 mV voltage pulse paradigm (Trube and Rorsman 1986) are "contaminated" by the contribution of the gap-junctional conductance which varies with the K_{ATP}-channel activity. However, with the information about the gap-junctional conductance in the intact islets we can now compensate for this contribution. We thus found that the corrected whole-cell K_{ATP}-conductance fell from ~2.5 nS in the absence of glucose to 0.1 nS at an elevated glucose concentration (15 mM) with an approximate IC_{50} of ~3.7 mM.

5.4.3 *Absence of dye coupling between β-cells*

Dye injection represents the traditional method for assessment of coupling between cells. However, contrary to previous reports (Meda, Santos et al. 1986; Charollais, Gjinovci et al. 2000), we were unable to find any sign of dye coupling even in cells that were definitely in electrical contact with its neighbours. Thus, dye coupling cannot be equated to electrical coupling.

5.4.4 *Propagation of $[Ca^{2+}]_i$ waves in an islet*

The role of electrical coupling in the islet may be to synchronise electrical and secretory activity in different parts of the islet (that may be 10-20 cells across). Is the electrical coupling sufficient to account for the synchronization observed in the islet? We used confocal imaging of $[Ca^{2+}]_i$-oscillations in intact islets to estimate how quickly an electrical signal spreads across the islet (remember that electrical activity leads to an increase in $[Ca^{2+}]_i$). The propagation and synchronization of the $[Ca^{2+}]_i$ waves have been suggested to reflect the electrical coupling between the β-cell (Valdeolmillos, Santos et al. 1989; Santos, Rosario et al. 1991). We observed that the $[Ca^{2+}]_i$ wave spread with an average speed of ~80 μm/s within the islet. This is in reasonable agreement with the value of ~60 μm/s that can be estimated from the analysis of the gap-junctional conductance.

6. General Discussion

Here I consider the particularly important aspects that have emerged during the course of this Ph.D. project.

6.1 *Speed of β-cell exocytosis estimated from capacitance measurements in intact islets suggests release rates similar to those observed hormone secretion experiments*

The application of capacitance measurements has revolutionized the study of cell exocytosis by allowing exocytosis to be monitored at the single-cell level with unprecedented temporal resolution (Neher 1998). Exocytosis in β-cells monitored as capacitance increases conforms to the properties of insulin secretion established by traditional biochemical assays (Rorsman 1997; Rorsman, Eliasson et al. 2000). The data obtained by the different techniques agree on several important aspects: (1) all results indicate that exocytosis is a Ca^{2+}-dependent process (Ammala, Eliasson et al. 1993); (2) cAMP enhances exocytosis via activation of protein kinase A (Ammala, Ashcroft et al. 1993; Ammala, Eliasson et al. 1994); (3) exocytosis/secretion is inhibited by adrenaline, galanin and somatostatin (Renstrom, Ding et al. 1996); (4) experimental temperature greatly influences both insulin secretion and β-cell exocytosis (Renstrom, Eliasson et al. 1996).

On the other hand, the exocytosis measured by capacitance measurements appear to proceed at the phenomenal speed of 1.2 pF/s, corresponding to 300-400 granules/s per cell (Barg, Ma et al. 2001) For comparison, biochemical assays from intact islets suggest a release rate in glucose-stimulated cells of 0.3 granule/s and cell (Bratanova-Tochkova, Cheng et al. 2002).

In *Paper I*, the application of capacitance measurements to β-cells within intact islets provided us with an opportunity to directly compare secretion detected by capacitance measurements and biochemical assays. The rate of exocytosis thus obtained from β-cells *in situ* by the onset of time course depolarizations is only ~40 fF/s (equivalent to 14 granules/s) which is < 10% of that reported from single cells (Barg, Ma et al. 2001). Still, this value is of

50-fold higher than the hormone secretion data. However, exocytosis at -20 mV (the peak of the β-cell action potential) proceeds at rates <30% of that observed at 0 mV; i.e. ~4 granules/s and β-cell. Finally, it should be noted that these experiments were done using an electrode solution containing 0.1 mM cAMP. Previous studies revealed that this cyclic nucleotide enhances β-cell exocytosis ~10-fold (Eliasson, Ma et al. 2003). We thus arrive at a value for β-cell exocytosis in the absence of cAMP and at -20 mV of 0.4 granules/s and β-cell. These considerations indicate that capacitance measurements do indeed report "true" exocytosis in the β-cell. Accordingly, whole-islet capacitance measurements should represent a valid and valuable tool for the elucidation of β-cell exocytosis. It will be interesting to apply this technique to β-cells in intact human islets.

6.2 *How does glucose regulate somatostatin secretion from δ-cells?*

Somatostatin-releasing δ-cells are electrically excitable, equipped with K_{ATP}-channels and respond to closure of these channels with membrane depolarization and action potential firing (Gopel, Kanno et al. 2000).

In *Paper II* it is described that although the K_{ATP} channel blocker tolbutamide is capable of initiating somatostatin release, it is a surprisingly weak stimulus. Moreover, glucose-induced somatostatin release was essentially normal in islets isolated from K_{ATP}-deficient mice (*Figure.9B*). Collectively, these observations indicate that glucose chiefly regulates somatostatin release downstream of closure of the K_{ATP}-channels. Indeed, in islets depolarized with high extracellular K^+ (70 mM), glucose retained a strong stimulatory effect (4-fold).

In *Paper II* we demonstrate that the K_{ATP}-independent component of secretion is mediated by Ca^{2+}-induced Ca^{2+}-release (CICR) and is blocked by dantrolene and ryanodine. We demonstrate that CICR is strongly dependent on Ca^{2+}-influx via R-type Ca^{2+}-channels. Thus, the stimulatory effect of glucose is abolished in $Ca_v2.3$ (R-type) knockout islets and in the presence of

SNX482 (an R-type Ca^{2+}-channel blocker). CICR was triggered with a latency as short as 10 ms and was able do discriminate between Ca^{2+}-influx between R- and L-type Ca^{2+}-channels situated within micrometres from each other. All these observations argue that the ryanodine receptors and the R-type Ca^{2+}-channels must be in close proximity to each other. However, they are not quite as close as in skeletal muscle where they actually associate into a functional complex (Ref). This is suggested by the observation that Ca^{2+}-influx is required and depolarization alone, unlike what is the case in skeletal muscle, is insufficient to trigger CICR (*Figure. 12C*).

The CICR-dependent component of somatostatin secretion gives rise to a "slow" component of capacitance increase that extends beyond the duration of the depolarization. Such responses are hardly ever observed in mouse β-cells. It is of interest, however, that in human β-cells (which express RyR2) (Johnson, Kuang et al. 2004) such a slow component is observed (Matthias Braun, personal communication) and it is tempting to propose that it reflects CICR and that this process contributes to glucose-induced insulin secretion in man.

6.3 *Cyclic AMP and the control of CICR and somatostatin secretion*

As reported in *Paper II*, glucose increases the magnitude of the CICR-dependent component of somatostatin secretion in a concentration-dependent fashion with an EC_{50} of ~10 mM. In *Paper III*, we addressed how glucose promotes CICR in δ-cells. As previously described for β-cells (Chow, Lund et al. 1995; Tengholm, Hellman et al. 2001), glucose promotes Ca^{2+} uptake into intracellular Ca^{2+} stores in δ-cells. This was visualized by the photoliberation of $InsP_3$ (which releases intracellular Ca^{2+} from the same stores as RyR-dependent Ca^{2+} mobilization); much larger $[Ca^{2+}]_i$ transients were observed in the presence of 20 mM glucose than with 1 mM glucose. In *Paper III*, we provide evidence that glucose stimulates CICR by a cAMP/PKA-dependent mechanisms. Thus, the stimulatory effect of glucose was antagonized by Rp-

cAMPS and no further stimulation by glucose was observed in the presence of 2 µM forskolin.

Collectively, the data of *Papers II-III* suggest that glucose-stimulated somatostatin secretion involves at least three discrete mechanisms. First, glucose triggers electrical activity via closure of K_{ATP} channels. This effect accounts for ~20% of the overall effect. Second glucose promotes intracellular cAMP production which in turn potentiates CICR. This effect explains ~50%. Finally, the remaining 30% of the stimulatory effect is due to glucose-induced sequestration of Ca^{2+} leading to larger CICR once electrical activity has been initiated and in the presence of elevated cAMP levels.

6.4 Cell-cell coupling of β-cells in intact islet

The β–cells are electrically coupled to each other via gap junctions (Orci, Malaisse-Lagae et al. 1975; Meissner 1976; Eddlestone, Goncalves et al. 1984; Mears, Sheppard et al. 1995). It is believed that electrical coupling accounts for the synchronization of β-cell electrical activity and Ca^{2+} oscillations in an islet, which explains the pulsatility of insulin secretion. In *Paper IV* we have documented the strength of cell coupling between β-cells in intact islets for the first time.

Mouse β-cells exposed to insulin-releasing glucose concentration exhibit $[Ca^{2+}]_i$ oscillations that can be resolved both at the single-cell and whole-islet level (Dryselius, Grapengiesser et al. 1999; Nadal, Quesada et al. 1999). The synchronization of this oscillation has been proposed to be due to both electrical coupling and diffusible factors co-released with insulin (like ATP) (Salehi, Qader et al. 2005) . In *Paper IV* we show that electrical coupling is sufficiently strong to allow a $[Ca^{2+}]_i$ wave to propagate at an average speed of ~60 µm/s, which is in reasonable agreement with the 80 µm/s observed experimentally.

However, the mathematical analysis revealed that coupling between adjacent β-cell is not particularly strong and that even a small decrease in the

amplitude of the action potential/bursts of action potentials can be expected to have a dramatic effect on cell coupling and islet synchronization with resultant impairment of islet rhythmicity. The loss of pulsatile insulin secretion is regarded as a hallmark of type-2 diabetes in both rodents model (Lin, Ortsater et al. 2001) and man (Lang, Matthews et al. 1981). Therefore, impaired electrical coupling should be considered in the aetiology of type-2 diabetes.

7. Conclusion

In this thesis I demonstrate that capacitance measurements in intact islets are feasible and represent a valuable complement to existing methods for monitoring islet secretion. They are of particular value when it comes to analysis of exocytosis in non-β-cells that only comprise a small fraction of the total islet cell number (as exemplified by the δ-cells). The following conclusions were reached:

1. Exocytosis in β-cells in intact islets proceed at rates 25-fold lower than those previously documented in isolated cells. When allowance is made for differences between the experimental conditions used for the biochemical (insulin release assays) and the capacitance measurements (membrane potential etc), a fair correlation between the rates of secretion detected by the two methods is obtained.

2. Glucose-induced somatostatin secretion involves Ca^{2+}-induced Ca^{2+}-release, is dependent on Ca^{2+}-influx through R-type Ca^{2+}-channels;

3. The effects of glucose on somatostatin secretion and ability to evoke CICR involve stimulation of electrical activity, intracellular Ca^{2+} sequestration and a cAMP/PKA-dependent mechanisms. Thus, cAMP may represent a trigger of glucose-induced somatostatin secretion.

4. Electrical coupling between β-cells in intact islets is strong enough – but only just - to explain the synchronization of β-cell electrical activity (and secretion) in intact islets.

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Addendum:

The immunostaining/confocal picture of pancreatic islet δ-cells which is used in the cover of this thesis and the picture of human islet on page 5 were kindly provided by Dr. Chris Partridge.

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