From the Rolf Luft Research Center for Diabetes and Endocrinology,
Department of Molecular Medicine and Surgery,
Karolinska Institutet, Stockholm, Sweden

ASSESSMENT OF PANCREATIC ISLET CELL FUNCTION AND SURVIVAL

Martin Köhler

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In memory of my father

ABSTRACT

Function and survival of pancreatic islet insulin-producing beta-cells (β-cells) and glucagon-producing alpha-cells (α-cells) were studied, and methods for this purpose were developed or refined.

Dynamic control of glucose metabolism is essential for β-cell stimulus-secretion coupling. ATP is an important metabolic parameter and therefore we set up a technique to monitor dynamic changes of ATP in insulin-producing cells using luciferase bioluminescence at the level of single cells or groups of cells. We could detect a decrease in ATP in response to the mitochondrial uncoupler FCCP in HIT M2.2 cells and an increase in ATP in response to glucose in intact mouse islets.

The glucose-induced stimulus-secretion coupling was also studied in a mutant mouse model with β-cell specific depletion of mtDNA and consequently disruption of the mitochondrial respiratory chain. In this model we could observe disrupted response in mitochondrial membrane potential (MMP), impaired response in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) as well as disrupted insulin release.

The interrelation between $[Ca^{2+}]_i$ and MMP was studied in response to glucose stimulation and to non-metabolic stimuli in mouse β-cells using a method for simultaneous detection of these two parameters. Our results indicate involvement of Ca^{2+}-dependent activation of mitochondrial dehydrogenases, under low glucose conditions. MMP depolarization due to Ca^{2+}-influx into mitochondria is consistent with effects under high glucose conditions. The latter phenomenon could also be observed subsequent to every peak of glucose-induced slow $[Ca^{2+}]_i$ oscillations.

A method for on-line detection of apoptosis at single-cell level was established with a FRET-based biosensor that was sensitive to caspase-3-like proteases, which act as executive caspases in several pathways of apoptotic cell-death. This was detected by two-photon laser scanning microscopy, a modality that is commonly used for *in vivo* microscopy. Therefore we conclude that the method has the potential to be used for *in vivo* detection of apoptosis.

Methods for enrichment of human and rat pancreatic α-cells were established or modified using fluorescence-activated cell sorting (FACS) based on only intrinsic cellular properties such as light scatter and autofluorescence. The sorted cells showed high purity, high viability and also demonstrated functional $[Ca^{2+}]_i$ responses.

In conclusion, the methods that I have developed or refined are successfully used in studies of islet cell function and survival, and they will be useful also for future *in vivo* experiments. My results contribute to our understanding how islet cells function in physiology and how their function becomes compromised in diabetes.

LIST OF PUBLICATIONS

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

I. **Martin Köhler**[*], Svante Norgren[*], Per-Olof Berggren, Bertil B.Fredholm, Olof Larsson, Christopher J. Rhodes, Terence P. Herbert, Holger Luthman.
Changes in cytoplasmic ATP concentration parallels changes in ATP-regulated K^+-channel activity in insulin-secreting cells.
FEBS Lett. (1998) 441, 97-102

II. José P. Silva, **Martin Köhler**, Caroline Graff, Anders Oldfors, Mark A. Magnuson, Per-Olof Berggren, Nils-Göran Larsson.
Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes.
Nat.Genet. (2000) 26, 336-340

III. Henrik Kindmark[*], **Martin Köhler**[*], Graham Brown, Robert Bränström, Olof Larsson, Per-Olof Berggren.
Glucose-induced oscillations in cytoplasmic free Ca^{2+} concentration precede oscillations in mitochondrial membrane potential in the pancreatic beta-cell.
J.Biol.Chem. (2001) 276(37), 34530-34536

IV. **Martin Köhler**[*], Sergei V. Zaitsev[*], Irina I. Zaitseva, Barbara Leibiger, Ingo B. Leibiger, Mikael Turunen, Iouri L. Kapelioukh, Linda Bakkman, Ioulia B. Appelskog, Jacques Boutet de Monvel, Gabriela Imreh, Per-Olof Berggren.
On-line monitoring of apoptosis in insulin-secreting cells.
Diabetes (2003) 52(12), 2943-2950

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

[*] These authors contributed equally.

Related publications:

Kindmark H., Köhler M., Nilsson T., Arkhammar P., Wiechel K.-L., Rorsman P., Efendic S. and Berggren P.-O. "Measurements of cytoplasmic free Ca^{2+} concentration in human pancreatic islets and insulinoma cells" (1991) FEBS Lett., 291, 310-314

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CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AM	acetoxymethyl ester
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	cytoplasmic free Ca^{2+} concentration
CCD	charge-coupled device
CLSM	confocal laser scanning microscopy
COX	cytochrome c oxidase
cps	counts per second
DM	diabetes mellitus
ECFP	enhanced cyan fluorescent protein
ER	endoplasmic reticulum
EYFP	enhanced yellow fluorescent protein
FACS	fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
FCCP	carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
FSC	forward scatter
GSIS	glucose-stimulated insulin secretion
IFNγ	interferon-γ
IL-1β	interleukin-1β
K_{ATP} channel	ATP-sensitive K^+ channel
MMP	mitochondrial membrane potential
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAD(P)H	NADH and NADPH
NIDDM	non-insulin dependent diabetes mellitus (same as T2DM)
PARP1	Poly(ADP ribose) Polymerase 1
PMT	photomultiplier tube
Rh123	rhodamine 123
SDH	succinate dehydrogenase
SSC	side scatter
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus (previously called NIDDM)
TFAM	mitochondrial transcription factor A
TNFα	tumor necrosis factor-α
TPLSM	two-photon laser scanning microscopy

1 INTRODUCTION

Insulin is the main blood glucose lowering hormone and therefore it has a decisive role in blood glucose homeostasis. This hormone is secreted from the pancreatic β-cell that is the predominant type of cell in the pancreatic islet. Another pancreatic islet cell type is the α-cell that secretes the hormone glucagon which serves to increase the blood glucose level. Insulin stimulates glucose uptake by skeletal muscle and fat. Glucagon mobilizes glucose from the liver into the circulation, whereas insulin inhibits this mobilization. Diabetes mellitus (DM), or simply diabetes, is a group of metabolic diseases that are characterized by elevated blood glucose, hyperglycemia. These disease states involve dysfunctional secretion of both insulin and glucagon. Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of the pancreatic β-cells whereas Type 2 diabetes mellitus (T2DM) is associated with a gradual loss of β-cell function during the progression of the disease. Secretion of glucagon from α-cells is also often deranged both in T1DM and T2DM, where a paradoxical elevation of glucagon in hyperglycemic subjects exacerbates the disease.

Both the pancreatic β-cell and α-cell serve as metabolic glucose-sensors where glucose metabolism converts a glucose-concentration signal into a secretory response, a process referred to as the 'stimulus-secretion coupling'. Metabolic sensing in these cells is dynamically mediated by glycolysis and mitochondrial respiration in response to glucose. Stimulated metabolism leads to an increased free cytoplasmic ATP/ADP ratio and polarization of mitochondrial membrane potential in addition to many other changes. A severe alteration of β-cell mitochondrial metabolism can lead to β-cell dysfunction, to β-cell death and consequently also to diabetes.

The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) serves as an important coupling factor between metabolism and insulin release from the β-cell. Closure of ATP-regulated K^+ channels leads to depolarization of the β-cell and consequent opening of voltage-gated Ca^{2+} channels in the plasma membrane with resulting increase in $[Ca^{2+}]_i$. High $[Ca^{2+}]_i$ triggers exocytosis of insulin granules. Moreover $[Ca^{2+}]_i$ can alter mitochondrial metabolism leading to possible feed-back loops.

The mechanism for auto-immune destruction of β-cells in the pathogenesis of T1DM can either be apoptosis or necrosis. Factors found in the serum from newly diagnosed T1DM patients can initiate β-cell apoptosis.

The metabolic status, as detected from cellular autofluorescence, can be used as a cell-specific fingerprint to distinguish between and separate different cell types, like β-cells and α-cells.

In the current thesis, the general aim has been to develop and use light microscopy techniques, based on fluorescence and luminescence, for functional assessment of pancreatic islet cells.

2 BACKGROUND

2.1 THE PANCREATIC ISLET AND ISLET CELLS

Paul Langerhans, as a German medical student, discovered in 1869 discrete cell-clusters dispersed in the rabbit pancreas [1]. These microorgans are now known as the 'islets of Langerhans', or 'pancreatic islets'. Their role for glucose homeostasis emerged from work in the late 19th century and onwards.

The pancreatic islets constitute approximately 1-2% of the mammalian pancreas. In humans this corresponds to 1-2 million islets [2], whereas the rat pancreas contains 4000-5000 islets [3; 4] and mouse pancreas contains about 900-4500 islets [5; 6]. The islet consists of at least five types of endocrine cells; alpha (α) cells producing glucagon, beta (β) cells producing insulin, delta (δ) cells producing somatostatin, PP cells (gamma cells) producing pancreatic polypeptide and epsilon (ε) cells producing ghrelin [7]. Out of these cells, the most predominant cell type is the β-cell that represents 60-80% of the rodent endocrine islet cells, and around 50% of the adult human islet cells [8]. α-cells represent 15-20% of the rodent endocrine islet cells and around 40% of the human islet cells [8].

The islet cells are stimulated by nutrients, hormones and other factors via the islet vasculature network [9; 10]. The islet cells are also modulated by neural regulation [11]. Intra-islet coordination of cells is carried out by intercellular [12], autocrine [13; 14] and paracrine [15; 16] mechanisms leading to the islet output of hormones that serve as main systemic regulators of blood glucose concentration [17; 18].

2.2 GLUCOSE METABOLISM IN PANCREATIC BETA CELLS

Glucose is transported into cells via glucose transporters, a process that is normally not rate limiting in β-cells [19]. Thereafter glucose is oxidized through the regulated process of glycolysis to generate pyruvate or lactate. Pyruvate enters mitochondria and converts into Acyl-CoA that is the main substrate for the mitochondrial Krebs cycle that feeds high-energy electrons into the mitochondrial respiratory chain via the reduced coenzymes NADH and $FADH_2$. Respiration is a key way for the cell to gain energy in the form of the phosphorylated molecule ATP.

The respiratory chain takes place within the inner mitochondrial membrane and consists of a number of steps, reactions within the complexes named I to V, where the reducing potential of NADH and $FADH_2$ is eventually converted to ATP. O_2 is the final electron acceptor when O_2 and H^+ form H_2O by the electron transport chain complex IV. Protons (H^+) are transported into the mitochondrial intermembrane space by complexes I, III and IV, which creates the so called proton motive force across the inner mitochondrial membrane. The proton motive force has two components, namely the mitochondrial membrane potential (MMP or $\Delta\psi_m$) and the proton concentration

gradient. The proton motive force drives complex V, also known as ATP synthase, where ADP is phosphorylated to ATP.

MMP can be monitored in intact cells with fluorescent indicators. The MMP becomes more negative (polarizes) as a consequence of enhanced respiration after addition of glucose.

Metabolism plays a central role for β-cell function and survival and therefore metabolic dysfunction (either glycolytic [20] or mitochondrial [21-25]) can act as a main or as a contributing factor for T2DM.

In β-cells it has been shown that glucose metabolism can be regulated by feed-back inhibition and stimulation whereby dynamic cyclic fluctuations, oscillations, of metabolites are generated. In this context, most attention has been given to oscillations in the glycolytic pathway [26; 27].

2.3 BETA CELL MEMBRANE POTENTIAL, Ca^{2+} AND EXOCYTOSIS

The resting β-cell membrane potential, at low glucose conditions, is around -60 to -70 mV. Stimulation of glucose metabolism increases the cytoplasmic ATP concentration that leads to closure of ATP-regulated K^+ channels (K_{ATP} channels), resulting in depolarization of the β-cell plasma membrane [28-30]. Depolarization to a threshold potential of -40 mV leads to activation of voltage gated L-type Ca^{2+} channels and thereby an increase in $[Ca^{2+}]_i$, that ultimately triggers exocytosis of insulin [31].

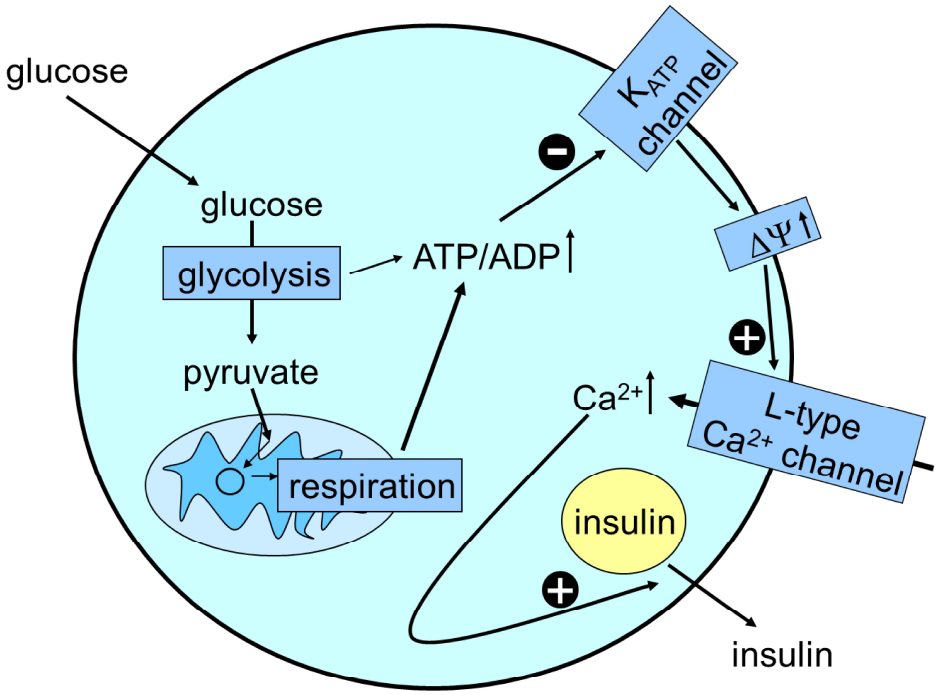

Figure 1. **The main mechanism of glucose-stimulated insulin secretion (GSIS) in the pancreatic β-cell.** Glucose that enters into the β-cell is metabolized to form ATP. The increased ATP/ADP ratio leads to the closure of ATP-sensitive K^+ channels and subsequent depolarization of the plasma membrane. This in turn leads to opening of voltage-dependent L-type Ca^{2+} channels and influx of Ca^{2+}, which triggers insulin exocytosis.

Insulin release is known to be pulsatile, oscillating, both *in vitro* and *in vivo* in normal subjects [32-35]. Lack of oscillatory insulin secretion is one of the earliest defects observed in T2DM [36; 37]. These oscillations are associated with both bursting electrical activity and also with metabolic oscillations in β-cells [38]. Bursting electrical activity [39] leads to oscillating $[Ca^{2+}]_i$ [40] that induces oscillations in insulin secretion [41-43].

The mechanism of islet β-cell bursting electrical activity and oscillating $[Ca^{2+}]_i$ has been studied extensively, both experimentally [40; 44-46] and theoretically [38; 47; 48]. The main components are known, but there are still some details that remain to be understood, in particular for other species than rodents. A constant glucose concentration of 10 mM given to intact islets typically evokes a slow wave electrical activity pattern on which action potentials, bursts, are superimposed [28]. After the depolarization phase of the burst, that is associated with Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels, there is a phase where action potentials are initiated from a plateau potential. During both these phases there is a substantial influx of Ca^{2+} that accumulates in the cytoplasm and in intracellular Ca^{2+} stores like endoplasmic reticulum (ER). Every burst is terminated by a rapid repolarization to slightly below the threshold potential. This repolarization is mediated by opening of Ca^{2+} activated K^+ channels [49-52], where Ca^{2+} from ER plays an active role [53]. Repolarization also leads to closure of L-type Ca^{2+} channels whereby $[Ca^{2+}]_i$ decreases. Oscillating $[Ca^{2+}]_i$ that is associated with islet β-cell bursting electrical activity, we denote as 'fast oscillations'. Typical period for the fast oscillations is in the range 6-30 s, and this oscillatory pattern has been described to be disturbed in islets from DM models and in ageing [25; 54].

$[Ca^{2+}]_i$ oscillations associated with metabolic oscillations are typically slower, with periods in the order of minutes. These slow oscillations can be observed both in single β-cells and in islets. The periods of slow oscillations coincide with the periods observed for insulin oscillations in man and rodents *in vivo* [34; 35].

2.4 INTERPLAY BETWEEN Ca^{2+} AND GLUCOSE METABOLISM

Mitochondrial metabolism and $[Ca^{2+}]_i$ are both intimately involved in glucose-stimulated insulin secretion (GSIS). In this context it is important to understand how these parameters are interrelated, a broad topic that has been studied both in general [55-57] and specifically in β-cells [58-60]. Some direct or indirect mechanisms identified until now are listed below.

Examples how mitochondrial metabolism can regulate β-cell $[Ca^{2+}]_i$:

- Removal of Ca^{2+} from cytoplasm:
  ATP stimulates Ca^{2+} ATPases that remove Ca^{2+} from cytoplasm over the plasma membrane or into organelles and thereby lowers $[Ca^{2+}]_i$ [61; 62].

- Influx of Ca^{2+} from extracellular space:
  ATP binds to K_{ATP} channels leading to depolarization of plasma membrane [28], opening of voltage-gated Ca^{2+} channels and influx of Ca^{2+}.
- $[Ca^{2+}]_i$ modulation by mitochondria:
  Dynamic Ca^{2+} responses can be modulated by mitochondrial MMP-dependent Ca^{2+} uptake [63] and Ca^{2+} extrusion.
- $[Ca^{2+}]_i$ modulation by other organelles:
  Dynamic Ca^{2+} release from other organelles, like sarcoplasmic reticulum and ER, is modulated by ATP. Examples are the potentiating effect of ATP on the sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor [64] and enhancement of Ca^{2+} release through IP3 receptors [65].
- Other ATP-dependent mechanisms:
  ATP can control formation of signaling molecules like cAMP [66; 67] that modulates $[Ca^{2+}]_i$.

Examples how $[Ca^{2+}]_i$ can regulate β-cell mitochondrial metabolism:

- Loss of proton motive force:
  MMP can depolarize in response to an increased influx of Ca^{2+} into mitochondria [68-70].
- Stimulation of Krebs cycle:
  Ca^{2+} activates dehydrogenases in the Krebs cycle leading to increased respiration and MMP polarization [71].
- Regulation through rate of glycolysis:
  Glycolysis can be partly regulated by Ca^{2+} and thereby regulate mitochondrial respiration [72].
- Regulation of mitochondria by Ca^{2+}-binding proteins:
  Signaling through calmodulin [73] or other Ca^{2+}-binding proteins.

One should bear in mind that mechanisms differ in terms of kinetics, localization and substrate sensitivity. These factors determine when and how one particular mechanism will be activated.

2.5 DIABETES MELLITUS AND BETA CELL APOPTOSIS

Apoptosis, programmed cell death, plays a critical role to maintain physiological homeostasis of cell turnover throughout the life of a multicellular organism [74; 75]. The β-cell mass is dynamic and regulated by neogenesis, proliferation, dedifferentiation [76] and apoptosis in order to meet the long-term demand of insulin to maintain blood normoglycemia.

However, during a prolonged period of diet-induced hyperglycemia and hyperlipidemia the β-cell mass will eventually be reduced in diabetes prone gerbil ‘sand rat’

Psammomys obesus [77-79]. Like in humans these animals with increased insulin resistance go through an initial phase of partial adaptation with increased insulin secretion. The reduction of β-cell mass is associated with apoptosis.

Reduction of β-cell mass is also observed in human T2DM, even if the values of human β-cell masses varies within an extraordinary wide range [80]. No study has reported increased β-cell mass in T2DM [81]. Also in humans the process of β-cell mass reduction in T2DM has been associated with increased apoptosis [82].

T1DM is caused by a selective autoimmune destruction of β-cells where the β-cell mass can be reduced already by 70-80% at the time of diagnosis [83]. The process of β-cell destruction is associated with inflammation caused by pro-inflammatory cytokines that are released from activated macrophages and T-cells infiltrating the islets [84; 85]. In particular the three pro-inflammatory cytokines interleukin-1β (IL-1β), interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) have been extensively studied because of their induction of β-cell apoptosis or necrosis [83; 86-88]. IL-1β is able to promote β-cell destruction on its own in isolated islets [89], but it is unlikely that any of these three cytokines will act alone in human diabetes. Their signaling pathways are complex but these converge at the final step of execution, namely the activation of caspase-3-like proteases [90-92]. Caspase-3 is the principal executor of apoptosis and caspase-3-mediated β-cell apoptosis is a key initiating event in T1DM [93]. Caspase-3-like proteases selectively cleave the amino acid sequence DEVD in a number of target proteins leading to a controlled dismantling of intracellular components while avoiding inflammation. One early identified target protein for Caspase-3 was PARP1 [94].

Reduction of β-cell mass during the progression of T2DM appears to be associated with apoptosis through islet inflammatory mechanisms that were suggested to have common denominators with cell death in T1DM [88; 95-98]. However, this is possibly an oversimplification due to the known differences between pathogenesis of T1DM and T2DM [83]. As a consequence, caspase-3 may not always be activated during reduction of β-cell mass in T2DM.

2.6 THE PANCREATIC ALPHA CELL

Glucose homeostasis is mainly controlled by the balance between glucagon and insulin concentrations in the blood. Hypoglycemia stimulates glucagon secretion from the pancreatic α-cells, which leads to increased hepatic glucose output and will therefore counteract the glucose lowering effect of insulin [99]. This balance is dysfunctional in both T1DM and T2DM where glucagon secretion response can be either too low to reverse hypoglycemia [100-102] or too high which aggravates hyperglycemia [103-105]. The α-cell function under healthy and diabetic conditions has been studied over the last decades, but the understanding is still limited [99].

α-cells can be studied within the intact islet. However, under these conditions direct effects on the α-cells can be difficult to distinguish from effects due to indirect involvement of other islet cell types. To clearly reveal direct effects on α-cells they

have to be studied in pure preparations. In order to separate one cell type from another one need to identify the unique cellular characteristics of that cell, and these parameters must be measurable. The metabolic response of α-cells to glucose differs from that of β-cells [106] and therefore it is not surprising that the levels of fluorescent oxidized flavin FAD, as well as the fluorescent reduced pyridine nucleotides NADH and NADPH, can be used to distinguish between these cell types. Other detectable parameters are forward and side scatter reflecting cell size and granularity that can also be expected to differ between the islet cell types. Additional cell specific criteria have been used, but these normally require staining of cell-specific surface receptors [107; 108] or staining of intracellular targets [109].

3 AIMS

The overall objective of this thesis was to develop and apply methods based on fluorescence and luminescence for functional assessment of islet cells.

The specific aims were to:

- Develop and evaluate a method for on-line monitoring of intracellular ATP in single insulin-producing cells.

- Characterize islet function in a mouse model with severe depletion of mitochondrial DNA.

- Characterize the interrelation between changes in $[Ca^{2+}]_i$ and MMP in pancreatic β-cells.

- Develop and demonstrate a method for on-line monitoring of apoptosis based on a fluorescent protein sensitive to caspase-3 activation.

- Develop, optimize and evaluate a new procedure to sort functional human and rat pancreatic α-cells.

4 MATERIALS AND METHODS

4.1 MATERIALS

Materials used in the experiments reported in the work are described in detail in the papers (I-V).

4.2 CELLS

4.2.1 Mouse and rat primary cells

Adult obese non-diabetic mice (gene symbol *ob/ob*) were obtained from a local colony [110]. Control heterozygous C57BL/KsJ-db/+ mice were obtained from a local colony. Wistar rats were obtained either from Scanbur AB (Sollentuna, Sweden) or from Charles River (Germany).

Rodent pancreatic islets were isolated by collagenase digestion and hand-picked essentially as previously described [111]. The variation of isolation methodology between the studies is described in papers I-V. Dissociation of islets into cell suspension was performed as described in papers III-V. Mouse islets were normally cultured in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin unless otherwise stated. Culture of rat islets is described in paper V.

4.2.2 Human islets

Human pancreata were obtained within the Nordic Network for Islet Transplantation from deceased donors with total brain infarction after appropriate consent. The islets were isolated at the Division of Clinical Immunology at the University of Uppsala, as previously described [112].

4.2.3 Cell lines

The clonal rat β-cell line RINm5F [113; 114] was cultured in RPMI 1640 medium, supplemented with 10% FCS, 100 μg/ml streptomycin and 100 IU/ml penicillin.

The clonal hamster β-cell line HIT M2.2 [115; 116] used in paper I was cultured in DMEM medium supplemented with 2 mM L-glutamine, 10% FCS and 50 μg/ml gentamycin.

The clonal mouse β-cell line MIN6 [117] was cultured in DMEM medium containing 25 mM glucose supplemented with 10% (vol/vol) FCS, 50 μM β-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin.

4.3 BIOLUMINESCENCE MICROSCOPY

Bioluminescence is light produced by a chemical reaction which originates in an organism [118-120]. Several types of bioluminescent reactions have been used for chemical analysis and instruments for this have evolved during the last few decades [119-122]. The most well-known bioluminescent assay is based on the firefly luciferase found in the light-emitting organ of the American firefly *Photinus pyralis* [123]. The reaction uses ATP and luciferin as substrates and this assay is considered to be one of the most sensitive analytical tools for detection of ATP. One challenge for detection is that bioluminescence generates much less light than fluorescence. However, there is no background signal in bioluminescence except for background (dark) signal from the detector or contaminating signal from ambient light in the room. We used (in paper I) a standard inverted fluorescence microscope (Zeiss Axiovert 135TV, Zeiss, Germany) for the task to detect bioluminescence signal from cells expressing luciferase. The luminescence signal was detected by either a photon-counting photomultiplier tube (PMT) or an intensified integrating CCD camera (Luminescence Imager, Photonic Science, EastSussex, UK). Special care had to be taken to reduce background signal by cooling the detector and to minimize light in the room. Since year 2008 there is one microscope model on the market that is optimized for single-cell bioluminescence microscopy [124] with more than 10x increase in light-collection efficiency, a fact that illustrates the challenge to use standard microscopes for this purpose. This optimized microscope was also used for single-cell detection in radioluminescence microscopy [125]. In this context it is also worth mentioning the term bioluminescence imaging (BLI) that refers to the noninvasive technology developed over the past decade to study ongoing biological processes by bioluminescence in small animals [126; 127].

4.4 FLUORESCENCE MICROSCOPY AND CYTOMETRY

4.4.1 Live-cell fluorescence microscopy, indicators and sensors

Fluorescence is the emission of light by a substance that has absorbed light. Fluorescence microscopy utilizes the wavelength difference between exciting and emitted wavelengths to separate fluorescent emitted light from a non-fluorescent background [128]. This technique can be used to study dynamic processes in living cells [129] using a variety of fluorescent probes [130; 131] and fluorescent proteins [132].

One commonly used fluorescent indicator for dynamic monitoring of $[Ca^{2+}]_i$ is fura-2 [133] that was used in papers I, II, III and IV. Ca^{2+} binds selectively to fura-2 which shifts its peak absorbance wavelength when bound. Therefore fura-2 fluorescence intensity, measured around 510 nm, increases in response to Ca^{2+} binding when excited at 340 nm, while fluorescence from 380 nm excitation will decrease. The ratio between these two signals (340/380) will reflect the level of $[Ca^{2+}]$ irrespective of the number of fura-2 molecules detected. This indicator is therefore called ‘ratiometric’ in contrast to

'non-ratiometric' indicators. For overview of available Ca^{2+} sensitive chemical indicators see [134]. In our studies we loaded cells with fura-2/AM, which is the acetoxymethyl (AM) ester form of the hydrophilic fura-2 molecule. Fura-2/AM is highly lipophilic and thereby membrane permeable. Once this molecule entered into a cell, intracellular esterases cleave the AM ester group by hydrolysis, and the molecule is trapped in the cytoplasm.

Changes in MMP were monitored in papers II and III using the fluorescent indicator Rh123 [135-137]. Rh123 is a lipophilic cation dye that loads directly into mitochondria due to its charge. The Rh123 distribution between cytosol and mitochondria is determined by the MMP, where depolarization of MMP leads to redistribution from mitochondria to cytoplasm. Redistribution of Rh123 into mitochondria at hyperpolarized MMP leads to a concentration-dependent quenching of the dye. Therefore the Rh123 fluorescent signal decreases when MMP polarizes.

Cellular autofluorescence in mammalian cells is dominated by two distinct molecular species, one with fluorescence emission around 520 nm and the other one with emission around 440 nm [138]. The first species originates from naturally occurring oxidized flavins and flavin nucleotides based on the FAD cofactor, especially FMN, FAD and riboflavin [139]. We denote this species simply as FAD. FAD fluorescence was used in paper V. The second autofluorescent molecular species originates from the reduced pyridine dinucleotides NADH and NADPH [140]. We denote this species as NAD(P)H because NADH and NADPH have a significant spectral overlap, and are therefore difficult to separate. NAD(P)H fluorescence was used in papers III and V.

FRET is a non-radiative energy transfer mechanism that occurs when two fluorophores are in sufficient proximity (<100 Å). The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance [141]. FRET efficiency can be measured in several ways, and we use the mode called 'sensitized emission' where the FRET donor is excited and fluorescence intensity is monitored both from the FRET donor and the FRET acceptor molecules. In this way an increased FRET efficiency will result in a stronger emission from the acceptor molecule and lower emission from the donor molecule, due to the transfer of energy from donor to acceptor.

The fluorescent proteins ECFP and EYFP can be spectrally separated. However, the emission spectrum of ECFP overlaps with the excitation spectrum of EYFP which implies that ECFP and EYFP are suitable to use as a 'FRET pair' [142]. This FRET pair has been used in numerous studies, one early example being the Ca^{2+} sensor Yellow Cameleon [143]. We utilized this protein construct as a basis for our construction of C-DEVD-Y in paper IV.

4.4.2 Fluorescence microscopy

Epi-fluorescence microscopy [144] is the most commonly used setting for fluorescence microscopy. An epi-fluorescence microscope is configured to excite and detect fluorescence through the same objective, and the light from the excitation light path is separated from light to the emitted light path by a wavelength-specific dichroic mirror.

Additional filters are normally used both in the excitation and in the emission light paths in order to excite and detect only the specific fluorophore and to reduce non-specific background signal.

The term 'widefield microscope' refers to an epi-fluorescence microscope with simultaneous illumination of the whole sample where the detected fluorescence signal includes both in-focus and out-of-focus information. The dynamic experiments with fura-2 (in papers I, II, III and V) and Rh123 (in papers II and III), as well as some experiments with C-DEVD-Y (in paper IV) were performed on widefield microscopes using either PMT detectors or CCD camera for fluorescence detection.

4.4.3 Confocal and two-photon laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is another modality of fluorescence microscopy, where one single point of the specimen is illuminated at a time and out-of-focus signal is rejected by a pinhole in front of the detector. The specimen is scanned point-by-point and the fluorescence image is reconstructed in the computer, which results in a confocal fluorescence image. The word confocal means that all points have the same focus. CLSM was used in paper III.

Two-photon laser scanning microscopy (TPLSM) is yet another modality of fluorescence microscopy, which also results in confocal images [145-147]. This modality is based on the concept of two-photon excitation that was first described in 1931 [148] and verified experimentally in 1963. In normal fluorescence excitation, as used with CLSM, one photon is absorbed to excite one fluorophore molecule from a ground state to an excited state which thereafter emits one photon when the molecule relaxes down to the ground state. In two-photon excitation, a near-simultaneous absorption of two low-energy photons will result in excitation by the combined energy of these two photons. Based on this principle TPLSM was invented in 1990 [145]. Two-photon excitation has a quadratic dependence of absorption in light intensity which limits excitation to a small focal volume, thus avoiding excitation of out-of-focus structures. This means that all fluorescence emission will originate from the focal plane and that photobleaching and phototoxic effects will be limited to the focal plane. One advantage of TPLSM is the use of low-energy, long-wavelength, photons that can penetrate deeper into tissues thanks to lower absorbance and less scatter at these wavelengths. This is a main reason why TPLSM is now widely used as a modality for *in vivo* fluorescence imaging. TPLSM was used in paper IV.

4.4.4 Fluorescence-activated cell sorting

Cytometry is the measurement of physical/chemical characteristics of cells or other biological particles. Flow cytometry measures characteristics of cells in a flow system which delivers cells, one by one, past a point of measurement in an instrument called flow cytometer [149; 150]. These values can be used in order to define what cell that should be sorted or not, in a fluorescence-activated cell sorter (FACS) [151; 152]. We used a FACS system equipped with 5 lasers and 16 PMT detectors in total, including fluorescence, forward scatter and side scatter detectors. FACS was used in paper V.

5 RESULTS AND DISCUSSION

5.1 DEVELOPMENT AND EVALUATION OF A METHOD FOR ON-LINE MONITORING OF INTRACELLULAR ATP (PAPER I)

Dynamics of glucose metabolism play an essential role in the stimulus-secretion coupling of the pancreatic β-cell [153]. One important metabolic parameter required for insulin release is ATP. As a consequence, the intracellular ATP concentration was expected to change dynamically in the β-cells. In order to investigate this we wanted to monitor ATP dynamics in β-cells in response to metabolic stimuli. One may note that at the time when we performed this work, no other investigator had reported real-time dynamic monitoring of ATP in intact insulin-producing cells. A report of our early work in this context was published in 1995 [154].

A well-established and sensitive way to detect the ATP concentration is to use the protein luciferase to catalyze a reaction that generates luminescence using ATP, D-luciferin and oxygen as substrates. When ATP is the limiting factor, the intensity of light generated will be proportional to the ATP concentration.

With the aim to monitor intracellular ATP in single insulin-producing cells, we therefore set to express the luciferase protein in an insulin-producing cell line named HIT M2.2. A number of challenges had to be solved in order to accomplish this:

1. A plasmid had to be introduced into the cells in order to express luciferase.
2. A sensitive method to monitor single-cell luminescence had to be established.
3. A procedure had to be established that alters the cellular ATP such that we could detect a luminescence response.
4. We had to demonstrate that the detected response correlated with an alternative readout method that also reflects dynamic ATP changes.

First: In order to transiently express luciferase we tried to transfect the HIT M2.2 cells with the plasmid pRSVL in different ways including the methods of calcium phosphate precipitation, Lipofectamin transfection and electroporation. Despite the massive cell death caused by the electroporation procedure, we found this method most useful because it produced the highest and most reproducible luciferase expression in the HIT cells that survived the procedure.

Second: A microscope, which was primarily configured for epi-fluorescence, was used to detect luciferase luminescence in single cells or in clusters of cells. The signal from luciferase bioluminescence is some orders of magnitude lower than a normal fluorescence signal. Therefore we made a cooling device for the PMT detector in order to reduce the dark count background signal from ~ 800 cps (counts per second) down to ~ 3 cps. This was essential because the luminescence signal detected from the cells could be as low as a few tens of cps.

Third: The HIT M2.2 cells did not respond well to glucose stimulation, possibly due to a predominant low K_m hexokinase I glucose phosphorylation and defective glucose

transport [155]. Therefore we used a non-physiological method to alter intracellular ATP in these cells, namely mitochondrial blockers that are expected to lower the intracellular ATP concentration. In a first series of experiments we used sodium azide (NaN_3), but after performing a control titration experiment on cell extracts we concluded that azide had a direct effect on the luciferase luminescence at the concentration used in our experiments. A titration with FCCP showed that this compound did not affect the luciferase reaction in the preferred range of concentrations. Therefore we used FCCP, a mitochondrial uncoupler that resulted in a clear ATP-dependent decrease in the luminescent signal.

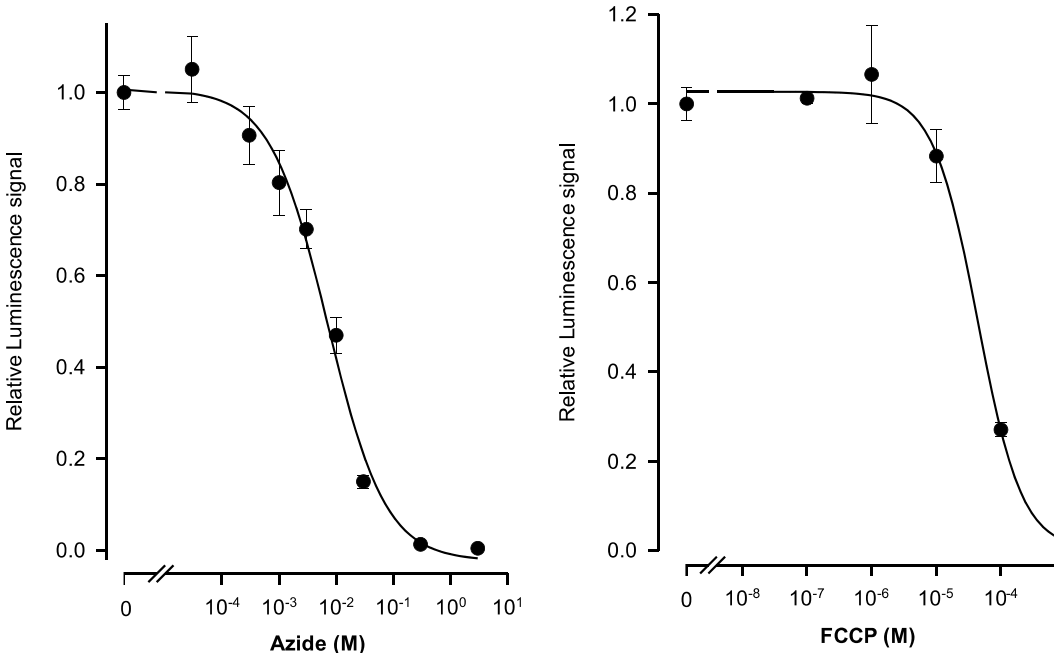

Figure 2. **Effect of sodium azide and FCCP on luciferase luminescence.**

Fourth: We wanted to use an alternative method to sense dynamic changes of intracellular ATP, in order to verify the method. Since the K_{ATP} channel closes in response to an increased intracellular ATP concentration, we could use single channel K^+ currents as an alternative sensor for ATP. The responses of the K_{ATP} channel was therefore compared with the dynamics of luminescence signal in response to the same inhibition of ATP production by 1 μM FCCP.

In order to demonstrate this method in primary cells, we expressed luciferase in mouse islets. In these islets we could clearly detect increased luminescence signal in response to 20 mM glucose, thereby confirming that this method can be used to detect dynamics of ATP in response to physiological stimuli in primary pancreatic islets. This experiment also demonstrated that we could not only detect a decrease in ATP, in response to FCCP, but also an increase in ATP as we showed after glucose stimulation.

Other investigators have now published a number of studies on ATP dynamics in living pancreatic β-cells [156-158], as well as pancreatic acinar cells [159], using bioluminescence. Also an ATP sensitive fluorescent biosensor has been developed [160] that has been used in β-cell studies [60; 161].

5.2 CHARACTERIZATION OF ISLET FUNCTION IN A MOUSE WITH MITOCHONDRIAL DIABETES (PAPER II)

Mitochondrial dysfunction in pancreatic β-cells is an important factor that can cause or contribute to diseases like DM [23]. This is illustrated by the growing list of specific mtDNA point mutations that have been associated with DM [162-164]. In order to study this we used a mouse model that was generated by my co-authors with a pancreatic β-cell specific disruption in the mitochondrial respiratory chain. This mouse model had a tissue-specific mutation of the nuclear gene encoding for mitochondrial transcription factor A (TFAM). This leads to a β-cell specific severe depletion of mitochondrial DNA (mtDNA) that results in deficient expression of proteins encoded by mtDNA, including essential subunits within the respiratory chain complexes I, III, IV and V. This was confirmed by enzyme histochemical double staining for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities. The staining showed that COX (complex IV) activity was deficient in mouse islets with mtDNA depletion as expected, since essential subunits of COX are encoded by mtDNA. In contrast, the SDH (complex II) activity appeared normal as expected, since this entire complex is encoded by nuclear DNA. Abnormally appearing mitochondria could also be observed with electron microscopy in these cells, which is a typical observation in tissues with severe respiratory chain dysfunction. Histochemical analyses showed a β-cell loss over time, which was still normal in 7-week old mutant mice but severe in 33-39-week old mutant mice.

Consistent with these characteristics, the mutant mouse showed an age-dependent diabetic phenotype beginning from approximately the age of 5 weeks and progressing up to older age, with decreased blood insulin concentrations and impaired glucose tolerance. However, from 20 weeks of age the animals partly recover from the diabetic phenotype as seen from the improved non-fasting glucose concentration and the increased non-fasting insulin concentration. This recovery appears to correlate with an expansion of β-cell mass, most likely originating from normal β-cells that did not express *cre* and therefore escaped the mutation, as seen by the mosaic pattern of normally appearing β-cells.

Based on these findings, we concluded that 7-8-week old mutant mice had normal β-cell mass but these β-cells appeared to be dysfunctional. Therefore we performed three types of experiments to assess β-cell function in islets isolated from this age group, in comparison to islets from littermate control mice:

1. MMP in response to addition of glucose.
2. $[Ca^{2+}]_i$ in response to addition of glucose.
3. Insulin release in response to addition of glucose.

These three types of experiments represent major sequential steps in the mechanism of glucose-stimulated insulin secretion, namely glucose metabolism, $[Ca^{2+}]_i$ responses and insulin release. Addition of glucose in these experiments was performed as an increase from 3 mM to 11 mM glucose.

First: MMP changes in response to addition of glucose were monitored with the fluorescent indicator Rh123 loaded to intact single islets. Rh123 fluorescence was plotted relative to the baseline signal prior to glucose stimulation and thereby the relative responses were visualized. We found decreased MMP polarization in mutant islets in response to glucose, an expected consequence of respiratory chain dysfunction.

Second: Changes in $[Ca^{2+}]_i$ were monitored in response to addition of glucose. The observed kinetics in mutant islets was slower and amplitude was lower for the initial response. An oscillatory pattern was observed in control islets, consistent with our observations in other normal mouse islets. These oscillations were almost completely absent in mutant islets, indicating a disturbed islet function. However, the response to stimulation with K^+ appeared similar in control and mutant islets, indicating that $[Ca^{2+}]_i$ increase in response to depolarization was not disturbed. These observations are consistent with a disturbed mitochondrial function.

Third: Dynamic insulin release was measured in islet perifusion experiments both in response to addition of glucose and to arginine. Insulin release from mutant islets was lower, both under basal and stimulatory conditions, even if the total insulin content was not significantly altered in the mutant islets. This suggests that respiratory chain dysfunction is associated with dysfunctional insulin exocytosis. Stimulation with 20 mM of the cationic amino acid arginine leads to sustained depolarization due to its positive charge [165-167] and therefore triggers insulin release from the total releasable pool of insulin granules. Arginine stimulation also resulted in lower insulin release from mutant islets.

This study thus confirms that a β-cell specific mitochondrial respiratory chain dysfunction is associated with both a diabetes-like phenotype and disturbed β-cell mitochondrial function, $[Ca^{2+}]_i$ responses and insulin release. In a recent study, a similar phenotype was described in a mouse with β-cell specific loss of the mitochondrial transcription factor TFB1M, which was identified as a T2DM risk gene [168].

5.3 STUDY OF INTERRELATION BETWEEN INTRACELLULAR Ca^{2+} AND MITOCHONDRIAL MEMBRANE POTENTIAL (PAPER III)

The two parameters mitochondrial metabolism and $[Ca^{2+}]_i$ have been shown to be closely interrelated [55-57]. We wanted to investigate this phenomenon in mouse pancreatic β-cells, using simultaneous detection of MMP and $[Ca^{2+}]_i$.

To accomplish this we first had to establish the method using Rh123 for MMP detection and Fura-2 for $[Ca^{2+}]_i$ detection at the same time, even if both of these indicators had already been used separately for experiments in β-cells. We had to solve the following questions to establish the method:

- Loading of Rh123.
  Loading cells with Rh123 concentrations below 8 μM resulted in cellular and mitochondrial uptake of the dye, but the dye did not report changes in MMP under this condition. Therefore we used either 16 μM or 32 μM for the remaining experiments.
- Possible toxic effects of Rh123.
  Cells loaded with Rh123 need to have an intact response to glucose under the conditions used. Therefore we performed experiments to confirm that the glucose-induced membrane potential response was normal in cells loaded with 32 μM Rh123.
- Rh123 signal sensitivity to the plasma membrane potential.
  In order to rule out that a minor part of the Rh123 signal changes originated from changes in the plasma membrane potential, we performed a control experiment. For this purpose we blocked MMP responses by FCCP and clamped membrane potential applying the perforated whole-cell configuration of the patch-clamp method.
- Simultaneous detection with Rh123 and Fura-2.
  Spectrally Rh123 and Fura-2 is a suitable combination of fluorophores because they are quite well separated by their excitation wavelengths whereas they have well overlapping emission spectra. However, we had to compensate for a minor spectral bleed-through from Rh123 in order to monitor the true Fura-2 signal.

Thereafter we applied this method to study the dynamic glucose-stimulated response in the mouse pancreatic β-cell. In β-cells, both MMP and $[Ca^{2+}]_i$ had been studied separately by other groups prior to our experiments. Glucose stimulation leads to glucose entry into the cell and formation of pyruvate via the glycolytic pathway. Pyruvate enters to mitochondria and activates Krebs cycle which increases respiration and polarizes MMP. This elevates the ATP/ADP ratio that closes K_{ATP} channels which leads to depolarization of the cell and influx of Ca^{2+} through voltage-gated Ca^{2+} channels.

The main question in paper III is how MMP changes during glucose stimulation and how MMP is modulated by dynamic changes in $[Ca^{2+}]_i$. As already described in section 2.4, there are some mechanisms whereby $[Ca^{2+}]_i$ can modulate mitochondrial metabolism. Out of these, the following two mechanisms appear to play significant roles in our study.

Mechanism 1: Ca^{2+} activates dehydrogenases in the Krebs cycle leading to increased respiration and MMP polarization [71].

Mechanism 2: MMP becomes more positive (depolarizes) in response to an increased influx of Ca^{2+} into mitochondria [68-70].

We observed the following sequence of events for changes in MMP and $[Ca^{2+}]_i$ in response to glucose, which mainly agrees with other studies:

- Increased respiration in response to glucose leads to MMP polarization. One may note that omission of extracellular Ca^{2+} resulted in a slower polarization of MMP, possibly due to less activation of mitochondrial dehydrogenases, i.e. mechanism 1.
- Decrease in $[Ca^{2+}]_i$ that is a result of increased ATP availability from respiration that drives removal of $[Ca^{2+}]_i$ [169; 170]. This decrease is initiated on average 6.5 ± 2.4 s after MMP starts to polarize. MMP continues to polarize during this phase.
- Increase of $[Ca^{2+}]_i$ that is due to influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. This increase was followed by a slight depolarization of MMP, possibly due to mechanism 2.
- When $[Ca^{2+}]_i$ oscillates in the presence of 10 mM glucose we observe a slight MMP depolarization subsequent to every $[Ca^{2+}]_i$ peak. That effect may also be attributed to mechanism 2.
- Addition of non-metabolic $[Ca^{2+}]_i$-increasing stimuli at 10 mM glucose give the same result, namely a MMP depolarization. We believe that this observation can also be attributed to mechanism 2.
- At low glucose, the same type of addition of non-metabolic $[Ca^{2+}]_i$-increasing stimuli result in MMP polarization. We believe that this observation can be attributed to mechanism 1.

When we forced the cell to move from an oscillating $[Ca^{2+}]_i$ pattern into a high sustained level of $[Ca^{2+}]_i$, this resulted in the cessation of MMP oscillations. This is consistent with a model of oscillations dependent on a feedback-loop where $[Ca^{2+}]_i$ causes temporary inhibition of mitochondrial metabolism.

Later publications confirm a strong link between mitochondrial function and Ca^{2+} handling in pancreatic β-cells [58; 60; 171]. Dynamic measurements of NAD(P)H oscillations combined with $[Ca^{2+}]_i$ and MMP dynamics in islets [172] fits with the conclusions in paper III. The link between $[Ca^{2+}]_i$ and metabolic oscillations in β-cells was also discussed and applied in mathematical models [173-175].

5.4 DEVELOPMENT AND EVALUATION OF A METHOD FOR ON-LINE MONITORING OF APOPTOSIS (PAPER IV)

Apoptotic β-cell death plays an important role in the development of T1DM and also during the progression of T2DM [88]. Toxic agents that play an important role in the pathogenesis of T1DM are known to induce apoptosis, where the execution step is carried out by caspase-3-like proteases. Therefore monitoring of caspase-3-like protease activation is of major interest, in particular with microscopy in real time both *in vitro* but also *in vivo* to resolve apoptosis with single cell spatial resolution and with sufficient temporal resolution. The aim of paper IV was to develop a fluorescent probe that eventually could be used *in vivo*.

Caspase-3-like proteases selectively cleave the amino acid sequence DEVD in target proteins like PARP1 [94]. This DEVD-specific proteolytic action can also be used as a sensor for selective detection of caspase-3 activity, as shown in some articles prior to our work [176; 177]. Our study demonstrated this type of technique for the first time in insulin-producing cells. In addition, we showed that this can be monitored with TPLSM, which is the preferred microscope modality for experiments when fluorescence should be visualized in deep tissues with single-cell resolution. Therefore our study paves the way to perform on-line single-cell monitoring of apoptosis *in vivo*.

We created a new fusion protein called C-DEVD-Y where C is short for ECFP and Y is short for EYFP. The DEVD sequence was placed in the middle of a 24-amino acid string that linked ECFP with EYFP. When C-DEVD-Y was intact, FRET between ECFP and EYFP was clearly detectable. Cleavage of DEVD resulted in separation of ECFP and EYFP, and thereby irreversible loss of FRET. When using a control construct where DEVD was replaced by the sequence KEAF, the construct remained intact and FRET was not lost during our experimental conditions. A modified variant, called C-DEVD-Y2, was created at a later stage of the project. Y2 denotes another brighter version of YPF, called Venus. Expression of C-DEVD-Y2 was driven by a β-cell specific promoter, the rat insulin-2 promoter, such that it could be used for selective β-cell expression in islets and tissues.

First my co-authors made experiments to justify the use of caspase-3-like proteolytic activity as a marker for pancreatic β-cell apoptosis. In these experiments they could confirm that β-cell apoptosis induced by high glucose, cytokines or staurosporine was inhibited by an inhibitor for caspase-3-like proteases, called Z-DEVD-fmk [178-180].

Main experiments for apoptosis on-line detection in this study were performed with RINm5F cells transiently transfected with plasmids for expression of C-DEVD-Y, C-KEAF-Y or C-DEVD-Y2. The initial temporal fluorescence characterization of these constructs was performed on single cells detected with microfluorometry, after stimulation with 6 μM staurosporine. Thereafter the same type of experiment was performed with TPLSM in order to demonstrate that apoptosis can be monitored in multiple cells with single cell resolution.

Our experiments show that time from staurosporine addition to activation of caspase-3-like proteases varies a lot, ranging from a few minutes up to almost 400 minutes. This protease activation results in loss of FRET within just a few minutes, reflecting a rapid apoptotic execution signal. Thereafter cells rounded up, died and detached from their substrate, but time between loss of FRET and time for detachment appears to be less variable. This indicates that cell death after caspase-3-like protease activation follows a predestined irreversible route, but this may of course be different *in vivo*. Fortunately this technique has the potential to be applied also *in vivo*.

Other investigators have used similar techniques for on-line detection of apoptosis both in mammalian cells [181-183] and in plant cells [184]. It was demonstrated that FRET loss and MMP depolarization occur in parallel [183]. A recent study has reported the use of light sheet fluorescence microscopy to monitor apoptosis in 3-dimensional cell

cultures [185] using a similar FRET probe. Caspase-3/7 activation was also detected *in vivo* with a bioluminescence technique based on DEVD proteolysis [186].

5.5 DEVELOPMENT, OPTIMIZATION AND EVALUATION OF A PROCEDURE TO SORT FUNCTIONAL HUMAN AND RAT PANCREATIC ALPHA CELLS (PAPER V)

In order to assess function of one specific cell-type rather than the complex combination of cells in an intact islet, there is an ultimate need for methods to enrich specific islet cells. FACS is a method that has been used since mid-1980th to separate β-cells from α-cells and from other islet cells in rat islet cell suspensions [187]. The sorting criteria to separate cell types have been based mainly on measured parameters like scatter and autofluorescence. We used intrinsic cellular parameters because we wanted to have non-labelled cells suitable for experimental work following the sorting procedure. These measured parameters reflect for example cell size, granularity and metabolic status. It turns out that at least some islet cell types can be separated based on these parameters, but their characteristics depend on the species and on environmental conditions like buffer and temperature.

Cell-type-specific separation of rat islet cells is more straightforward than the same kind of separation of human islet cells. This is partly due to the size difference between α-cells and β-cells in rat [187; 188], whereas human α-cells and β-cells have a more similar size [189], meaning that cell size cannot be used alone as a parameter for separation of human islet cells. In this study we primarily wanted to enrich α-cells from both rat and human islets with FACS and characterize the α-cell purity, viability and function after enrichment. These parameters should all be maximized, and therefore our first goal was to renew and optimize the quality of islet cell preparation and the sorting procedure for rat islet cells. We also wanted to enrich β-cells that could be used for comparison.

The first goal was to identify optimal sorting criteria, a new gating strategy for sorting and to generate best yield and purity of viable α-cells. In this process we started with all available excitation and emission combinations that we had in our FACS-system, and aimed to identify all cell populations that appear to be separable based on the plots. Thereafter cells from these populations were gated, sorted and characterized for their cell-type identity by PCR and/or immunostaining. The results were used to refine the sorting gates and then perform another round of sorting and characterization, a process that was iterated several times. The final iteration resulted in a new gating strategy for rat α-cells that is unique but also shares details with previously published strategies for rat islet α-cell sorting [187; 190-194]. However, we could achieve a high purity of α-cells by using one single step of sorting instead of two, an improvement that resulted in higher yield and better quality of α-cells after sorting. One explanation for this improvement is that we used a combination of more parameters for the gating compared with previous attempts. We used the four parameters side scatter (SSC),

forward scatter (FSC), FAD fluorescence and NAD(P)H fluorescence for the gating strategies as shown in paper V.

We had to make yet another main improvement of our protocol before reaching the goal of well functional rat α-cells, namely to change the type of culture medium from SMEM to Improved MEM Zn^{++} Option (Richter's Modification). The latter medium has been reported to be used in some other studies for culture of rat pancreatic cells [195], but the explanation for the functional improvement in comparison with SMEM is mainly unknown.

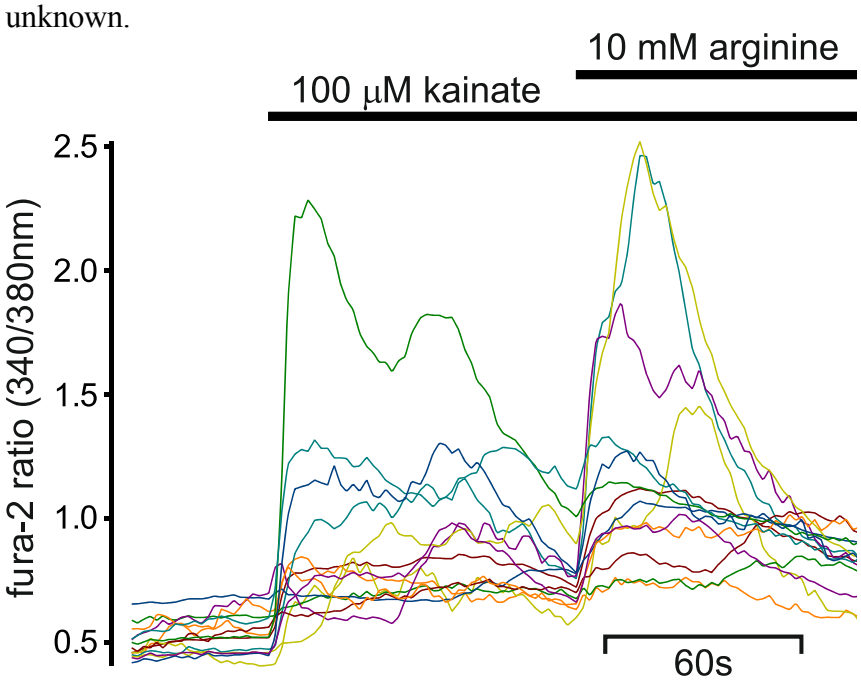

Figure 3. **$[Ca^{2+}]_i$ responses in human sorted α-cells stimulated with kainate and arginine.**

The same kind of procedure, with iterative refinement of sorting gates, was successfully used to find a novel gating strategy for enrichment of human α-cells. We managed to get a quite high purity of human α-cells after sorting. But human islet quality and function is highly variable between batches and donors that may partly explain why the human α-cell enrichment gives a quite low and variable yield of cells. Therefore this procedure is mainly useful to prepare cells for experiments based on low numbers of human α-cells.

We assessed the enriched α-cell and β-cell purity, viability and function in a series of experiments. Our results confirm a good purity and viability after sorting. Kainate stimulation led more frequently to $[Ca^{2+}]_i$ responses in human α-cells than in rat α-cells, whereas arginine stimulated most of the α-cells from both species. Increasing $[Ca^{2+}]_i$ in response to arginine is expected because of its membrane depolarizing effect. The response to kainate in human α-cells is consistent with earlier studies [13], and a reported low number of dispersed rat islet cells responding to kainate [196] is consistent with our observation that only a subpopulation of rat α-cells responded to kainate. However, further studies are required to fully explain why.

5.6 FUTURE PERSPECTIVES

The results in papers I-V all represent valuable progress in the fields of diabetes research and cell biology. In a future perspective these results and techniques, in combination with other studies, build a knowledge-base that we and others will use.

The ultimate goal will be to understand normal physiology and pathology of islet cells in the intact living organism, *in vivo*. We have developed a technique for non-invasive optical *in vivo* microscopy on islets transplanted and engrafted in the anterior chamber of the eye using the cornea as a natural body window [197; 198]. In this setting we can monitor the *in vivo* function of islet cells that mirrors the normal function of islets *in situ* in the pancreas [199]. We will continue to develop this *in vivo* technique and implement measurements of [Ca^{2+}] in cytoplasm and other cellular locations as well as measurements of parameters associated with mitochondrial metabolism, like in papers I-III. One example of this is our recently published paper [200] where we measure NAD(P)H and FAD fluorescence *in vivo* using TPLSM. We will also study cell death *in vivo*, as a continuation of paper IV. FACS sorting and analysis will also be used to both enrich cell populations for further experiments and for end-point analysis in various experiment.

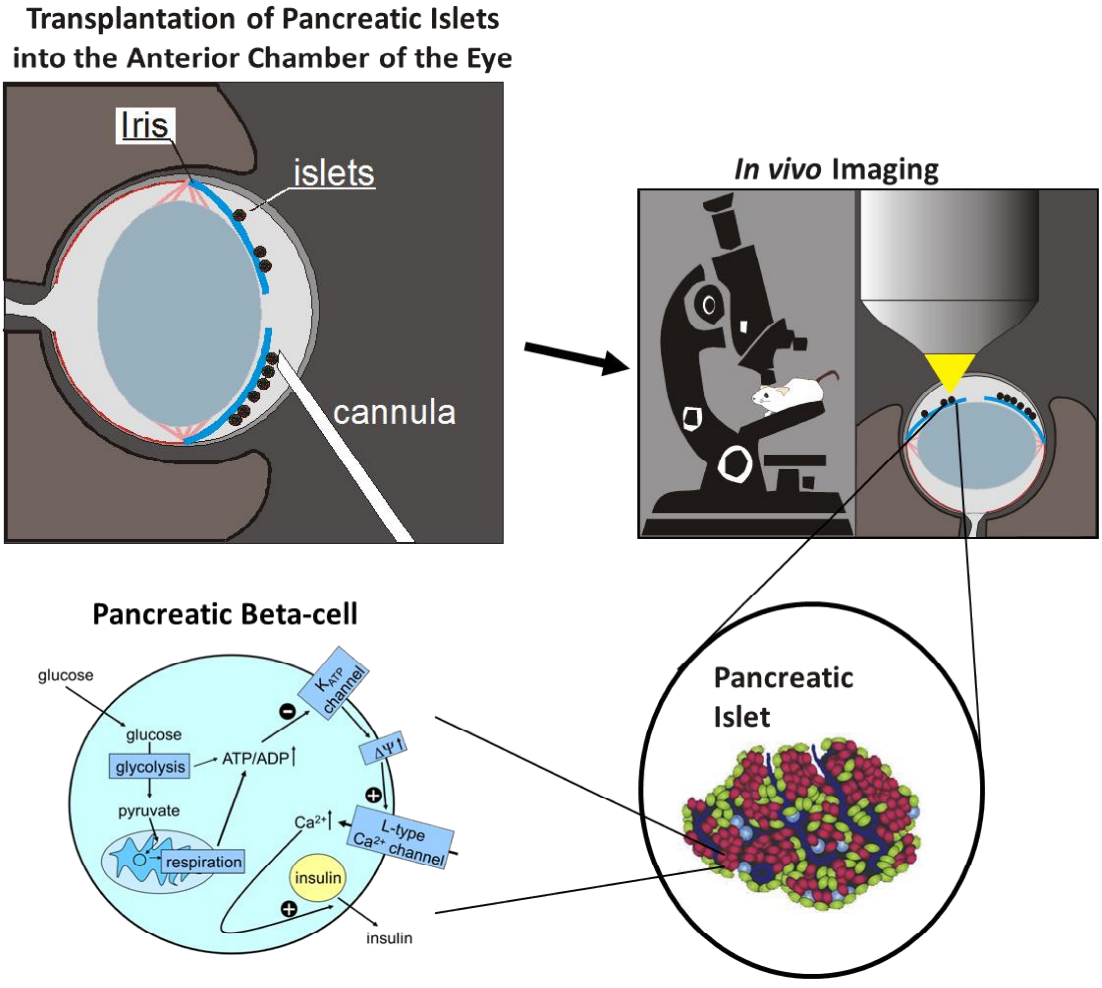

Figure 4. **Non-invasive *in vivo* microscopy to monitor islets transplanted to the anterior chamber of the eye.**

6 CONCLUDING REMARKS

1. Dynamic changes in intracellular ATP were monitored in single insulin-producing cells using luciferase luminescence as a biosensor. This worked both to monitor a decrease in ATP in HIT M2.2 cells in response to a mitochondrial uncoupler as well as an increase in ATP in mouse islets in response to glucose stimulation. However, the technique has a limited temporal resolution due to the limited output level of photons. The level for detection of small ATP changes may also be limited due to the low signal to noise ratio.
2. The stimulus-secretion coupling was dysfunctional in pancreatic islets from a mutant mouse model with β-cell specific depletion of mtDNA and consequently disruption of the mitochondrial respiratory chain. Islets showed an impaired response to glucose when monitored dynamically. Readouts for this dysfunction were MMP, $[Ca^{2+}]_i$ and insulin release. This dysfunctional pattern can be expected in mitochondrial diabetes.
3. MMP and $[Ca^{2+}]_i$ were measured simultaneously in mouse β-cells in response to glucose and in response to non-metabolic $[Ca^{2+}]_i$-increasing stimuli. We observed effects consistent with Ca^{2+}-dependent activation of mitochondrial dehydrogenases under low glucose conditions with depolarized MMP and low prevailing $[Ca^{2+}]_i$. In contrast, under high glucose conditions where MMP is polarized at high prevailing $[Ca^{2+}]_i$ levels we observed effects consistent with MMP depolarization due to Ca^{2+}-influx into mitochondria. During glucose-induced slow $[Ca^{2+}]_i$ oscillations MMP was depolarized subsequent to the $[Ca^{2+}]_i$ peaks, showing that MMP follows $[Ca^{2+}]_i$ under these conditions.
4. Apoptosis could be monitored on-line in single insulin-producing cells using a FRET-based biosensor sensitive to activation of caspase-3-like proteolytic activity. The loss of FRET appeared as a sign of sudden controlled cell-death activation in an individual cell. The FRET signal could be detected with TPLSM, which suggests that this technique can be successfully applied for *in vivo* imaging.
5. New procedures were developed to sort rat and human pancreatic α-cells with FACS, using a one-step sorting protocol. Parameters of purity, viability and function of the sorted cells were investigated after sorting. We could confirm a high purity and viability of α-cells as well as good $[Ca^{2+}]_i$ responses to arginine as an indication of basic function. Kainate stimulation more frequently led to responses in human α-cells as compared to rat α-cells.
6. Overall, the results in this thesis contribute to the understanding of metabolism and $[Ca^{2+}]_i$ in the function and survival of pancreatic β-cells. The techniques that were developed or refined are at the present successfully used *in vitro* by us and other investigators. We are optimistic that the actual techniques will also be highly applicable for *in vivo* experiments within the near future. Cell-type-specific cell-sorting of functional cells will continue to be a valuable complementary tool.

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