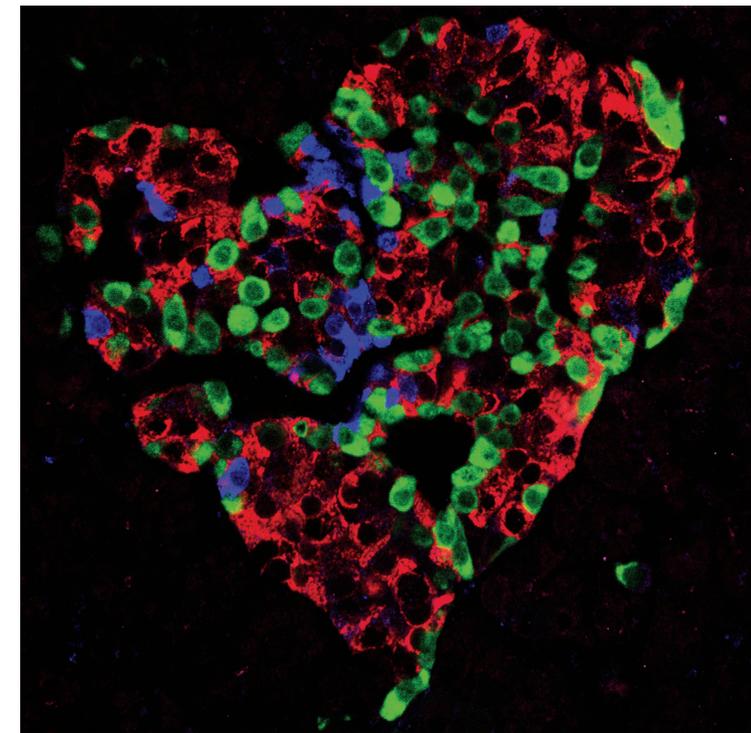


Thesis for doctoral degree (Ph.D.)
2007

AUTOCRINE/PARACRINE INTERACTIONS MODULATING HORMONE RELEASE IN THE ENDOCRINE PANCREAS



Over Cabrera

Thesis for doctoral degree (Ph.D.) 2007

Autocrine/paracrine Interactions Modulating Hormone Release In The Endocrine Pancreas
Over Cabrera



**Karolinska
Institutet**



**Karolinska
Institutet**

COVER

A section of a normal human pancreas showing an islet of Langerhans with three endocrine cells immune-labeled; α cells (green), β cells (red), and δ cells (blue).



Karolinska Institutet

**From the Rolf Luft Research Center for Diabetes and
Endocrinology**

Department of Molecular Medicine and Surgery

Karolinska Institutet, Stockholm, Sweden

Autocrine/paracrine interactions modulating hormone release in the endocrine pancreas

AKADEMISK AVHANDLING

som för avläggande av medicine doktorexamen vid Karolinska
Institutet offentlig försvaras i Astrid Lindgren/Skandiasalen,
Building Q3:01, Solna.

Onsdag den 28 november 2007, kl 09.00

av

Over Cabrera

MSc

Huvudhandledare:

Prof. Per-Olof Berggren,
Karolinska Institutet
Institutionen för molekylär medicin

Bihandledare:

Doc. Ingo Leibiger
Karolinska Institutet
Institutionen för molekylär medicin

Fakultetsopponent:

Prof. Piero Marchetti
University of Pisa
Faculty of Medicine
Department of Endocrinology and
Metabolism, Italy

Betygsnämnd:

Doc. Stefan Sjöberg
Karolinska Institutet
Institutionen för medicin Huddinge

Doc. Peter Bergsten

Uppsala Universitet
Institutionen för medicinsk cellbiologi

Prof. Gunnar Norstedt

Karolinska Institutet
Institutionen för molekylär medicin

Stockholm 2007

ABSTRACT

Human islet transplantation is emerging as an alternative to pancreas transplantation or insulin therapy in the treatment of type I diabetes. It has been possible to achieve euglycemia by transplanting isolated human islets in patients who suffer from the long term side effects of diabetes and insulin therapy. However, the islet transplantation procedure is still experimental. To obtain FDA approval, each batch of islets will have to be labeled with its potency to meet the transplantation criteria. Consequently, finding an *in vitro* test that predicts the quality of the islets prior to transplantation will require a better understanding of the human islet physiology.

In this thesis we report on the generation of an *in vitro* perfusion machine with high throughput capabilities which integrates with other commercial high content screening systems. These technologies were successfully applied to measure $[Ca^{2+}]_i$ and hormone release from batches of damaged islets with decreased viability, and it was possible to differentiate those islets from their undamaged counterparts. Pharmacological profiling of individual batches of islet cells proved feasible, measuring both $[Ca^{2+}]_i$ and hormone release. Moreover, we showed that the hormone release assay can be used to distinguish batches of human islets from healthy donors and donors with type II diabetes.

Our data on human islets revealed a cytoarchitecture that differs from that of other animal models used to study islet physiology and diabetes. In the human islet, all endocrine cells are intermingled throughout the islets without the mantle-core segregation observed in the rodent islet. The endocrine cells are facing blood vessels and they appear without a specific pattern. Additionally, $[Ca^{2+}]_i$ handling in human islets is different from that in mouse islets. While the entire human islet does not show oscillations in $[Ca^{2+}]_i$, as described for the mouse islet, single human β cells show oscillations in $[Ca^{2+}]_i$ that resemble those found in mouse.

Our data showed that glutamate is a potent stimulus for glucagon secretion but not for insulin secretion. While glutamate induced increases in $[Ca^{2+}]_i$ in α cells through activation of the voltage gated Ca^{2+} channels, it does not cause any change in $[Ca^{2+}]_i$ in β cells. We provided evidences that α cells express the machinery needed for glutamatergic signaling. We propose that glutamate released from the α cell activates the glutamate receptors in the α cell plasma membrane to allow more Ca^{2+} into the α cells and further increase glucagon release.

Finally, we demonstrated that ATP exerts different effects in human and in rodent islets. In human islets, ATP potentiated insulin release, at basal and at high glucose concentration, but it did not do so in mouse, rat, or pig islets. This potentiation most likely occurs by activation of purinergic receptors of the P2X₃ type located in the β cell plasma membrane. Upon activation, these receptors become permeable to Ca^{2+} , allowing an influx of this ion into the β cell cytoplasm, which stimulates further insulin release. Hence, ATP serves as an autocrine signal that forms a positive feedback loop stimulating insulin release in a glucose independent manner.

The suggestion that both α and β cells utilize positive feedback loops to potentiate the secretion of their respective hormones might indicate that glucose alone is insufficient to achieve adequate glucagon and insulin release from the α and β cells and that other additional autocrine/paracrine signals are required to achieve fine-tuned exocytosis.

From the Rolf Luft Research Center for Diabetes and Endocrinology
Karolinska Institutet, Stockholm, Sweden

**AUTOCRINE/PARACRINE
INTERACTIONS MODULATING
HORMONE RELEASE IN THE
ENDOCRINE PANCREAS**

Over Cabrera



**Karolinska
Institutet**

Stockholm 2007

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

© Over Cabrera, 2007

ISBN 978-91-7357-378-8

Printed by



www.reproprint.se

Gårdsvägen 4, 169 70 Solna

ABSTRACT

Human islet transplantation is emerging as an alternative to pancreas transplantation or insulin therapy in the treatment of type I diabetes. It has been possible to achieve euglycemia by transplanting isolated human islets in patients who suffer from the long term side effects of diabetes and insulin therapy. However, the islet transplantation procedure is still experimental. To obtain FDA approval, each batch of islets will have to be labeled with its potency to meet the transplantation criteria. Consequently, finding an *in vitro* test that predicts the quality of the islets prior to transplantation will require a better understanding of the human islet physiology.

In this thesis we report on the generation of an *in vitro* perfusion machine with high throughput capabilities which integrates with other commercial high content screening systems. These technologies were successfully applied to measure $[Ca^{2+}]_i$ and hormone release from batches of damaged islets with decreased viability, and it was possible to differentiate those islets from their undamaged counterparts. Pharmacological profiling of individual batches of islet cells proved feasible, measuring both $[Ca^{2+}]_i$ and hormone release. Moreover, we showed that the hormone release assay can be used to distinguish batches of human islets from healthy donors and donors with type II diabetes.

Our data on human islets revealed a cytoarchitecture that differs from that of other animal models used to study islet physiology and diabetes. In the human islet, all endocrine cells are intermingled throughout the islets without the mantle-core segregation observed in the rodent islet. The endocrine cells are facing blood vessels and they appear without a specific pattern. Additionally, $[Ca^{2+}]_i$ handling in human islets is different from that in mouse islets. While the entire human islet does not show oscillations in $[Ca^{2+}]_i$ as described for the mouse islet, single human β cells show oscillations in $[Ca^{2+}]_i$ that resemble those found in mouse.

Our data showed that glutamate is a potent stimulus for glucagon secretion but not for insulin secretion. While glutamate induced increases in $[Ca^{2+}]_i$ in α cells through activation of the voltage gated Ca^{2+} channels, it does not cause any change in $[Ca^{2+}]_i$ in β cells. We provided evidences that α cells express the machinery needed for glutamatergic signaling. We propose that glutamate released from the α cell activates the glutamate receptors in the α cell plasma membrane to allow more Ca^{2+} into the α cells and, as a consequence, a further increase in glucagon release.

Finally, we demonstrated that ATP exerts different effects in human and in rodent islets. In human islets, ATP potentiated insulin release, at basal and at high glucose concentration, but it did not do so in mouse, rat, or pig islets. This potentiation most likely occurs by activation of purinergic receptors of the P2X₃ type located in the β cell plasma membrane. Upon activation, these receptors become permeable to Ca^{2+} , allowing an influx of this ion into the β cell cytoplasm, which stimulates further insulin release. Hence, ATP serves as an autocrine signal that forms a positive feedback loop stimulating insulin release in a glucose independent manner.

The suggestion that both α and β cells utilize positive feedback loops to potentiate the secretion of their respective hormones might indicate that glucose alone is insufficient to achieve adequate glucagon and insulin release from the α and β cells and that other additional autocrine/paracrine signals are required to achieve fine-tuned exocytosis.

LIST OF PUBLICATIONS

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

- I. **Over Cabrera**, M. Caroline Jacques-Silva, Dora M. Berman, Alberto Fachado, Felipe Echeverri, Ramon E. Poo, Aisha Khan, Norma S. Kenyon, Camillo Ricordi, Per-Olof Berggren, Alejandro Caicedo (2007). Automated, High-Throughput Assays for Evaluation of Human Pancreatic Islet Cell Function. *Accepted for publication in Cell Transplantation*.
- II. **Over Cabrera**, Dora M. Berman, Norma S. Kenyon, Camillo Ricordi, Per-Olof Berggren, and Alejandro Caicedo (2006). The Unique Cytoarchitecture of Human Pancreatic Islets has Implications for Islet Cell Function. *Proc Natl Acad Sci U S A. 103(7):2334-9*.
- III. **Over Cabrera**, M. Caroline Jacques-Silva, Martin Köhler, Dora M. Berman, Norma S. Kenyon, Camillo Ricordi, Alejandro Caicedo, Per-Olof Berggren (2007). Glutamate is an Autocrine Signal Essential for Glucagon Release. *Submitted*.
- IV. M. Caroline Jacques-Silva, **Over Cabrera**, Natalia Makeeva, Dora Berman, Norma S. Kenyon, Camillo Ricordi, Antonello Pileggi, Damaris Molano, Per-Olof Berggren, Alejandro Caicedo (2007). ATP is a Positive Autocrine Signal in the Human Pancreatic Beta Cell. *Submitted*.

Table of Contents

1	<i>Introduction</i>	1
2	<i>Aims</i>	6
3	<i>Materials and methods</i>	7
3.1	Pancreas procurement	7
3.2	Immunofluorescence.....	7
3.3	Islets isolation	8
3.4	Islets culture and cell culture	8
3.5	Preparation of islet cells	9
3.6	Assay Buffer	9
3.7	Islets perfusion	9
3.8	Quantification of Hormones	10
3.9	[Ca ²⁺] _i	10
3.10	Microfluorometric determination of glutamate.....	10
3.11	Real time determination of glucagon release with biosensor cells	10
3.12	RT-PCR	11
3.13	Single cell RT-PCR	11
3.14	Statistical analysis	11
4	<i>Results and discussion</i>	12
4.1	Paper I	12
4.1.1	Automated, High-Throughput Assays for Evaluation of Human Pancreatic Islet Cell Function	12
4.2	Paper II	15
4.2.1	The Unique Cytoarchitecture of Human Pancreatic Islets has Implications for Islet Cell Function	15
4.3	Paper III	18
4.3.1	Glutamate is an Autocrine Signal Essential for Glucagon Release	18
4.4	Paper IV	21
4.4.1	ATP is a Positive Autocrine Signal in the Human Pancreatic beta-Cell.....	21
5	<i>Conclusions</i>	23
6	<i>Acknowledgments</i>	24
7	<i>References</i>	27

LIST OF ABBREVIATIONS

$\Delta[\text{Ca}^{2+}]_i$	Variations in cytoplasmic free calcium concentration
$[\text{Ca}^{2+}]_i$	Cytoplasmic free calcium concentrations
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
cGMP	current Good Manufacturing Practice
CNG	Cyclic Nucleotide-Gated
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DAPI	4 ,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DRI	Diabetes Research Institute
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HBSS	Hanks' Balanced Salt Solution
HCS	High Content Screening
HT	High Throughput
ITS	Insulin-Transferrin-Selenium
PS	Penicillin-Streptomycin
VGCC	Voltage Gated Calcium Channels
vGluT1	Vesicular Glutamate Transporter 1
vGluT3	Vesicular Glutamate Transporter 3

1 INTRODUCTION

Glucose homeostasis is achieved by the interplay of hormones secreted by the endocrine pancreas, the islets of Langerhans (hereafter “islets”) [1]. Within these islets, four different types of endocrine cells specialize in producing a specific hormone, α cells glucagon, β cells insulin, δ cells somatostatin, and PP cells pancreatic polypeptide. Insulin and glucagon directly influence glucose homeostasis, while somatostatin and pancreatic polypeptide have modulatory functions [2-7]. After a carbohydrate-rich meal, β cells secrete insulin that facilitates glucose uptake by muscle, liver, and adipose tissue, thereby reducing glucose concentration in blood. If glucose concentration decreases, α cells secrete glucagon, which activates glycogenolysis and gluconeogenesis, increasing glucose concentration in blood. In this way, the action of glucagon and insulin on their respective receptors maintains a post-absorptive blood glucose concentration of $5 \pm 1\text{mM}$ [8]. Failure to restore this blood glucose level after a meal causes a disease known as diabetes.

Based on the etiology of the disease, diabetes has been classified into two types. Type I diabetes is an autoimmune disease occurring very often at an early age characterized by nearly complete destruction of the β cells by the immune system, and a present, yet functionally impaired α cell mass. Type II diabetes most often occurs at a more advanced age and is characterized by a deficient insulin release and/or insulin action in response to glucose. Type I diabetes is treated with insulin administration. Type II diabetes is treated with a combination of life style changes, drugs that stimulate insulin secretion, and insulin administration or a combination of the above therapies in severe cases. Because the physiological range of glucose in blood is very narrow, proper glycemic control in type I diabetes using insulin therapy is challenging at this time. Achieving euglycemia over long periods of time requires an aggressive insulin therapy. This, coupled with the impaired functionality of the α cells in type I diabetes, may lead to a decrease in blood glucose levels and perhaps even a life threatening condition known as hypoglycemia. Understanding the signals that regulate glucagon secretion from the α cell is of paramount importance for diabetes management and is a major focus for this thesis.

An alternative therapy for type I diabetes is islet transplantation. The procedure involves the isolation of islets from the pancreas from a donor and their infusion into the diabetic patient. Islets are normally infused into the portal vein of the liver, but

alternative sites are being investigated. To its credit, islet transplantation has the advantage of achieving a regulated glycemic control, without the long term complications arising from aggressive insulin therapy. The issue as to whether islet transplantation also solves hypoglycemia is still contested [9-12]. Nevertheless, when compared to insulin therapy alone, transplanted patients achieve better glucose homeostasis and overall hormonal control. This also holds true when combined islet transplantation and insulin therapy is required [13, 14]. The benefits of islet transplantation warrant the study of human islets physiology and human islets transplantation (I). Many aspects of islet physiology are already known, but the majority of the studies have been conducted in rodent or other animal model systems. It is imperative to reassess in human islet what has been learned from animal model systems, as shown in this thesis (II).

Traditionally, the study of biological systems, including islet cell physiology, has been undertaken using specific approaches. Individual characteristics of the system are studied separately, and the information gathered about the system is integrated to build models. Recent technological advances have made it possible to approach complex biological systems from a more multi-parametrical point of view. High throughput (HT) and high content screening (HCS) technologies are revolutionizing experimental biology [15-20]. Instead of collecting information from a single cell or studying a single process at a time, HT and HCS allow researchers to acquire information from many cells and to monitor multiple cellular processes simultaneously with very high temporal resolution. Consequently, a larger amount of information about the object under study is generated in a shorter period of time which is an advantage to traditional methods. Additionally, since the processes are studied simultaneously, their interacting components tend to be preserved. Consequently, the HT-HCS approach dramatically increases the overall amount of knowledge and accuracy of this knowledge about the biological system in question. Technologies to measure $[Ca^{2+}]_i$ have been developed for the HT and HCS setup, and fully self sufficient systems for such a purpose are commercially available [21]. $[Ca^{2+}]_i$ influences a variety of intracellular processes and $\Delta[Ca^{2+}]_i$ is used as an indicator of the functionality of the cell. In the islets, $[Ca^{2+}]_i$ acts as a second messenger for hormone release [22]. In this thesis, a perfusion system was built to include HT and automation capabilities, and progress was made in the implementation of a methodology to study $\Delta[Ca^{2+}]_i$ of islets cells in a HCS setup (I).

As discussed above, the physiological blood glucose concentration varies very little. Therefore, in order to understand how to achieve glucose homeostasis, it is crucial to comprehend how these relative little variations in blood glucose concentration are able to induce a response at the β or α cell level, respectively, to restore euglycemia from a high or low blood glucose state. The molecular mechanisms by which this may happen have been studied, but the explanations offered appear to be contradictory and difficult to reconcile. Nutrients or glucose alone have been proposed as a sufficient signal on α and β cells alike to achieve islets functionality [23-25]. Factors of neuronal origin are also thought to be the master signal controlling hormone secretion in the islets [26-29]. Additionally, autocrine/paracrine interactions inside the islets have been proposed as another way of signaling to achieve glucose homeostasis [30-34]. The fact that α and β cells are always clustered together seems to indicate that there is a functional relationship between them. There are architectural differences between rodent and primate islets, but α and β cells are always together with δ and PP cells forming islets (II, and Fig. A). If nutrients alone and mainly glucose were able to provide the necessary and sufficient signaling for glucose homeostasis, individual islets cells might be scattered throughout the pancreas or somewhere else with sufficient blood supply. Additionally, how can one explain that glucose will have an opposing effect in α and β cells if both cell types are equipped with similar machinery to import and metabolize glucose? [24, 35]. The answer to this may indicate that low glucose concentration is not an optimal signal to induce glucagon release from the α cell and that other signals are needed to amplify the release of this hormone. It may also be the case that β cells are influenced by autocrine [36], and paracrine [37] feedback loops that further amplify the effect of glucose on them. Insulin has been suggested to form a positive feedback loop, which further stimulates β cells to release more insulin [38-41]. In such systems where a weak signal functions as a trigger, feedback mechanisms amplify the original stimuli to reach full functionality. The data presented in this thesis suggests that the insulin and glucagon stimulus-secretion-coupling pathways are both equipped with a positive feedback mechanism to amplify hormone release (III, IV). Within the physiological range of glucose concentrations, it is suggested that the combination of glucose as a metabolic fuel and autocrine/paracrine interactions as negative/positive feedback signals is what achieves glucose homeostasis (Fig. B).

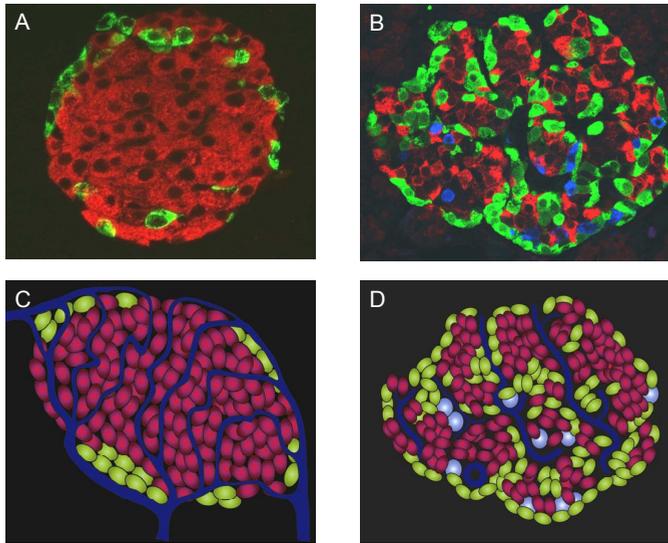


Figure A. The cytoarchitecture of the human islet differs from that of the mouse islet.

(A) In mouse islets, α cells (green) are segregated to the periphery surrounding the β cells (red).

(B) In human islets, all the endocrine cells are intermingled. α cells (green), β cells (red), and δ cells (blue).

(C) Functional model to explain autocrine/paracrine interactions in the mouse islet. This model assumes arterial blood enters the islet without having contact or exchange with α or δ cells, but with β cells. A consequence of this model is that the secretion products of the β cells are the only ones that can influence hormone secretion in α and δ cells. Blood vessels (blue), α cells (green), β cells (red), and δ cells (not shown).

(D) Functional model of the human islet. In the human islet, all endocrine cells can be found in close proximity to the same blood vessel, without a pattern. Therefore, in this model, a cell type is equally likely to influence hormone release in another endocrine cell type, including itself. Blood vessels (blue), α cells (green), β cells (red), and δ cells (cyan).

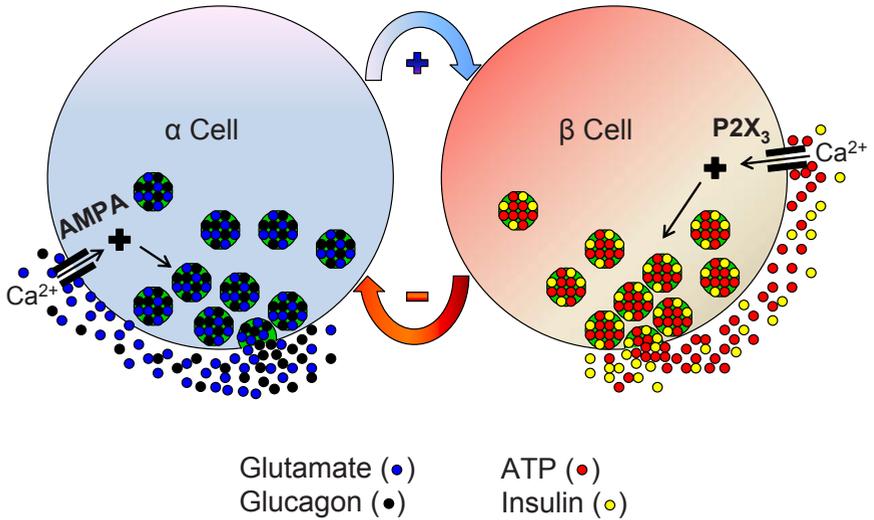


Figure B. Autocrine interactions modulate hormone release in α and β cells.

Glutamate, which is co-released with glucagon from the α cell, activates the AMPA/Kainate receptors on the α cell plasma membrane to form a positive feedback loop that potentiates glucagon secretion. The activation of these receptors leads to membrane depolarization and Ca^{2+} influx through VGCC, which stimulates further glucagon release. ATP activates the purinergic receptors making them permeable to Ca^{2+} and as a result an influx, of this ion that stimulates the β cell to secrete additional insulin.

2 AIMS

1. Utilize HT and HCS technologies to study the physiology of islets prior to transplantation and assess their potency.
2. Compare the cytoarchitecture of human islets with islets from animals used as model systems and analyze the implication of this cytoarchitecture in the context of human islet physiology.
3. Identify the signaling role of glutamate as molecular stimulus acting on α cells to increase glucagon secretion in human islets.
4. Identify the signaling role of ATP as molecular stimulus acting on β cells to increase insulin secretion in human islets.

3 MATERIALS AND METHODS

3.1 PANCREAS PROCUREMENT

Human pancreata were obtained from multi-organ donors (<http://www.aopo.org/aopo/>). Monkey pancreata were obtained from ≥ 4 year old male cynomolgus monkeys (*Macacca fascicularis*) from a local facility (Mannheimer Foundation; Homestead, FL), and Charles River Laboratories, Inc. (<http://www.criver.com>). Pig pancreata were procured from >1 year old male pigs from a local slaughter house (Cabrera's slaughter house, Miami, FL). Rat pancreata were obtained from 10-12 weeks Lewis rats (<http://www.harlan.com>).

Mouse pancreata were obtained from 10-15 weeks old male C57Bl/6J mice from Jackson Laboratories (<http://www.jax.org>).

3.2 IMMUNOFLOURESCENCE

Pieces of pancreatic tissue of about 0.5 cm^3 were fixed in Bouin's fixative solution (<http://www.sigmaaldrich.com>) for 4 to 6 h, dehydrated in 70 % ethanol, and embedded in paraffin. Tissue for detecting vesicular glutamate transporters was fixed in 4 % paraformaldehyde (<http://www.emsdiasum.com>) for 4 h, and islet cells were fixed in 4% paraformaldehyde for 10 min. Fixed tissues were sliced into $5 \mu\text{m}$ sections on a microtome, air dried overnight, deparaffinized, and rehydrated in HBSS. After a rinse in OptiMax Wash Buffer (<http://biogenex.com>), sections were incubated in Universal Blocker Reagent (<http://biogenex.com>) for 5 to 10 minutes, rinsed again three times in OptiMax Wash Buffer, and incubated in Protein Block (<http://biogenex.com>) for 20 minutes or longer. Thereafter, sections were incubated overnight with the primary antibodies diluted in Common Antibody Diluent (<http://biogenex.com>) (table 1).

Table 1. Antibodies.

Species	Antigen	Dilution	Source website
Chicken	Insulin	1:500	http://www.accuratechemical.com
Mouse	Glucagon	1:2000	http://www.sigmaaldrich.com
Sheep	Somatostatin	1:500	http://www.ab-direct.com
Rabbit	Pancreatic polypeptide	1:100	http://www.ab-direct.com
Mouse	CD34	1:500	http://biogenex.com
Mouse	Smooth muscle actin	1:2000	http://www.sigmaaldrich.com
Rabbit	vGluT1, vGluT3	1:20000	http://millipore.com

Blood vessels were labeled with a cocktail of anti- CD34 and anti-smooth muscle actin antibodies. All secondary antibodies were conjugated with Alexa Fluor[®] fluorophores (<http://www.invitrogen.com>) and used at a dilution of 1:400 or 1:500. Cell nuclei were stained with DAPI and ProLong Anti Fade was used as a mounting medium (<http://www.invitrogen.com>). Normal serum of the species used to raise the antibody was used as a negative control.

3.3 ISLETS ISOLATION

Human islets were isolated at the cGMP Human Islet Cell Processing Facility of the Cell Transplant Center of the DRI at the Miller School of Medicine, University of Miami. The pancreata were cold preserved in University of Wisconsin solution [42]. Islets were isolated using a modification of the automated method, [43] applying seven different lots of the enzyme Liberase HI (<http://www.roche.com>) and a standard purification step, as described previously [43].

Monkey, pig, rat, and mouse islet isolations were performed using modifications of the automated method for human islet isolation [43], adapted for isolation of monkey islets [44] and mouse islets [45].

3.4 ISLETS CULTURE AND CELL CULTURE

Islets and islet cells from all species were cultured under the same environmental condition (37°C and 5% CO₂) in CMRL-1066 medium from Invitrogen (<http://www.invitrogen.com>) supplemented with: niacinamide (10 mM) and Zn₂SO₄

(15 μ M) from Sigma (<http://www.sigmaaldrich.com>), ITS from BD Biosciences (<http://bd.com/us>), and Glutamax, Sodium Pyruvate, HEPES (25 mM), FBS (10%), and PS from Invitrogen.

HEK293H-CNG cells were grown in DMEM medium supplemented with FBS (10%) from Invitrogen, Puromycin from BD Biosciences, and G418 sulfate from Mediatech (<http://www.cellgro.com>).

3.5 PREPARATION OF ISLET CELLS

Islets were washed three times with HBSS without Ca^{2+} and Mg^{2+} , and once with Versene 1:5000, and then suspended in enzyme free Cell Dissociation Buffer (<http://www.invitrogen.com>). The suspension was gently aspirated several times with 1ml pipette to assist the dissociation of the islets into single cells. Sample aliquots were observed intermittently with a microscope to follow the dissociation process. When about 80-90% of the cells appeared as single cells, the dissociation was stopped and culture medium was added to wash away the chelating agents.

3.6 ASSAY BUFFER

All *in vitro* assays were performed in a HEPES-buffered solution containing (mM: 125 NaCl, 5.9 KCl, 2.56 CaCl_2 , 1 MgCl_2 , 25 HEPES), 0.1% BSA, and pH adjusted to 7.4. The basal glucose concentration was adjusted to 3mM.

3.7 ISLETS PERIFUSION

Islets from all species were perfused using the perfusion system V2.0.0 built for the purpose (<http://biorep.com>). A low pulsatility peristaltic pump from Ismatec (<http://www.ismatec.com>) pushed the assay buffer (containing the stimuli) through a sample container harboring 100 islets immobilized in Bio-Gel P-4 Gel from BioRad (<http://biorad.com>). The perfusate fractions were collected every minute (or one minute and half when somatostatin needed to be measured) in a 96 well plate kept below 4°C to preserve the integrity of the analytes. The sample containers and the perfusing solution were kept in a built in temperature controlled chamber at 37°C.

3.8 QUANTIFICATION OF HORMONES

Hormone concentrations in the perfusates were determined with the human, rat or mouse Endocrine LINCoplex Kits from Millipore (<http://www.millipore.com>), and the Bio-Plex protein array system (<http://biorad.com>).

3.9 $[Ca^{2+}]_i$

Imaging with perfusion was performed in an imaging workstation from Andor Bioimaging (<http://www.andor.com>) equipped with the following basic components: Light source (Cairn Research Optoscan Monochromator, <http://www.cairnweb.com>), image capture with a Hamamatsu Orca-ER2 camera (<http://www.hamamatsu.com>), an Axiovert 200M microscope from Carl Zeiss (<http://www.zeiss.com>), and the IQ imaging software Ver. 6.0.3.62 from Andor Bioimaging. Imaging without perfusion was performed in the BD Pathway 855 Bioimager [46]. Islets were loaded with 2 μ M of Fura-2AM (<http://www.invitrogen.com>) for 1h (or 30 minutes for dispersed islet cells) at 37°C in assay buffer. Stimuli were applied with the perfusing solution or with the built in pipette on the BD Pathway 855 Bioimager. In both systems, the sample was incubated in an environmentally controlled chamber and recordings were done at 37°C. $[Ca^{2+}]_i$ was determined as the fluorescence ratio (340/380) after the excitation of Fura-2 at 340 and 380 nm.

3.10 MICROFLUOROMETRIC DETERMINATION OF GLUTAMATE

Glutamate release from pancreatic islets was measured with the Amplex Red Glutamic Acid Assay Kit (<http://www.invitrogen.com>) adapted for microscopic microfluorometric detection format [47]. Islets were allowed to attach to a glass coverslip previously coated with poly-D-lysine. The coverslip was mounted in a 35 μ l RC-20 imaging chamber (<http://www.warneronline.com>, and perfused with assay buffer containing the stimuli. Resorufin was excited at 510 nm, and its emission was recorded at 590 nm, using the imaging workstation from Andor Bioimaging.

3.11 REAL TIME DETERMINATION OF GLUCAGON RELEASE WITH BIOSENSOR CELLS

HEK293H-CNG cells [48] from BD Biosciences (<http://bd.com/us>) were used to measure glucagon release in real time. These cells were grown on a coverslip and loaded with 2 μ M of Fura-2 AM for 30 minutes at 37°C. Thereafter, the remaining

extracellular Fura-2 AM was washed off with assay buffer. The coverslip was mounted in a 35 μ l RC-20 imaging chamber, and islets were placed on top of the Fura-2AM loaded cells. $[Ca^{2+}]_i$ was measured in the imaging workstation from Andor Bioimaging. The perfusion was stopped after the addition of the stimulus to allow the islet secretory products to accumulate and was resumed after 10 minutes.

3.12 RT-PCR

Total RNA from >90% pure human and monkey islets was isolated using the RNA Easy kit from Qiagen (<http://www1.qiagen.com>). Total mRNA (500 ng) was reverse transcribed using the SuperScript™ First-Strand Synthesis System from Invitrogen. Two micro liters of cDNA was used without further purification for a 35 cycles PCR reaction in the LightCycler and Roche amplification kit. Primers for human AMPA/Kainate (GluR1-7, Ka1 and KA2) and vesicular glutamate transporters (vGluT1-3) were purchased from Qiagen.

3.13 SINGLE CELL RT-PCR

Dispersed human or monkey islet cells were harvested using glass micropipettes (~20 μ m) and placed in centrifuge tubes. Total mRNA was reverse transcribed using the SuperScript First Strand Synthesis System from Invitrogen. Real time PCR was performed using the TaqMan Fast Universal PCR Master Mix and the 7500 or 7900HT Fast Real-Time PCR Systems from Applied Biosystems (<http://www.appliedbiosystems.com>). The TaqMan assays were chosen to span an exon junction and, therefore, they did not detect genomic DNA.

3.14 STATISTICAL ANALYSIS

For statistical comparisons in paper II and IV, one way ANOVA followed by Bonferroni multiple comparisons test was used. In paper III, student's t-test or one-way ANOVA, followed by multiple comparisons procedures with the Student-Newman-Keuls method was used.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Automated, High-Throughput Assays for Evaluation of Human Pancreatic Islet Cell Function

One of the challenges of islet research is the assessment of islet quality or potency that takes place post isolation and prior to transplantation. There are several factors that make it difficult to find a test for islet quality assessment. For practical purposes, the factors affecting the quality of islets can be divided into two categories. First, those that are inherent in the biology of the system, i.e. the substantial differences in lifestyle, age, and genetic makeup of different pancreas donors. Many pancreata are recovered from patients that suffered severe trauma and were under intensive drug treatment, and these factors may have a toxic effect on the endocrine pancreas. Second, those factors which are associated with our knowledge about the physiology of the endocrine pancreas, or our lack of understanding of which factors affect the viability of the islet endocrine cells and the factors that regulate hormone secretion from these cells. The factors in the first category are difficult to control and therefore this paper focuses on the factors in the second category, whose contribution to islets potency can be investigated under controlled laboratory research conditions.

Islets are micro-organs with several types of cells, each of which specializes in secreting a specific hormone. It is the interaction of these hormones which achieves glucose homeostasis, rather than the action of any one of them. In this paper, a multi-parametric approach is used to develop a methodology to study islets cell physiology with the objective of finding a test to assess islet quality prior to transplantation.

From the same batch of islets, $\Delta[\text{Ca}^{2+}]_i$ was measured as a marker of islet health. The pharmacological profile of each batch of islets was identified using glucose, kainate, ATP, and acetylcholine. Additionally, the dynamic profile of insulin, glucagon, and somatostatin release was obtained for the following stimuli: changes in glucose concentration, several purinergic and adrenergic receptor agonists, and KCl stimulation.

To test whether the amplitude of $\Delta[\text{Ca}^{2+}]_i$, and insulin, glucagon and somatostatin release could be used to detect differences in islet responses, batches of islets were incubated in an anoxic environment or cultured in the presence of Staurosporine. Both of these conditions induce cell death and impair the function of islet cells [49-52]. Incubation of the islets with Staurosporine for 2 h was enough to

reduce $\Delta[\text{Ca}^{2+}]_i$ amplitude after stimulation with high glucose. Untreated islet cells were classified as highly responsive, responsive, and non responsive according to $\Delta[\text{Ca}^{2+}]_i$ amplitude subsequent to stimulation with high glucose. In Staurosporine treated islet cells, there were no highly responsive cells, but instead the number of responsive cells decreased, and the number of non responsive cells increased. It is difficult to identify the specific pathway that has been damaged because Staurosporine is a very broad protein kinase inhibitor. Rather, it is expected that several key steps in the stimulus-secretion coupling pathway were compromised, i.e. mitochondrial function. Moreover, it will be more physiologically relevant to conduct these experiments using intact islets and not dissociated islets cells, but the HT-HCS technology and software for such experiments are yet to be developed. However, disregarding the specific mechanism by which $[\text{Ca}^{2+}]_i$ has been impaired, this study indicates that $\Delta[\text{Ca}^{2+}]_i$ can be used as a marker to measure islet cells function.

Another experiment with the same islet cells cultured in Staurosporine was conducted, and it was observed that the amount of insulin and glucagon release decreased proportionally in relation to the concentration of Staurosporine. Similarly, when the islets were incubated under anoxic conditions, it was also noted that the amount of insulin released decreased proportionally to the length of the incubation time. In this case, the amount of insulin released was compared with that of the untreated control islets. In addition, the amount of insulin released from the treated islets was also compared with the averaged insulin released from many untreated islet batches. In both, untreated control and averaged insulin release of many untreated islet batches, the amount of insulin release was higher than in the islets incubated under anoxic conditions. In the treated islets only a fraction of the original capacity to release insulin was preserved. This is because the amount of insulin release is proportional to the number of islets in the sample containers and in all cases the same number of islets was perfused.

It was determined that α , but not β cells, responded with an increase in $[\text{Ca}^{2+}]_i$ following kainate stimulation. Similarly, it was found that all islet cells responded with an increase in $[\text{Ca}^{2+}]_i$ following stimulation with ATP and acetylcholine. Insulin, somatostatin, and glucagon release were increased by application of purinergic and adrenergic receptor agonists. Conversely, glucagon release, but not insulin release, was largely increased by application of kainate. Pharmacological profiling of islets and islet cells will allow the identification of key signaling events in the stimulus-secretion

coupling pathway that may be damaged in a specific batch of islets, either due to a genetic predisposition or damage before and during islets isolation.

The amount of insulin released, in response to stimulation with high glucose and KCl, decreased proportionally with the progression of type II diabetes. This preliminary result might indicate that the perfusion assay is sensitive enough to detect batches of islets with decreased function, e.g. a batch of islets isolated from an undiagnosed type II diabetic donor.

Whether the *in vitro* output of the above physiological parameters correlates with the outcome of the transplantation remains to be tested. Their prediction capacity can be tested assessing aliquot of islets that will eventually be transplanted into human diabetic patients. A model using other animals, e.g. nude mice, might be inconclusive. Mice have different dynamics in glucose homeostasis, and human insulin is less potent in mice than their own insulin. Non-human primates may be an alternative. The *in vitro* glucose-stimulated insulin release profile in islets from non-human primates is indistinguishable from that of the human islets, but substantially differs from other model species such as mouse, rat, and pigs.

4.2 PAPER II

4.2.1 The Unique Cytoarchitecture of Human Pancreatic Islets has Implications for Islet Cell Function

Islets are small micro-organs which are scattered, but not randomly [53] distributed throughout the pancreas, and variations in their size and cell composition have been reported [54]. Islet cell distribution or islet cell architecture in rodents have been studied in detail. [54, 55]. It is established that islet cells in rodent are segregated [56]. In this model system, the β cells occupy the center of the islets and α , δ , and PP cells occupy the periphery of the islets [57]. This segregation has been used as a basis to hypothesize that cell to cell communication is important for islet function [55, 56, 58-62]. It has been demonstrated that islet cellular architecture is altered in diabetes [63-65], and that a disruption of β cell communication also impairs insulin secretion [66]. The model of segregated islet cells explains why the direction of the blood flow is important in the resultant amount of hormones released from the islets [67]. It was suggested, neutralizing specific hormones with antibodies, that insulin can control the release of the other islet endocrine hormones because the blood flow goes from the core (β rich area) to the mantle (α , β , and PP rich area) [68-71]. Overall, the segregated islet model implies that the direction of the blood flow will dictate what kind of endocrine interactions can be established in the islets, and therefore will regulate hormone release. This paper demonstrates that human islets do not have the segregated islet cell architecture that rodent islets do. Moreover, it shows ample variability in islet cell architecture among species used as model systems to study islet physiology and pathophysiology. It also establishes the relationship between endocrine cells and blood vessels and provides evidence that the architecture of the human islets predisposes them for different types of interaction than those observed in rodent islets.

Here, islet cell composition and islet architecture were compared for mouse, pig, non human primate, and human. In all of these species, β cells are still a majority, but the proportion β/α cells is less in non human primates and humans than in rodent islets. In all of these species, δ and PP cells constitute a minority. Endocrine cells in non human primates and humans were not segregated as in mouse islets. All endocrine cells in non human and human islets were intermingled throughout the islets. The typical pig islet forms clusters or subunits, each resembling the mouse islets. Inter species differences in islets cell composition and cytoarchitecture have been reported [72-74]. However, the topic has not gained much attention. This becomes especially

evident while examining very recent textbooks in endocrinology [1], which still depict the rodent islet as the model islet without emphasizing the striking cytoarchitectural differences between the rodent and the human islet. In order to support the islet transplantation field, more efforts need to be allocated to study the human islet or the islet of our closest evolutionary relatives, non human primates.

In order to determine the degree of association or segregation of the human islet cells, the percentage of cells adjacent to cells of the same type (homotypic associations) and the percentage of cells of different types adjacent to each other (heterotypic associations) were calculated. In the mouse islet, most of the β cells form homotypic contacts, contrary to the β cells in the human islet which form mostly heterotypic contacts. This confirms that the mouse β cells are more clustered than the human β cells. It also suggests that paracrine interactions in the human islet may occur more frequently than in the rodent islet. Serial sectioning of human islets demonstrated that the architecture and cell association described above remain the same, regardless of which part of the islet was sampled.

In addition, the association of the islet cells with the blood vessels was investigated. As discussed above and elsewhere [60, 75], islet microcirculation and blood flow is thought to influence islet cells hormone release. It was found that all islet cells, regardless of type, are closely associated with blood vessels, and all of them were aligned along blood vessels in no particular pattern. Many β cells were facing α or δ cells across a blood vessel. This finding strongly suggests that the direction of the blood flow in the human islet may not be as influential as it appears to be in the mouse islet. It is also a reminder that care should be taken when extrapolating the knowledge acquired from studies with rodent islets and applying this knowledge to the human system.

Glucose-stimulated changes in $[Ca^{2+}]_i$ were measured to investigate if the morphological differences described above also imply that the human islet is functionally different from the rodent islet. Mouse, non human primate, and human islets responded to high glucose stimulation with large increases in $[Ca^{2+}]_i$. However, only non human primate and human islets responded with an increase in $[Ca^{2+}]_i$ when the stimulus was a decrease in glucose concentration. No changes in $[Ca^{2+}]_i$ were observed in mouse islets when the glucose concentration was decreased. This indicates that $[Ca^{2+}]_i$ responses from the α cells in the whole mouse islet were probably below our detection limit. In contrast, $[Ca^{2+}]_i$ responses from the larger mass of α cells in non human and human islets is readily detected. Subsequently, the oscillatory behavior of

$[Ca^{2+}]_i$ in mouse and human islets after stimulation with high glucose was investigated. Whereas $[Ca^{2+}]_i$ measured from the whole mouse islets showed the typical oscillations reported in the literature [76-78], $[Ca^{2+}]_i$ measured from the whole non human primate and human islets did not oscillate. It was further investigated whether small regions inside the islets show oscillations in $[Ca^{2+}]_i$ after high glucose stimulation. Small clusters of β cells within the islets showed $[Ca^{2+}]_i$ oscillations resembling those reported in the literature [79-81], but these oscillations were not synchronized, which explains why the whole islet did not respond to high glucose with oscillations in $[Ca^{2+}]_i$ [82]. These results contradict those of a previous report showing $[Ca^{2+}]_i$ oscillations in human islets [83]. The discrepancies are likely a consequence of the technical difficulties in isolating intact human islets. It is possible that the reported $[Ca^{2+}]_i$ oscillations in human islets were obtained recording $[Ca^{2+}]_i$ from fragments of islets or islets with altered physiology. Also, many past studies with human islets have been conducted with islets isolated from pancreatic biopsies of patients suffering from cancer. It might be possible that the physiology of the islets isolated from such biopsies is altered. These issues notwithstanding, it is safe to conclude that the human islet is morphologically different from the rodent islet.

4.3 PAPER III

4.3.1 Glutamate is an Autocrine Signal Essential for Glucagon Release

It was already mentioned above that there is tight control of blood glucose concentration at the organism level. In this context, it is difficult to formulate a hypothesis of how so little variations in blood glucose concentration (about 1mM) will result in the stimulation of β cells to decrease, and α cells to increase blood glucose concentration. A decrease in blood glucose concentration is associated with stimulation of glucagon release, but a causal relationship has not been established. Activation of α cells by decreasing blood glucose concentration seems to be paradoxical because the same α cell needs glucose as an energy source. Therefore, it was hypothesized that under these suboptimal conditions, from the glucose concentration standpoint, the α cell will need a glucose independent positive feedback mechanism to amplify glucagon release. The data presented in this paper suggests that such a positive feedback mechanism exists, and that glutamate released from the α cell forms a positive autocrine feedback loop that stimulates glucagon release.

It was found that application of glutamate as well as other ionotropic glutamate receptor agonists had a profound stimulatory effect on glucagon secretion. This stimulation seems to be Ca^{2+} mediated because glutamate applications also induced an increase in $[\text{Ca}^{2+}]_i$ that is concentration dependent. Glutamate stimulated increases in $[\text{Ca}^{2+}]_i$ and glucagon secretion were both effectively suppressed by CNQX, a specific antagonist of the ionotropic glutamate receptor subtypes AMPA and Kainate. The concentrations of glutamate that stimulate glucagon secretion had no effect on insulin secretion. These data are in disagreement with others in the literature [84]. It was reported that glutamate inhibits glucagon release through the activation of a metabotropic glutamate receptor [85]. Since these studies were conducted using the rat model system, it is possible that species differences are one cause of the disagreement. The disagreement might also stem from technical differences. In this paper, glutamate stimulated glucagon release was detected using a perfusion system which is constantly changing the stimulating solution and washing off any secondary products from islet cells. The conflicting studies that suggested an inhibitory effect of glutamate on glucagon secretion used a static incubation (batch incubation) type of assay. In static incubations, islet cell secondary products accumulate over time with unclear consequences for a particular stimulus. For example, GABA, somatostatin, or Zn^{2+} could accumulate over time in the incubation buffer, creating a condition that might

mask the stimulatory effect of glutamate. Additionally, it is established that ionotropic glutamate receptors desensitize rather quickly [86, 87]. Hence, it is expected that glutamate will have no effect after a few seconds or minutes of application, but the static incubation type of assay will last for many minutes or hours. These results suggest that static incubation is not a good method to study receptor mediated mechanisms.

At the cellular level, it was possible to identify the α cells as those responding with an elevation in $[Ca^{2+}]_i$ after glutamate stimulation. It was found that the cells that responded to a high glucose stimulus do not respond to a glutamate stimulus, and conversely, those that respond to a glutamate stimulus do not respond to a high glucose stimulus. A subsequent labeling by immunofluorescence, revealed that the cells that responded to glutamate with an elevation in $[Ca^{2+}]_i$ also labeled positive for glucagon (α cells), and as expected, those responding with an elevation in $[Ca^{2+}]_i$ in response to high glucose labeled positive for insulin (β cells). As additional evidence for the presence of AMPA/Kainate receptors in α cells, islet cells positive for glucagon mRNA also expressed *gria2* and *3*, as well as *grik2* and *5* mRNAs, subunits of the AMPA and Kainate receptors, respectively.

This was followed by an investigation of the molecular mechanisms(s) by which glutamate stimulated glucagon release. The omission of Ca^{2+} from the assay buffer prevented increases in $[Ca^{2+}]_i$ subsequent to kainate application. This indicated that extracellular Ca^{2+} is required for increases of $[Ca^{2+}]_i$ following glutamate application. This Ca^{2+} enters the α cell through voltage activated Ca^{2+} channels, and part of it through the L-type Ca^{2+} channels. The inclusion of La^{3+} , a blocker of Ca^{2+} channels, in the assay buffer prevented increases in $[Ca^{2+}]_i$ following glutamate application, and the inclusion of nifedipine, a blocker of the L-Type Ca^{2+} channels, also led to substantially reduced increases in $[Ca^{2+}]_i$. The increases in $[Ca^{2+}]_i$ described above are specifically linked to the action of glutamate because CNQX also blocked them. Additionally, this influx of Ca^{2+} into the α cell cytoplasm is required for glucagon release. Removal of Ca^{2+} from the assay buffer prevented kainate induced glucagon secretion, and blocking the Ca^{2+} channels diminished glucagon secretion by 90%.

The effects of glutamate on the α cells, as discussed above, have been measured applying glutamate to the islet or islet cells, but glutamate's effect will only have physiological significance if an endogenous source of glutamate exists in the islet. The expression of vGluT1 was detected by immunofluorescence, and it colocalized with

glucagon immunofluorescence, indicating that the α cells store glutamate in vesicles. In fact, glutamate release subsequent to kainate stimulation of islets was detected. This is a strong indication that the α cells not only store glutamate in vesicles, but they also secrete glutamate in a regulated manner. Additional evidence in support of regulated secretion of glutamate from islets was acquired by detecting glutamate release with biosensor cells (HEK293H-CNG). These cells express the glucagon receptor which, following stimulation by glucagon, increases the intracellular concentration of cAMP, which in turn activates the CNG channels, also expressed by HEK293H-CNG cells. Islets were placed on top of a monolayer of HEK293H-CNG cells, and the application of glutamate as well as a reduction in glucose concentration induced an increase in $[Ca^{2+}]_i$ on the HEK293H-CNG cells. This was a consequence of the activation of the glucagon receptor on these cells by glucagon secreted from the islets. Therefore, glutamate promotes the amplification of glucagon release from the α cell. Because all of the components of the glutamatergic signal reside in the α cell, it is concluded that glutamate, presumably cosecreted with glucagon [88] from the α cells, establishes a positive feedback loop in the α cell to amplify the secretion of glucagon release. This allows the formulation of a model for efficient glucagon release under low glucose conditions. When blood glucose concentration decreases, the release of inhibitors of glucagon secretion from the β cell (Insulin, GABA, Zn, others) and the δ cell (somatostatin) decrease, which allows for an increase in glucagon release along with glutamate, the later feeding back on the glutamate receptors to stimulate more glucagon secretion. If blood glucose concentration increases again, β and δ cell secretion will again inhibit the α cell, and ultimately reduce glucagon secretion. This interplay among the islet cells is what achieves glucose homeostasis. Interestingly, this may also explain instances in type I diabetes where β cells are absent and blood glucagon concentration is higher than normal, despite higher blood glucose.

4.4 PAPER IV

4.4.1 ATP is a Positive Autocrine Signal in the Human Pancreatic beta-Cell

Purinergic receptors are ubiquitously expressed in the membrane of islet cells [89, 90]. ATP is stored in the same vesicles as insulin, and large quantities of it are secreted from the β cell in a regulated manner [91-93]. Once secreted, ATP is quickly hydrolyzed by extracellular ectonucleotidases [90, 94]. These characteristics make ATP a well suited neurotransmitter [95].

When isolated islets are stimulated with ATP, there is a marked influence on the insulin secreted by the islets. However, there is disagreement in the literature as to whether ATP increases or decreases insulin secretion [96-102]. Defining the role of ATP in islets has been very difficult because of the large variety of purinergic receptors, ionotropic and metabotropic, expressed in islet cells [89, 90]. In addition, purinergic receptors may be differentially expressed between rat and mice and also between rat/mice and humans [100, 102, 103], which complicates the translation of the research done in a rodent system to a human system. The fact that ATP hydrolysis will result in adenosine introduces another level of complexity because adenosine, through activation of its receptors on islets cells, can in itself modify islet hormone release [104, 105].

The objective of this paper was to use a variety of *in vitro* functional assays to dissect the role of ATP in modulating insulin release in human islets. It was hypothesized that under physiological conditions glucose alone is not enough to promote sufficient insulin release to achieve glucose homeostasis. Therefore, ATP may act on the purinergic receptors in the β cell membrane, as an autocrine positive signal, to sustain a basal insulin concentration in blood or participate in glucose-induced amplification of insulin release. The interest was to focus on the role of the endogenous ATP secreted during basal and glucose-stimulated insulin release.

Application of ATP to human islets promoted insulin secretion, and the effect was concentration dependent. ATP as low as 1 μ M promoted measurable increases in insulin release, and concentrations of 1mM promoted large increases in insulin release. Concentrations larger than 1mM did not cause further increases in insulin release. Interestingly, the same concentrations of ATP tested above had no effect on insulin release in mouse, rat, or pig islets. It might be that the differential expression of purinergic receptors in different species also translates into functional differences.

Subsequently, the approach was to distinguish whether the stimulating effect of ATP on insulin secretion was mediated by a purinergic or an adenosine receptor. Rodent and human islets were perfused with several purinergic receptor agonists and adenosine. All the purinergic receptor agonists largely stimulated insulin release in human islets, with adenosine having a minor effect. The stimulatory effect of ATP on insulin release was independent of the glucose concentration because it occurred at both, 3 and 11 mM of the sugar. However, neither the purinergic receptor agonists nor adenosine stimulated insulin release in rodent islets. This reinforces the notion that ATP signaling in rodent and human islets is different.

To identify and localize the type of purinergic receptors expressed in human islets, western blot and immunofluorescence were performed. The former established the expression of P2X_{1, 3, and 4} in human islets, and the latter localized P2X₃ to β cells, P2X₄ to α cells, and P2X₁ to δ cells. It was found that all β cells, but only a subpopulation of α cells responded with an increase in $[Ca^{2+}]_i$ to ATP applications. The effect of ATP was dependent on extracellular Ca^{2+} , which appears to enter the cell through the P2X receptor channel.

To investigate if endogenously released ATP influences insulin secretion, the hydrolysis of extracellular ATP was either inhibited or promoted, and the action of ATP on its receptors was blocked. At basal glucose concentration, inhibiting ATP degradation resulted in more insulin release and the contrary was observed when ATP hydrolysis was promoted. Similarly, blocking the action of ATP with purinergic receptor antagonist reduced glucose-induced insulin release. This last effect seems to be mediated by P2X type of receptors because their pharmacological blockade significantly reduced glucose-stimulated insulin release. In contrast, antagonizing the P2Y receptor had no effect on glucose-stimulated insulin release. This indicates that endogenous ATP, coreleased with insulin, activates a subset of the P2X type of purinergic receptors to potentiate glucose-stimulated insulin release.

It is proposed that ATP acts as a positive autocrine signal in β cells to potentiate insulin release. At basal glucose concentration, ATP most likely activates the P2X type of purinergic receptors to maintain a basal level of insulin release, and at stimulatory glucose concentrations potentiates glucose-induced insulin release. This potentiation allows the β cell to translate small variations in glucose concentration into large quantities of insulin being released.

5 CONCLUSIONS

1. It is feasible to study human islet cell physiology in the HT and HCS set up.
2. The *in vitro* hormone release profile and $\Delta[\text{Ca}^{2+}]_i$ can be used to differentiate the health status of human islets.
3. In the human islet:
 - a. All endocrine cells are intermingled throughout the islet without the segregation mantle/core observed in rodent islets.
 - b. Nearly all, if not all, endocrine cells are facing blood vessels and appear without a specific pattern.
 - c. Oscillations in $[\text{Ca}^{2+}]_i$ is limited to individual endocrine cells.
 - d. α -cells express ionotropic glutamate receptors of the AMPA/Kainate type.
 - e. Glutamate activation of AMPA/Kainate receptors stimulates α cells to secrete large quantities of glucagon.
 - f. Glutamate stimulates α cells through membrane depolarization and opening of the voltage gated Ca^{2+} channels, which allow extracellular Ca^{2+} to enter the cell.
 - g. Glutamate-induced mobilization of extracellular Ca^{2+} into the α cell cytoplasm is essential for glucagon release.
 - h. Glutamate signaling in α cells forms an autocrine positive feedback loop to potentiate glucagon release.
4. Extracellular ATP potentiates insulin release in human islets at basal and stimulatory glucose concentration, but has no effect in rodent islets.
5. ATP secreted from β cells, stimulates purinergic receptors in the β cell plasma membrane to form a positive autocrine feedback loop, which sustains insulin release at basal glucose concentration and potentiates insulin release at high glucose concentrations.

6 ACKNOWLEDGMENTS

I feel an enormous pleasure writing the last part of my thesis. First, it allows me to put an end to one of my most challenging endeavors. Also, it gives me the opportunity to thank all of those special people that in way or another have contributed to this effort.

The first group that deserves the greatest amount of love is my family, both in Cuba and the United States. My mother, Caridad Pérez, my father, Ernesto Cabrera, and my brother Osniel Cabrera have always supported my decisions to move forward and, for this, they deserve all of my heart. My wife, Elvira Gonzalez, my eldest daughter, Solange Cabrera, and my youngest daughter, Selene Cabrera have spent many days on the “front lines”, handling affairs at home while I grew to love Stockholm (my home away from home) and, for this, I promise that our family will share much more quality time together.

My uncle, Rene Perez, currently retired just outside of Cuba, was insistent on his desire that I obtain an upper level education. There were many moments throughout my education where I believed I did not measure up to fellow classmates who might have had their education in the city as opposed to my country experience. On many occasions, he provided inspiration which was just enough to allow me to continue. My uncle always led by example and showed me an important concept... every person counts, and every person is useful when they are guided properly.

My grandfather, Jose Cabrera, now deceased was a tremendous influence to me. Although I was just a boy, I will never forget the many times he would prop me on his knee, and later walk alongside me to pass on his little nuggets of inspiration or advice. If you can imagine this, he was speaking to a young Over who grew up in a rural area of Cuba with little, if at all, modern inventions. He was a visionary of sorts. On many occasions, he told me that it was possible for a man to live on a piece of land and nothing else. In his honor, I try to pass on many of his simple teachings to my daughters and am proud to say that they are developing a beautiful understanding of nature, thanks in large part to him.

The second group that I remember with love is a broad selection of personal and professional friends. These are the friends that I consider my family and will always hold dear to me. This esteemed list begins with Silvia Montero, the sister I always wanted who was taken from all of us way too soon, and Joachim Rodríguez de

Miranda, the brother who I cherish. This list also includes my Cuban/Swedish Ambassadors of Peace, Abiel Orrego and Mercedes Rodriguez.

The third group that deserves heartfelt recognition is certain distinguished professors that I have had throughout the years. These include: Maria E. Montero Cabrera, Gabriel Macaya, Benigno Aguirre, Marilyn Roossinck, and Ulrich Melcher. My bond with Marilyn Roossinck is a special one. I am forever grateful for her guidance and support which have been instrumental in my career. Marilyn always trusted me and encouraged me to move forward during decisive times. I continue to be at awe with her selfless personality and her honest dedication to scientific research. I am also grateful to Ulrich Melcher. Despite the fact that I went to his laboratory when it was not ready and will always wish I could have accomplished more there, I will always be grateful for his continued communication throughout the years.

The fourth group that deserves heartfelt recognition is certain supervisors and colleagues that I have had throughout the years, particularly abroad. I express my sincere gratitude to my current supervisors Per-Olof Berggren and Ingo Leibiger. In addition to their scientific guidance, they have made many other important contributions to this thesis and to me as a scientist. Both of them lead by example and I now understand that being a supervisor is much more than enforcing hard work, learning laboratory techniques, and writing a paper. It is also about discussing what is next, it is about life, and it is about looking beyond a thesis or a laboratory technique. P-O has been a great inspiration in teaching me how to bring people together and achieve efficiency.

Through these words, I want to thank all of my colleagues at the lab in Stockholm. Please bear in mind that by mentioning the following, I am in no way excluding any other colleagues that I may have overlooked. Great thanks to Martin Kohler for assisting me with the imaging business and many more topics, Shao-Nian Yang and Stephan Speier for helping with Patch clamp, Daniel Nyqvist and Nancy Wenna for many hours of talking and fruitful discussions, also for being a friend and host, Lisa Juntti-Berggren, Katarina Breitholtz, Christina Bremer, Annika Lindgren, Hannelore Rotter, Essam Refai, Rebecka Holmberg, Gabriela Imreh, Sabine Uhles, Sergei Zaitsev, Irina Zaitseva, Barbara Leibiger, Jenny Johansson, Slavena Mandic, Christopher Barker, Christina Bark, Dominic-Luc Webb, Tilo Moede, Jelena Petrovic, Pilar Vaca Sanchez, Vladimir Sharoyco, Christopher Illies, Jia Yu, Lou-Sheng Li, and Juliette Janson.

I also want to extend a great “thanks a million” to some special persons at the lab in Umea. Ingela Berglun-Dalh, Ulf Dalh, and Helena Edlund. I feel very fortunate to have lab mates and friends like the three of you.

The fifth group that I wish to recognize are colleagues at the Diabetes Research Institute in Miami. Please bear in mind that by mentioning the following, I am in no way excluding any others that I may have overlooked. First and foremost is Dr. Camillo Ricordi. His continued, inspirational passion in the field of diabetes research leaves little room for any of us to “take it easy”. I am extremely grateful for his vision, and also for the leadership of Dr. Norma Kenyon. I also extend a special thank you to my lab mates, Alberto Fachado, Rayner Rodriguez, M. Caroline Jacques-Silva, and special thanks to Alejandro Caicedo for being extremely helpful. My gratitude goes also to Dora Berman-Weinberg, Ramon Poo, Felipe Echeverri, Luca Inverardi, Antonello Pileggi, Mitra Zehtab, Damaris Molano, Margaret Collado, Elizabeth Fenjves, Kevin Johnson, Rodolfo Alejandro, Gloria Allende, Silvia Alvarez, Sabrina Boulazreg, Aisha Khan, Armando Mendez, Alexander Rabassa, Aimee Siegel-Harris, Sofia Ochoa, Ricardo Pastori, and Melina Ribeiro. A special thanks to Carlos Garcia, Mariolga Fernandez, and Mario Valiente.

Many thanks to the Diabetes Research Foundation, and in special to Robert (Bob) Perelman and Gary Kleiman.

Finally, I would like to thank those persons and entities that provided funding for the research presented in this thesis. The NIH grants for General Clinical Research Center MO1RR16587, 1R01-DK55347-IU42RR016603, Islet Cell Resources grant 5U42RR016603, 1R03DK075487-01, Juvenile Diabetes Research Foundation International grant 4-2004-361, Juvenile Diabetes Research Foundation International grant 3-2006-853, The European Foundation for the Study of Diabetes, EuroDia grant FP6-518153, the Diabetes Research Institute Foundation, the Swedish Research Council, Novo Nordisk Foundation, Karolinska Institutet, the Swedish Diabetes Association, the Berth von Kantzow’s Foundation, the Family Erling-Persson Foundation, and the Knut and Alice Wallenberg Foundation.

Kindest thanks to all.

7 REFERENCES

1. DeGroot, L.J. and J.L. Jameson, *Endocrinology*. 5th ed. 2006, Philadelphia: Elsevier Saunders.
2. Krowicki, Z.K. and P.J. Hornby, *Pancreatic polypeptide, microinjected into the dorsal vagal complex, potentiates glucose-stimulated insulin secretion in the rat*. *Regul Pept*, 1995. **60**(2-3): p. 185-92.
3. Degano, P., et al., *Effects of rat pancreatic polypeptide on islet-cell secretion in the perfused rat pancreas*. *Metabolism*, 1992. **41**(3): p. 306-9.
4. Szecowka, J., et al., *Effects of PYY and PP on endocrine pancreas*. *Acta Physiol Scand*, 1983. **119**(2): p. 123-6.
5. Moldovan, S., et al., *Somatostatin inhibits B-cell secretion via a subtype-2 somatostatin receptor in the isolated perfused human pancreas*. *Journal of Surgical Research*, 1995. **59**(1): p. 85-90.
6. Strowski, M.Z., et al., *Somatostatin inhibits insulin and glucagon secretion via two receptors subtypes: an in vitro study of pancreatic islets from somatostatin receptor 2 knockout mice*. *Endocrinology*, 2000. **141**(1): p. 111-7.
7. Sherwin, R.S., et al., *Glucose homeostasis during prolonged suppression of glucagon and insulin secretion by somatostatin*. *Proc Natl Acad Sci U S A*, 1977. **74**(1): p. 348-52.
8. Cryer, P.E., S.N. Davis, and H. Shamoon, *Hypoglycemia in diabetes*. *Diabetes Care*, 2003. **26**(6): p. 1902-12.
9. Kendall, D.M., A.U. Teuscher, and R.P. Robertson, *Defective glucagon secretion during sustained hypoglycemia following successful islet allo- and autotransplantation in humans*. *Diabetes*, 1997. **46**(1): p. 23-7.
10. Paty, B.W., et al., *Intrahepatic islet transplantation in type 1 diabetic patients does not restore hypoglycemic hormonal counterregulation or symptom recognition after insulin independence*. *Diabetes*, 2002. **51**(12): p. 3428-34.
11. Rickels, M.R., et al., *Islet cell hormonal responses to hypoglycemia after human islet transplantation for type 1 diabetes*. *Diabetes*, 2005. **54**(11): p. 3205-11.

12. Gustavson, S.M., et al., *Islet auto-transplantation into an omental or splenic site results in a normal beta cell but abnormal alpha cell response to mild non-insulin-induced hypoglycemia*. Am J Transplant, 2005. **5**(10): p. 2368-77.
13. Pileggi, A., R. Alejandro, and C. Ricordi, *Islet transplantation: steady progress and current challenges*. Curr Opin Organ Transplant, 2006. **11**: p. 7-13.
14. Froud, T., et al., *Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience*. Am J Transplant, 2005. **5**(8): p. 2037-46.
15. Gribbon, P., et al., *Evaluating real-life high-throughput screening data*. J Biomol Screen, 2005. **10**(2): p. 99-107.
16. Neumann, B., et al., *High-throughput RNAi screening by time-lapse imaging of live human cells*. Nat Methods, 2006. **3**(5): p. 385-90.
17. Glory, E. and R.F. Murphy, *Automated subcellular location determination and high-throughput microscopy*. Dev Cell, 2007. **12**(1): p. 7-16.
18. Pepperkok, R. and J. Ellenberg, *High-throughput fluorescence microscopy for systems biology*. Nat Rev Mol Cell Biol, 2006. **7**(9): p. 690-6.
19. Huang, S.G., *Development of a high throughput screening assay for mitochondrial membrane potential in living cells*. J Biomol Screen, 2002. **7**(4): p. 383-9.
20. Morelock, M.M., et al., *Statistics of assay validation in high throughput cell imaging of nuclear factor kappaB nuclear translocation*. Assay Drug Dev Technol, 2005. **3**(5): p. 483-99.
21. Monteith, G.R. and G.S. Bird, *Techniques: high-throughput measurement of intracellular Ca(2+) -- back to basics*. Trends Pharmacol Sci, 2005. **26**(4): p. 218-23.
22. Yang, S.N. and P.O. Berggren, *The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology*. Endocr Rev, 2006. **27**(6): p. 621-76.
23. Gopel, S.O., et al., *Regulation of glucagon release in mouse α -cells by KATP channels and inactivation of TTX-sensitive Na⁺ channels*. J Physiol, 2000. **528**(Pt 3): p. 509-20.
24. Olsen, H.L., et al., *Glucose stimulates glucagon release in single rat alpha-cells by mechanisms that mirror the stimulus-secretion coupling in beta-cells*. Endocrinology, 2005. **146**(11): p. 4861-70.

25. Gromada, J., I. Franklin, and C.B. Wollheim, *{alpha}-Cells of the Endocrine Pancreas: 35 Years of Research but the Enigma Remains*. Endocr Rev, 2007. **28**(1): p. 84-116.
26. Taborsky, G.J., Jr., et al., *Autonomic mechanism and defects in the glucagon response to insulin-induced hypoglycaemia*. Diabetes Nutr Metab, 2002. **15**(5): p. 318-22; discussion 322-3.
27. Taborsky, G.J., Jr., B. Ahren, and P.J. Havel, *Autonomic mediation of glucagon secretion during hypoglycemia: implications for impaired alpha-cell responses in type 1 diabetes*. Diabetes, 1998. **47**(7): p. 995-1005.
28. Havel, P.J. and G.J. Taborsky, Jr., *The contribution of the autonomic nervous system to changes of glucagon and insulin secretion during hypoglycemic stress*. Endocr Rev, 1989. **10**(3): p. 332-50.
29. Ahren, B., *Autonomic regulation of islet hormone secretion--implications for health and disease*. Diabetologia, 2000. **43**(4): p. 393-410.
30. Kisanuki, K., et al., *Expression of insulin receptor on clonal pancreatic alpha cells and its possible role for insulin-stimulated negative regulation of glucagon secretion*. Diabetologia, 1995. **38**(4): p. 422-429.
31. Franklin, I., et al., *Beta-cell secretory products activate alpha-cell ATP-dependent potassium channels to inhibit glucagon release*. Diabetes, 2005. **54**(6): p. 1808-15.
32. Ishihara, H., et al., *Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells*. Nat Cell Biol, 2003. **5**(4): p. 330-5.
33. Rorsman, P., et al., *Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels*. Nature, 1989. **341**(6239): p. 233-6.
34. Ravier, M.A. and G.A. Rutter, *Glucose or insulin, but not zinc ions, inhibit glucagon secretion from mouse pancreatic alpha-cells*. Diabetes, 2005. **54**(6): p. 1789-97.
35. Heimberg, H., et al., *Differences in glucose transporter gene expression between rat pancreatic alpha- and beta-cells are correlated to differences in glucose transport but not in glucose utilization*. J Biol Chem, 1995. **270**(15): p. 8971-5.
36. Leibiger, I.B., B. Leibiger, and P.O. Berggren, *Insulin feedback action on pancreatic beta-cell function*. FEBS Lett, 2002. **532**(1-2): p. 1-6.

37. Ashcroft, F.M. and S.J.H. Ashcroft, *Insulin : molecular biology to pathology*. 1992, Oxford ; New York: IRL Press at Oxford University Press. xxiii, 421 p.
38. Aspinwall, C.A., J.R. Lakey, and R.T. Kennedy, *Insulin-stimulated insulin secretion in single pancreatic beta cells*. J Biol Chem, 1999. **274**(10): p. 6360-5.
39. Borelli, M.I., F. Francini, and J.J. Gagliardino, *Autocrine regulation of glucose metabolism in pancreatic islets*. Am J Physiol Endocrinol Metab, 2004. **286**(1): p. E111-5.
40. Srivastava, S. and H.J. Goren, *Insulin constitutively secreted by beta-cells is necessary for glucose-stimulated insulin secretion*. Diabetes, 2003. **52**(8): p. 2049-56.
41. Khan, F.A., et al., *Insulin activates ATP-sensitive K(+) channels in pancreatic beta-cells through a phosphatidylinositol 3-kinase-dependent pathway*. Diabetes, 2001. **50**(10): p. 2192-8.
42. Kuroda, Y., et al., *A new, simple method for cold storage of the pancreas using perfluorochemical*. Transplantation, 1988. **46**(3): p. 457-60.
43. Ricordi, C., et al., *Automated method for isolation of human pancreatic islets*. Diabetes, 1988. **37**(4): p. 413-20.
44. Kenyon, N.S., et al., *Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154*. Proc Natl Acad Sci U S A, 1999. **96**(14): p. 8132-7.
45. Berney, T., et al., *Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis*. Transplantation, 2001. **71**(1): p. 125-32.
46. Chan, G.K., et al., *High content kinetic assays of neuronal signaling implemented on BD pathway HT*. Assay Drug Dev Technol, 2005. **3**(6): p. 623-36.
47. Akagi, Y., et al., *Enzyme-linked sensitive fluorometric imaging of glutamate release from cerebral neurons of chick embryos*. J Biochem (Tokyo), 2003. **134**(3): p. 353-8.
48. Llorente, I., et al., *BD™ Calcium Assay Kits and BD ACTOne™ Biosensor Technology*. HotLines, 2006. **10**(3): p. 7-12.

49. Klein, D., et al., *Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets*. *Biochem Biophys Res Commun*, 2004. **323**(2): p. 473-8.
50. Persaud, S.J., P.M. Jones, and S.L. Howell, *Staurosporine inhibits protein kinases activated by Ca²⁺ and cyclic AMP in addition to inhibiting protein kinase C in rat islets of Langerhans*. *Mol Cell Endocrinol*, 1993. **94**(1): p. 55-60.
51. Li, X., H. Chen, and P.N. Epstein, *Metallothionein Protects Islets from Hypoxia and Extends Islet Graft Survival by Scavenging Most Kinds of Reactive Oxygen Species*. *Journal of Biological Chemistry*, 2004. **279**(1): p. 765-771.
52. Papas, K.K., et al., *High-density culture of human islets on top of silicone rubber membranes*. *Transplant Proc*, 2005. **37**(8): p. 3412-4.
53. Malaisse-Lagae, F., et al., *Identification of a lobe in the adult human pancreas rich in pancreatic polypeptide*. *Diabetologia*, 1979. **17**(6): p. 361-5.
54. Baetens, D., et al., *Endocrine pancreas: three-dimensional reconstruction shows two types of islets of langerhans*. *Science*, 1979. **206**(4424): p. 1323-5.
55. Orci, L. and R.H. Unger, *Functional subdivision of islets of Langerhans and possible role of D cells*. *Lancet*, 1975. **2**(7947): p. 1243-4.
56. Orci, L., et al., *A morphological basis for intercellular communication between alpha- and beta-cells in the endocrine pancreas*. *J Clin Invest*, 1975. **56**(4): p. 1066-70.
57. Nussey, S.S.a.W., S.A., *Endocrinology: An Integrated Approach*. 2001, Oxford: BIOS Scientific Publishers, Ltd.
58. Gepts, W. and D. Pipeleers, *The endocrine pancreas. Functional morphology and histopathology*. *Acta Med Scand Suppl*, 1976. **601**: p. 9-52.
59. Samols, E. and J.I. Stagner, *In the Human Islet Vascular Perfusion Is Core to Mantle and B- a- D*. *Clinical Research*, 1991. **39**(2): p. A246-A246.
60. Bonner-Weir, S., *Morphological evidence for pancreatic polarity of beta-cell within islets of Langerhans*. *Diabetes*, 1988. **37**(5): p. 616-21.
61. Samols, E., S. Bonner-Weir, and G.C. Weir, *Intra-islet insulin-glucagon-somatostatin relationships*. *Clin Endocrinol Metab*, 1986. **15**(1): p. 33-58.
62. Weir, G.C. and S. Bonner-Weir, *Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes*. *J Clin Invest*, 1990. **85**(4): p. 983-7.

63. Stefan, Y., et al., *Quantitation of endocrine cell content in the pancreas of nondiabetic and diabetic humans*. *Diabetes*, 1982. **31**(8 Pt 1): p. 694-700.
64. Clark, A., et al., *Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes*. *Diabetes Res*, 1988. **9**(4): p. 151-9.
65. Rahier, J., R.M. Goebbels, and J.C. Henquin, *Cellular composition of the human diabetic pancreas*. *Diabetologia*, 1983. **24**(5): p. 366-71.
66. Ravier, M.A., et al., *Loss of connexin36 channels alters beta-cell coupling, islet synchronization of glucose-induced Ca²⁺ and insulin oscillations, and basal insulin release*. *Diabetes*, 2005. **54**(6): p. 1798-807.
67. Bonner-Weir, S. and L. Orci, *New perspectives on the microvasculature of the islets of Langerhans in the rat*. *Diabetes*, 1982. **31**(10): p. 883-9.
68. Marks, V., et al., *Intra-islet cellular interrelationships*. *Biochem Soc Trans*, 1990. **18**(1): p. 103-4.
69. Stagner, J.I., E. Samols, and V. Marks, *The anterograde and retrograde infusion of glucagon antibodies suggests that A cells are vascularly perfused before D cells within the rat islet*. *Diabetologia*, 1989. **32**(3): p. 203-6.
70. Samols, E., et al., *The order of islet microvascular cellular perfusion is B---A--D in the perfused rat pancreas*. *J Clin Invest*, 1988. **82**(1): p. 350-3.
71. Samols, E., et al., *The Order of Islet Microvascular Cellular Perfusion Is B- a- D in the Perfused Rat Pancreas*. *Journal of Clinical Investigation*, 1988. **82**(1): p. 350-353.
72. Wieczorek, G., A. Pospischil, and E. Perentes, *A comparative immunohistochemical study of pancreatic islets in laboratory animals (rats, dogs, minipigs, nonhuman primates)*. *Exp Toxicol Pathol*, 1998. **50**(3): p. 151-72.
73. Greenspan, F.S. and D.G. Gardner, *Basic & Clinical Endocrinology*. Sixth ed. 2001, New York, NY: Lange Medical Books/McGraw-Hill. 891.
74. Brissova, M., et al., *Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy*. *J Histochem Cytochem*, 2005. **53**(9): p. 1087-97.
75. Brunnicardi, F.C., et al., *Microcirculation of the islets of Langerhans. Long Beach Veterans Administration Regional Medical Education Center Symposium*. *Diabetes*, 1996. **45**(4): p. 385-92.

76. Valdeolmillos, M., et al., *Glucose-induced oscillations of intracellular Ca²⁺ concentration resembling bursting electrical activity in single mouse islets of Langerhans*. FEBS Lett, 1989. **259**(1): p. 19-23.
77. Santos, R.M., et al., *Widespread synchronous [Ca²⁺]_i oscillations due to bursting electrical activity in single pancreatic islets*. Pflugers Arch, 1991. **418**(4): p. 417-22.
78. Nunemaker, C.S., et al., *Individual mice can be distinguished by the period of their islet calcium oscillations: is there an intrinsic islet period that is imprinted in vivo?* Diabetes, 2005. **54**(12): p. 3517-22.
79. Kindmark, H., et al., *Measurements of cytoplasmic free Ca²⁺ concentration in human pancreatic islets and insulinoma cells*. FEBS Lett, 1991. **291**(2): p. 310-4.
80. Kindmark, H., et al., *Oscillations in cytoplasmic free calcium concentration in human pancreatic islets from subjects with normal and impaired glucose tolerance*. Diabetologia, 1994. **37**(11): p. 1121-31.
81. Martin, F. and B. Soria, *Glucose-induced [Ca²⁺]_i oscillations in single human pancreatic islets*. Cell Calcium, 1996. **20**(5): p. 409-14.
82. Quesada, I., et al., *Glucose induces opposite intracellular Ca²⁺ concentration oscillatory patterns in identified alpha- and beta-cells within intact human islets of Langerhans*. Diabetes, 2006. **55**(9): p. 2463-9.
83. Soria, B. and F. Martin, *Cytosolic calcium oscillations and insulin release in pancreatic islets of Langerhans*. Diabetes Metab, 1998. **24**(1): p. 37-40.
84. Moriyama, Y. and M. Hayashi, *Glutamate-mediated signaling in the islets of Langerhans: a thread entangled*. Trends Pharmacol Sci, 2003. **24**(10): p. 511-7.
85. Uehara, S., et al., *Metabotropic glutamate receptor type 4 is involved in autoinhibitory cascade for glucagon secretion by alpha-cells of islet of Langerhans*. Diabetes, 2004. **53**(4): p. 998-1006.
86. Hansen, K.B., H. Yuan, and S.F. Traynelis, *Structural aspects of AMPA receptor activation, desensitization and deactivation*. Curr Opin Neurobiol, 2007. **17**(3): p. 281-8.
87. Quirk, J.C., E.R. Siuda, and E.S. Nisenbaum, *Molecular determinants responsible for differences in desensitization kinetics of AMPA receptor splice variants*. J Neurosci, 2004. **24**(50): p. 11416-20.

88. Hayashi, M., et al., *Secretory granule-mediated co-secretion of L-glutamate and glucagon triggers glutamatergic signal transmission in islets of Langerhans*. J Biol Chem, 2003. **278**(3): p. 1966-74.
89. Coutinho-Silva, R., et al., *P2X and P2Y purinoceptor expression in pancreas from streptozotocin-diabetic rats*. Mol Cell Endocrinol, 2003. **204**(1-2): p. 141-54.
90. Burnstock, G., *Physiology and pathophysiology of purinergic neurotransmission*. Physiol Rev, 2007. **87**(2): p. 659-797.
91. Obermuller, S., et al., *Selective nucleotide-release from dense-core granules in insulin-secreting cells*. J Cell Sci, 2005. **118**(Pt 18): p. 4271-82.
92. Braun, M., et al., *Corelease and differential exit via the fusion pore of GABA, serotonin, and ATP from LDCV in rat pancreatic beta cells*. Journal of General Physiology, 2007. **129**(3): p. 221-31.
93. MacDonald, P.E., et al., *Release of small transmitters through kiss-and-run fusion pores in rat pancreatic beta cells*. Cell Metab, 2006. **4**(4): p. 283-90.
94. Zimmermann, H., *Extracellular metabolism of ATP and other nucleotides*. Naunyn Schmiedebergs Arch Pharmacol, 2000. **362**(4-5): p. 299-309.
95. Burnstock, G., *Historical review: ATP as a neurotransmitter*. Trends Pharmacol Sci, 2006. **27**(3): p. 166-76.
96. Poulsen, C.R., et al., *Multiple sites of purinergic control of insulin secretion in mouse pancreatic beta-cells*. Diabetes, 1999. **48**(11): p. 2171-81.
97. Salehi, A., et al., *Inhibition of purinoceptors amplifies glucose-stimulated insulin release with removal of its pulsatility*. Diabetes, 2005. **54**(7): p. 2126-31.
98. Chapal, J., et al., *Comparative effects of adenosine-5'-triphosphate and related analogues on insulin secretion from the rat pancreas*. Fundam Clin Pharmacol, 1997. **11**(6): p. 537-45.
99. Bertrand, G., et al., *P2 purinoceptor agonists and insulin secretion: Structure-activity relationship*. European Journal of Pharmacology, 1990. **183**: p. 579-82.
100. Petit, P., et al., *Evidence for two different types of P2 receptors stimulating insulin secretion from pancreatic B cell*. Br J Pharmacol, 1998. **125**(6): p. 1368-74.
101. Fernandez-Alvarez, J., et al., *P2 receptor agonists stimulate insulin release from human pancreatic islets*. Pancreas, 2001. **22**(1): p. 69-71.

102. Leon, C., et al., *The P2Y1 receptor is involved in the maintenance of glucose homeostasis and in insulin secretion in mice*. Purinergic Signal, 2005. **1**: p. 145-151.
103. Hillaire-Buys, D., et al., *Purinergic receptors on insulin-secreting cells*. Fundam Clin Pharmacol, 1994. **8**(2): p. 117-27.
104. Hillaire-Buys, D., et al., *Evidence for an inhibitory A1 subtype adenosine receptor on pancreatic insulin-secreting cells*. Eur J Pharmacol, 1987. **136**(1): p. 109-12.
105. Fields, R.D. and G. Burnstock, *Purinergic signalling in neuron-glia interactions*. Nat Rev Neurosci, 2006. **7**(6): p. 423-36.

