$P2Y_1$ RECEPTOR IN PANCREATIC BETA CELLS AND ITS ROLE IN INSULIN SECRETION

by

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is characterized by peripheral insulin resistance and an insufficiency of insulin secretion from the pancreatic β cell. The incidence of T2DM is rising worldwide at an alarming rate. Increased prevalence of obesity is a major contributor to this rise in T2DM incidence as it is estimated that about 90% of T2DM is attributable to excess weight. The economic burden of this disease is increasing globally; but more importantly, despite numerous treatment options, many patients with type 2 diabetes still suffer from a decreased life expectancy.

The mechanisms that regulate insulin secretion from the pancreatic β cell are still not fully understood. Thus, in order to understand β cell dysfunction in the disease state, it is essential to fully understand the mechanisms that regulate insulin secretion from the healthy pancreatic β cell. The present thesis thus investigates the role of the $P2Y_1$ receptor in the regulation of insulin secretion

There was considerable controversy regarding the dominant purinergic receptor subtype present and the proposed mechanisms involved in the human pancreatic β cells. The present work resolves this issue. It shows that, in human β cells, ATP acts as a positive autocrine signal by activating $P2Y_1$ receptors, stimulating electrical activity and coupling Ca^{2+} influx to Ca^{2+} release from endoplasmic reticulum stores. Positive autocrine signalling via $P2Y_1$ also activates PKD1 via the PLC/DAG pathway in mouse and human β cells which becomes more important in obesity. Both the pathways enhance exocytosis and increase insulin secretion.

Findings of the current thesis shed light on the understudied $P2Y_1$ and PKD1 signalling in pancreatic β cells and provide insights into the potential mechanisms responsible for regulation of

insulin secretion. A promising $P2Y_1$ receptor agonist has already been reported and may emerge as an effective drug in the treatment of T2DM (**295** words)

PREFACE

This thesis is an original work by Shara Khan

Chapter 2

This chapter is adapted from work published as Shara Khan, Richard Yan-Do, Eric Duong, Xichen Wu, Austin Bautista, Stephen Cheley, Patrick E. MacDonald. Autocrine activation of $P2Y_1$ receptors couples Ca^{2+} influx to Ca^{2+} release in human pancreatic β cells. Diabetologia, 2014 Dec; 57(12):2535-45. Late Matthias Braun was responsible for data collection, analysis, and manuscript composition. Shara Khan and Richard Yan-Do assisted with data collection and analysis. Eric Duong, Austin Bautista and Stephen Cheley was also responsible for data collection. Matthias Braun was the supervisory authority and was involved in concept formation. Patrick E. MacDonald was involved in edits to the manuscript.

Chapter 3

Shara Khan, Mourad Ferdaoussi, Austin Bautista and Nancy Smith were responsible for data collection and analysis. P.E. MacDonald and M. Ferdaoussi were the supervisory authorities and were involved in concept formation.

Research work in this thesis received research ethics approval from the University of Alberta Animal Ethics Board, protocols AUP00000291 and AUP00000405, and the Human Ethics Board, protocols Pro00013094 and Pro00001754. All families of organ donors provided informed consent for use of pancreatic tissue in research.

To everyone and everything that I lost in the last two years. Shaka and Papa, you two are missed every single day.

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GLOSSARY OF TERMS

[Ca2+]i	intracellular calcium concentration
α,β-meATP	α,β-methyleneATP
βPKD1KO	beta cell specific PKD1 knock-out
µg	microgram
µm	micron
µmol	micromole
Ad	adenovirus
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ANOVA	Analysis of variance
AU	arbitrary units
AUC	area under the curve
Beta-TC6	mouse insulinoma cell line
BMI	body mass index
Ca^{2+}	calcium
CaMKs	Calmodulin-dependent protein kinase
CICR	calcium induced calcium release
CGCC	calcium gated calcium channels
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EC_{50}	half-maximal effective concentration
EL	extracellular loop
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
ER	endoplasmic reticulum
F-actin	filamentous actin

FBS	fetal bovine serum
FFA	free fatty acid
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
GPR40	G-protein coupled receptor
GSIS	glucose stimulated insulin secretion
h	hour
HbA1c	glycated hemoglobin
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high fat diet
HIT cells	Hamster insulin secreting cell line
Gd^{3+}	gadolinium
IAPP	islet amyloid polypeptide
IC_{50}	half-maximal inhibitory concentration
IL-1Ra	IL-1R antagonist
INS-1	rat insulinoma cell line
INS-1E	rat insulinoma cell line
INS 832/13	rat insulinoma cell line
$InsP_3R$	inositol 1,4,5-trisphosphate receptor
K+	potassium
KATP	ATP-sensitive potassium channel
kg	kilogram
K_i	inhibitory constant
KO	knock-out
KRB	Krebs-Ringer bicarbonate buffer

l	litre
M3	muscarinic acetylcholine receptor
mg	milligram
min	minute
MIN6	mouse insulinoma cell line
ml	millilitre
mmol	millimole
mRNA	messenger ribonucleic acid
MRS2279	2-chloro-N6-methyl-(N)-methanocarba-20-deoxyadenosine 30,50-bisphosphate
MRS2365	(N)-methanocarba analog of 2-methylthio-ADP
MRS2500	2-iodo-N6-methyl-(N)-methanocarba-20-deoxyadenosine 30,50-bisphosphate
mV	millivolts
Na^+	sodium
NaCl	sodium chloride
NaHCO3	sodium bicarbonate
ng	nanogram
NMDG	N-methyl-D-glucamine
pA	picoAmpere
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PH domain	Pleckstrin homology domain
PKC	protein kinase C
PKD	protein kinase D
PLC	Phospholipase C
PPADS	pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid
PPARɣ	peroxisome proliferator activated receptor ɣ
RP	reserve pool

RPMI	Roswell Park Memorial Institute medium
RP	Reserve Pool
RRP	Readily releasable pool
RT-PCR	reverse-transcriptase PCR
RyR	ryanodine receptor
SDS PAGE	polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interfering RNA
SNAP-25	synaptosomal-associated protein, 25 kDa
SNARE	N-ethylmaleimide-sensitive factor attachment receptor
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TEM	transmission electron microscopy
TGN	trans-Golgi network
TIRF	total internal reflection fluorescence
TNP-ATP	2′,3′-O-(2,4,6-trinitrophenyl)-ATP
UDP	uridine diphosphate
UTP	uridine triphosphate
VGCC	voltage gated calcium channels
Vm	membrane potential
Zn^+	zinc

CHAPTER 1: INTRODUCTION

1.1 Type 2 diabetes and the pancreatic β cell

1.1a Diabetes

In 2017, International Diabetes Federation reported that there are 424.9 millions of people affected by diabetes worldwide and the number is expected to grow to 628.6 million in the year 2045 (1). One of the top 10 leading causes of death, globally diabetes claims 4.0 million deaths between 20 – 79 years of age and costs USD 727 billion in total healthcare expenditures (1). In Canada alone, roughly 2.1 million people who were 12 years or older are diagnosed with diabetes (2). Canada is also the country with the tenth highest spending on diabetes-related health expenditure, totaling 15 billion US dollars (1). On top of this severe economic burden, patients with diabetes are at increased risk of heart disease, stroke, and microvascular complications such as blindness, renal failure, and peripheral neuropathy (3). These figures help to emphasize that diabetes and its complications is not only a health crisis; it is a global societal catastrophe. This calls for a better understanding of the etiology of the disease.

Diabetes is characterized by high blood glucose levels as a result of absolute or relative loss of insulin secretory capacity. It is a heterogeneous disorder resulting from a combination of genetic and acquired factors that impair β cell function. Type 1 diabetes (T1DM), usually presented in childhood, is an autoimmune disease that results in β cell destruction. It accounts for 5%–10% of all diabetes (4); it will not be considered further here. Type 2 diabetes (T2DM), the most common form of the disease, is influenced by factors, such as age, pregnancy, and obesity and also has a genetic component. It accounts for 90% – 95% of all diabetes (4).

Impaired insulin secretion from pancreatic β cells plays the central role in T2DM. It is increasingly recognized that β cell failure develops during early stages of T2DM and is a major determining factor for its progression. Recent genome-wide association studies have identified over ~100 T2DM risk variants, the majority of which affect insulin secretion and β cell function rather than insulin sensitivity (5–9). The predominant role of reduced β cell sensitivity to glucose over insulin resistance in impaired glucose tolerance has also been shown (10). Thus, these evidence favour the idea that T2DM is a disease of impaired β cell function, resulting from a reduction in insulin content, and a failure of the β cell to respond to glucose stimulation with insulin secretion. However, the mechanisms underlying the impaired insulin secretion in response to glucose remains unclear. Since insulin is the body's only blood glucose-lowering hormone and is

secreted by the pancreatic β cells of the islets of Langerhans, therefore, a detailed knowledge about the mechanism of insulin secretion from the islets of Langerhans is imperative.

Extensive molecular research in the field of T2DM is paving the way for setting better ways of prevention, higher standards of diagnosis and improved management. A huge wealth of information has been gathered from basic research obtained from cell lines and in animal models. However, a burning question, whether the results and models can be translated into humans or not, remains. Human islet research is crucial to understanding the cellular biology of the pancreas in developing therapeutic options for diabetic patients and in attempting to prevent the development of this disease. Therefore, researchers are leaning towards collecting data from isolated pancreatic tissue, particularly islets of Langerhans, which contain the cells that produce the hormone insulin.

1.1b Islets of Langerhans and pancreatic β cells

There are ~1 million pancreatic islets in a human pancreas (11). The islets consist of several types of endocrine cells of which 50% of the cells in human islets are insulin-secreting β cells, 35% – 40% the glucagon-releasing α cells, and 10% – 15% are the somatostatin-releasing δ cells (12). The islets receive a rich systemic vascular supply originating from the splenic and superior mesenteric arteries and they empty their effluent into the portal vein. Although the islets compose only 1% – 2% of the pancreas, they receive ~10% of the pancreatic blood flow (13). The pancreatic islets of rodents are extensively innervated whereas human islet cells are more sparsely innervated (14). The architecture of rodent islets is a clearly demarcated core of β cells surrounded by a mantle of non – β cells. In contrast, human islets have a more complex architecture where β cells are directly juxtaposed to non – β cells, and non – β cells are also found in the center of the islet core, an arrangement that facilitates paracrine interactions (15).

1.1c Glucose Stimulated Insulin Secretion

Glucose is the most important physiological stimulus for insulin secretion. The stimulus – secretion coupling model consists of relaying chemical signals to electrical signals to mechanical signals. In brief, GLUT1 and GLUT3 are the predominant glucose transporters in human β cells and GLUT2 is the predominant glucose transporter in rodent β cells (16,17). At low glucose, the β cells are hyperpolarized (-70 mV) and electrically silent (18,19). Once glucose is transported into the cell, the glycolytic pathway is initiated by phosphorylation of glucose, a reaction catalyzed

by the enzyme glucokinase (20). This phosphorylation of glucose by glucokinase is rate limiting to insulin secretion, and therefore loss-of-function mutations in glucokinase result in reduced glucose induced insulin secretion and in glucose intolerance or diabetes (21). Glucose produces a concentration-dependent plasma membrane depolarization, and once the membrane potential exceeds -60 mV, initiates electrical activity. The action potentials of human β cells are also triggered from a more negative membrane potential in human than in mouse β cells (-60 mV rather than -50 to -40 mV) and often overshoot, peaking at potentials above zero mV (22). This concentration-dependent plasma membrane depolarization is achieved through stimulation of glycolytic and oxidative metabolism of glucose that is central to glucose sensing by β cells (23,24). It ultimately causes enhanced mitochondrial ATP synthesis from ADP by ATP synthase. Mitochondrially generated ATP is crucial for glucose-stimulated electrical activity and insulin secretion. An important aspect of ATP production is that it leads, via ATP sensitive K^+ (K_{ATP}) channel closure and initiation of β cell electrical activity (25,26). The unbalanced influx of positively charged ions, notably Na^+, then leads to plasma membrane depolarization (27), the firing of action potentials and the opening of voltage-gated Ca^{2+} channels (28). This, in turn, prompts the activation of secretory granules and fusion with the plasma membrane (29) (Figure 1).

Calcium release from intracellular organelles including the ER (endoplasmic reticulum) (30) and Golgi (31) as well as secretory granules (32,33) and other acidic stores including lysosomes (34), are also known to be involved in GSIS. The ER is a multifunctional organelle that serves as the most important Ca^{2+} store in the cell. This organelle is able to accumulate Ca^{2+} at millimolar levels in both free and protein-buffered forms (35–37). The Ca^{2+} stored in the ER lumen is essential for the regulation of protein posttranslational processing, folding, and export and can be rhythmically released to the cytosol, providing sustained and precise Ca^{2+}-mediated responses. Among the channels that release Ca^{2+} from the ER or the secretory vesicles, the roles of the inositol 1,4,5-trisphosphate receptors ($InsP_3R$) in the β-cells are well accepted (38,39). Ryanodine receptors (RyR) are also expressed in ER of islets (40). The ER stores large amounts of Ca^{2+} which are mobilized through the $InsP_3R$ and RyR ion channels. Just as there are voltage-gated Ca^{2+} channels (VGCC) in the plasma membrane, there are Ca^{2+}-gated Ca^{2+} channels (CGCC) on the intracellular Ca^{2+} stores. Both $InsP_3Rs$ and RyRs are CGCCs (41,42) and both can mediate Ca^{2+} Induced Ca^{2+} Release (CICR), making the process a universal one (40). One important function of CICR in the β cells is that it amplifies Ca^{2+}-dependent exocytosis (43,44).

The release of insulin induced by a step increase in glucose concentration from a low basal value (~3 mmol/l) to 10–20 mmol/l is biphasic both in vitro and in vivo (45). An initial rapid, transient, 3–10 min peak aka first phase is followed by a nadir and a subsequent, gradually-increasing second phase which may last 60 min more (46–48). A feature of T2DM is the first phase is almost abolished and second-phase secretion is reduced following a bolus of glucose injection (49,50). It is generally accepted that there are at least two populations of insulin secretory granules, the readily releasable pool (RRP) that is responsible for the first phase of the insulin secretion and a second reserve pool (RP) that is responsible for the second phase of the insulin secretion (47,51–53). (Figure 2) The readily releasable granule pool is apparently pre-docked at the cell surface membrane in a complex with SNARE and calcium-regulated proteins that allow for the rapid calcium-dependent fusion of primed insulin granules with the plasma membrane (54). This is achieved through the coordinated interaction and recruitment of SNARE proteins including SNAP-25, VAMP-8 and syntaxins 1A and 3 (55). In addition, Munc18 and syntaxin isoforms play critical modulatory role in the granule fusion/docking process (56,57). Assembly of the granule fusion/exocytic site in pancreatic β cells includes the association of insulin granules with L-type Ca^{2+} channels (58). This ensures efficient delivery of Ca^{2+} to the secretory vesicle Ca^{2+} sensor, synaptotagmin VII. Another player is known to be involved in controlling the availability of insulin secretory granules to the plasma membrane in the precisely timed biphasic secretion of insulin. Cortical F-actin is known to regulate the partitioning and mobility of insulin secretory granules by acting as a barrier between syntaxin based fusion sites and the insulin secretory granules. Upon step increase in glucose concentration, the cortical actin undergoes remodelling which not only plays a passive role in fusion of the predocked secretory granules to the plasma membrane but plays an active and important role in the second phase of the insulin secretion (55,59,60). A few general fusion processes have been proposed. The first is a complete fusion of the granule membrane with the plasma membrane that results in the emptying of the granule contents and complete mixing of the granule membrane contents (membrane proteins and lipids) with the plasma membrane (61,62). The second is a “kiss and run” type mechanism in which transient pores open between the granule membrane and the plasma membrane allowing for the partial or full release of the granule contents followed by a closure of the membrane fusion pore (63–65).

Many other nutrients (amino acids, fatty acids, hormones and neurotransmitters) are capable of enhancing insulin secretion in the presence of glucose and are referred to as

'potentiators' of insulin secretion. The potentiators enhance insulin release only at permissive glucose concentrations. A few pertinent examples to this thesis include acetylcholine, acting through muscarinic M3 receptors (66), and ATP (via P2X and P2Y purinergic receptors) (67), as well as fatty acids (via GPR40) (68,69). One of the mechanisms by which ATP acts is autocrine action and this thesis will explore potentiation of insulin secretion by autocrine action in the next section. Inhibitors of secretion include somatostatin, which acts in part via an inhibitory G protein (70), adrenaline (epinephrine) and noradrenaline (norepinephrine), the latter acting through α2 receptors to open K_{ATP} channels and hyperpolarize the cell (71).

Figure 1: Glucose Stimulated insulin secretion

Glucose is transported from the extracellular space into the β cell via a GLUT transporter (GLUT2 in rodent cells, GLUT1 and 3, in human cells.) Glucose is subsequently metabolized via the glycolytic pathway, and enters the mitochondria to generate ATP. This rise in cellular ATP levels leads to the closure of ATP-sensitive potassium (K_{ATP}) channels (which set the resting membrane potential, by passing a small outward positive current), and causes membrane depolarization. Membrane depolarization leads to the opening of voltage-gated calcium channels (VGCCs), which further leads to membrane depolarization, and subsequent rise in $[Ca^{2+}]_i$, which is the trigger for insulin granule exocytosis.

Figure 1.

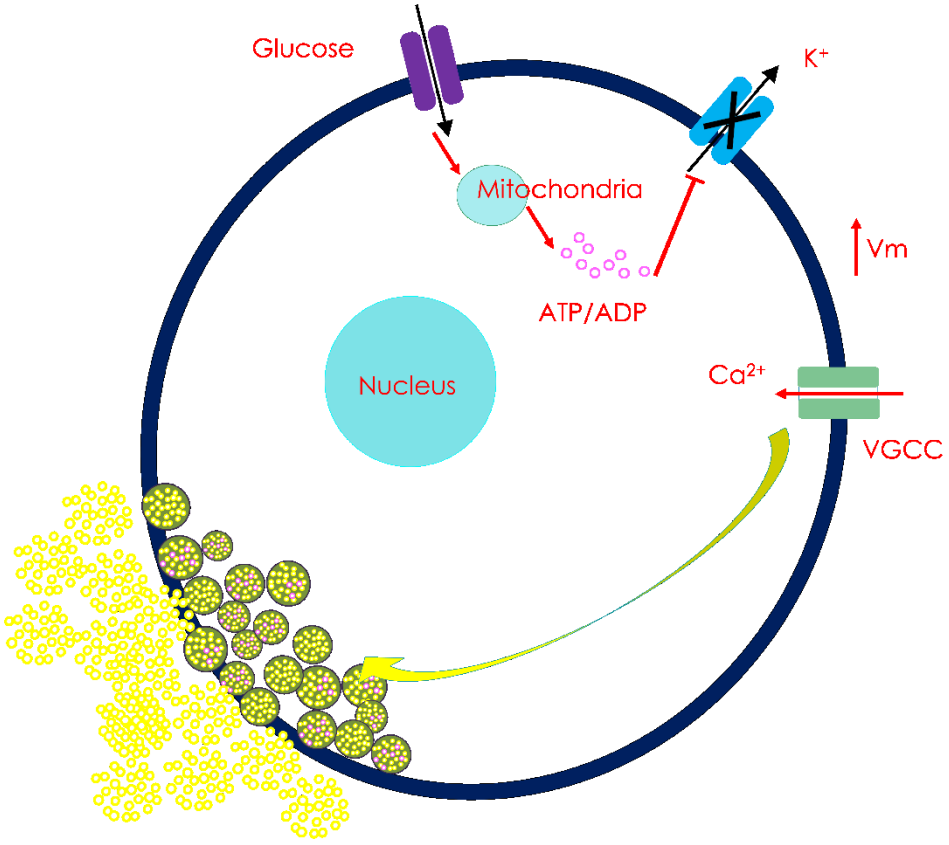
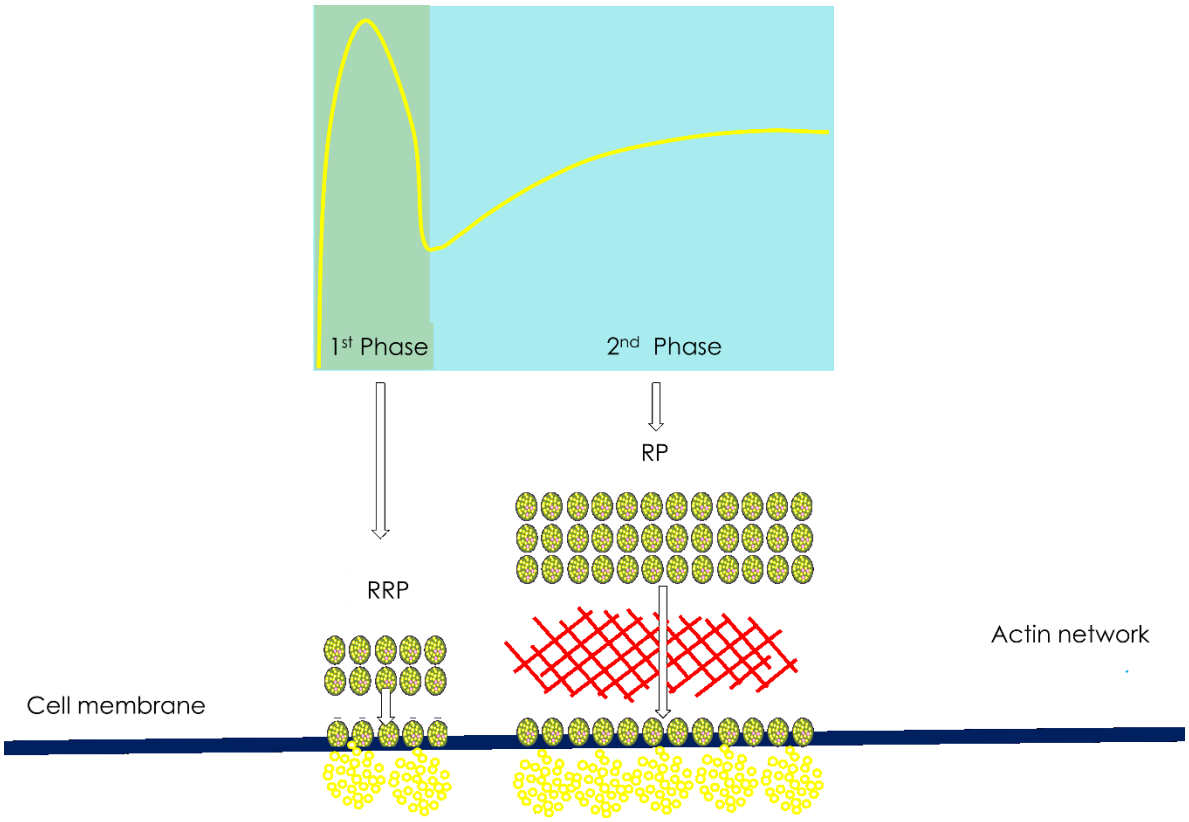


Figure 2: Phases of insulin secretion in response to glucose stimulation

Insulin is released in two phases. The first phase involves rapid release of insulin from docked and primed granules from the readily releasable pool (RRP) peaking at 3-10 minutes. The second phase involves mobilization of reserve pool (RP) secretory granules associated with the actin network regulated by glucose-evoked signals with a sustained release of insulin lasting up to 60 minutes.

Figure 2.



1.2 Autocrine regulation of insulin secretion

1.2a Neurotransmitter modulation of insulin secretion

In their natural environment, β cells are typically exposed to a mixture of many important physiological cues, rather than glucose alone. And therefore, GSIS is modulated by a plethora of agents including circulating nutrients, hormones, neurotransmitters or paracrine signalling molecules released locally within the islets, and ions and other molecules released by the β cell that may exert autocrine effects on insulin release. These may potentiate or inhibit GSIS. These factors assist to shape appropriate insulin secretion dynamics. Some contribute to synchronize the β cells within and between islets in the pancreas (72). The effects of neurotransmitter stimuli are mediated via cell surface receptors and typically do not initiate insulin release, but rather modulate secretion at permissive stimulatory concentrations of glucose (73). Neurotransmitters interact with receptors that are located in the plasma membrane. In terms of their effects on β cell electrical activity, these receptors can broadly be divided into ionotropic (ligand gated cation channels) and metabotropic (G-protein coupled receptors). The ionotropic receptors contain an ion channel and receptor activation therefore directly modulates ion flux. Metabotropic receptors are mostly G protein-coupled receptors and receptor activation results in indirect activation of ion channels via G proteins or an intracellular second messenger (such as Ca^{2+}). In the following section, the molecular action and effect on secretion of the major putative autocrine transmitters will be discussed briefly.

1.2b Overview of autocrine regulators

In autocrine signalling, a cell secretes a diffusible hormone or messenger molecule that binds to receptors on the same cell. This definition of autocrine signalling can be extended to reflect functional features of islet. Thus, released molecules that affect other cells of the same cell type (e.g. β cell to β cell communication) are also considered autocrine signals because these cells work together to produce a common, synchronized hormonal output. Paracrine communication can be defined as cellular signalling in which a factor secreted by a cell affects other cells in the local environment. In the context of the pancreatic islet, it is convenient to consider secreted molecules as paracrine signals if they affect neighbouring cells of a different type within the same islet, either via diffusion through interstitial spaces or via local microcirculation. And juxtacrine

signalling involves membrane-bound molecules in close contact (74,75). Autocrine signalling partly explains why development of cells in isolation and within groups differ. It also plays an important role during embryonic development (76). In the central nervous system, autocrine signals influence the regulation of neurotransmitter release (77). Effects of exogenous insulin on insulin secretion from the pancreas have been first studied some 50 years ago. And recent study indicate that an autocrine signalling role on insulin receptor activation inhibits insulin secretion from human islets of Langerhans (78). The following section delves into autocrine signalling in islets mediated by insulin, adenosine-5′-triphosphate (ATP) and some other factors secreted by the β cells that exert control on stimulus-secretion coupling/GSIS.

(i) Insulin: It has been proposed that autocrine insulin signalling may influence β cell function both acutely and in the longer term through the two isoforms of the insulin receptor (A and B) present on the pancreatic β cell with the different isoforms producing slightly different functional effects (79,80). Changes in gene transcription, cell proliferation and apoptosis may be mediated by the longer term effects of autocrine insulin action (80). The acute effects of insulin on stimulus–secretion coupling are less clear. Experiments on β cell specific insulin receptor knockout mice (BIRKO mice) created an insulin secretory defect similar to that in T2DM (81). Moreover, pancreas-specific deletion of insulin receptor substrate 2 lead to impaired glucose tolerance and reduced GSIS (82).

In vitro studies in isolated rodent islets or β cells have reported inhibitory, stimulatory, no, or mixed responses to exogenous insulin or C-peptide (75). Acute application of insulin to mouse β cells was found to activate the K_{ATP} channels resulting in β cell hyperpolarization, cessation of glucose induced electrical activity and inhibition of islet $[Ca^{2+}]_i$ oscillations (82). It was proposed that this inhibitory effect of insulin enables the β cell to match its energy requirements to its energy production. It is therefore of interest that BIRKO mice are glucose intolerant and exhibit impaired GSIS (characterized by the loss of first phase insulin secretion) but retain normal arginine induced insulin secretion (81). This phenotype recapitulates the insulin secretion defects seen in T2DM.

In isolated human islets, insulin stimulates moderate release of C-peptide (75). A similar small but transient stimulation of C-peptide release in response to exogenous insulin was also observed in isolated human islets (83). However, the effects of insulin in human islets still remain unclear.

Rhodes et al. agree that multiple downstream elements of the insulin signal transduction pathway critical for normal β cell function, growth, survival, and general well-being are established. But by considering the in vivo physiological context, they question the autocrine action of insulin based on different insights, other studies, and circumstantial evidence (84).

(ii) γ -Aminobutyric Acid: γ -aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the nervous system. Its presence at high levels in pancreatic β cells has led to the proposal that GABA may function as an autocrine or paracrine signal within the islet (71). There are two types of GABA receptor on β cells: $GABA_A$ receptors are ligand-gated Cl^- channels, while $GABA_B$ receptors are G-protein coupled. Activation of $GABA_A$ receptors in human β cells leads to a transient increase in Cl^- permeability. The effect is transient because $GABA_A$ receptors rapidly desensitize. GABA-induced action potential firing has been observed in human β cells and application of GABA stimulates insulin secretion (85). In human β cells, activation of $GABA_B$ receptor leads to inhibition of insulin secretion in mice via a pertussis-toxin sensitive mechanism (86,87). $GABA_B$ antagonists stimulate insulin secretion in human islets (88). Thus, in human islets, GABA appears to exert both stimulatory ($GABA_A$) and inhibitory ($GABA_B$) effects, and which effect prevails will depend on the concentration of GABA and the duration of exposure.

(iii) Glycine: Glycine is stored within the secretory granules and co-released with insulin from β cells, providing a mechanism for autocrine stimulation of insulin secretion (89). Pentamaric ligand-gated ion channels comprise of five subunits - two α-subunits and three β-subunits - that form a glycine-activated Cl^- selective channel (90). In human β cells, activation of glycine receptors gives rise to sustained inward Cl^- currents (89). Activation of glycine receptors Cl^- channels results in membrane depolarization because of the high intracellular Cl^- and thereby increases action potential firing in

human β cells exposed to permissive stimulatory concentrations of glucose (89). As expected, GSIS in human islets is reduced by the glycine receptor antagonist strychnine (90).

(iv) Islet Amyloid Polypeptide: Islet Amyloid Polypeptide (IAPP)/Amylin is co-secreted with insulin from β-cells in response to glucose stimulation (91). IAPP has been proposed to function as an autocrine/paracrine factor. IAPP-deficient male mice exhibit increased GSIS and improved glucose tolerance (92), suggesting that IAPP may be involved in feedback control of insulin secretion. Its mechanism of action on the β cell includes activation of K_{ATP} channels (93).

(v) Adenosine-5′-Triphosphate: Adenosine-5′-Triphosphate (ATP) is present in insulin granules (94,95). Studies using 'sniffer' cells overexpressing P2X receptors have shown that rodent pancreatic β cells secrete ATP in response to glucose stimulation, thereby increasing the ATP concentration close to the cell surface sufficiently high enough to enhance insulin secretion from the pancreatic β cells (96). The insulin secretory granules contain millimolar concentrations of ATP and other purine nucleotides (95,97). Upon being co-released with insulin during vesicle exocytosis, the local concentration of ATP at the β cell surface may then reach micromolar levels (96). The ATP co-released with insulin may be expected to result in autocrine activation of β cell purinergic receptors. Indeed, GSIS is reduced in both human and mouse β cells when purinergic receptor activation is prevented (98–101). (Figure 3)

Extracellular ATP binds to two types of plasma membrane receptors (purinergic/P2 receptors): P2X receptors are ligand-gated, non-selective cation-conducting channels, while P2Y receptors are G-protein-coupled receptors that influence cell function via interaction with intracellular signal transduction mechanisms. These vary in their structure, relative sensitivity to adenine nucleotides and the mechanism by which they modulate electrical activity (direct for P2X, indirect for P2Y). The next section of this thesis will cover in detail the receptor subtypes expressed in pancreatic β cells and the role each P2X or P2Y subtype plays in insulin secretion. Therefore here, very concisely, extracellular ATP has been found to amplify glucose stimulated insulin secretion in both rodent and human studies (102), but inhibitory effects have also been

demonstrated, at least in the mouse (103,104). A negative effect of ATP is also supported by the observation that glucose-induced insulin secretion is enhanced in P2Y1-knockout mice and that a $P2Y_1$ receptor antagonist increases insulin secretion in the perfused rat pancreas (105,106). However, recent studies support a stimulatory autocrine effect in human β cells, although these reached different conclusions regarding receptor involvement, favouring either $P2X_3$ or $P2Y_1$ receptors (100,107). This thesis seeks to characterise the effects of extracellular ATP on membrane currents and membrane potential in human β cells and identify the receptor subtype involved and the possible mechanism of action.

Stimulation of human β cells by ATP is associated with activation of a small Na^+-dependent inward current (108). This current need not necessarily reflect activation of a ligand-gated cation-conducting purinergic receptor, as activation of $P2Y_1$ receptors leads to activation of phospholipase C, which may culminate in opening of Trpm4 or 5 channels by a mechanism similar to GLP-1 (109)

1.2c Roles of acute autocrine regulation of insulin secretion

Mechanisms regulating insulin secretion from the islet often depend on feedback regulation. Many of the autocrine signals in the islet are involved in regulatory circuits that use positive or negative feedback. Autocrine signals reinforce the effects produced by the initial perturbation (i.e., a change in glucose concentration). As a result, a small perturbation at the input causes a much larger effect at the output. Small deviations in plasma glucose concentration (~10%) are thus counteracted by sharp changes in insulin and glucagon secretion (74).

However, autocrine regulation is clearly a complex process as it involves a variety of neurotransmitters and pathways. The overall significance of this process for insulin secretion and glucose homeostasis is therefore difficult to establish. Chronic effects of autocrine signalling are beyond the scope of this thesis and therefore, the following section considers the physiological role(s) of acute autocrine signalling in islets with a focus on ATP wherever evidence and examples exist.

(i) Pulsatile insulin secretion in single β cells

Apart from generating the exocytosis triggering Ca^{2+} signal, glucose promotes insulin secretion by amplifying signals generated by glucose metabolism, but these signals are not entirely understood (72). The discovery that glucose evokes oscillations of $[Ca^{2+}]_i$ with similar periodicity as the insulin pulses indicated that each individual β cell has the capacity to generate signals that can trigger pulsatile insulin secretion (110). The $[Ca^{2+}]_i$ oscillations in single β cells are caused by periodic entry of the ion through the voltage-dependent Ca^{2+} channels. The $[Ca^{2+}]_i$ oscillations correlate with oscillations in electrical activity, with bursts of action potentials alternating with silent intervals that correspond to the nadirs of the oscillations (111). The oscillations in electrical activity in turn most likely arise from oscillations in cell metabolism. Periodic metabolism in islets may be due to oscillatory activity of the rate-limiting glycolytic enzyme, phosphofructokinase, and/or primary oscillations in mitochondrial metabolism (72).

Determination of insulin release kinetics from single β cells is challenging. However, the observations that Zn^{2+} co-release with insulin from β cells is coordinated with oscillations of $[Ca^{2+}]_i$, and that glucose triggers oscillations of plasma membrane phosphatidylinositol-3,4,5-trisphosphate, apparently reflecting autocrine activation of β cell insulin receptors, have provided compelling evidence for pulsatile insulin secretion at the level of individual β cells (112–114).

As described above, ATP influences β cells via purinergic receptors. Purinergic effects on β cells have been extensively studied and involve both stimulatory and inhibitory effects on insulin secretion, to some extent reflecting species differences (102). It will be discussed in detail in the immediate next section. In short, inhibitory effects of ATP on exocytosis in mouse β cells are probably related to activation of the phosphatase calcineurin (103). The dominating stimulatory effect of ATP in β cells is due to $P2Y_1$ receptor-mediated activation of phospholipase C with mobilization of Ca^{2+} from $InsP_3$-sensitive stores (115). The resulting $[Ca^{2+}]_i$ spikes activate hyperpolarizing currents and reduce cytosolic ATP to provide negative feedback on the slow $[Ca^{2+}]_i$ oscillations (111,116). Phospholipase A_2-dependent closure of K_{ATP} channels contributes to $[Ca^{2+}]_i$ elevation by ATP (103). In human, there is debate about the predominant receptor subtype. Jacques-Silva et al. proposed that in human pancreatic

β cells, ATP exerts positive feedback on secretion via P2X receptors. Activation of these purinergic receptors likely produces large inward currents and depolarizes the β cell membrane (100). Wuttke et al. on the other hand proposed that in human β cells, ATP exerts its positive feedback via P2Y. Autocrine activation of $P2Y_1$ receptors and phospholipase C results in local, brief, increases of diacylglycerol in the plasma membrane that translate into increased protein kinase C activity, which is known to stimulate insulin release (72,99).

(ii) Intra-islet synchronization of β cells

Glucose stimulated insulin secretion from isolated individual pancreatic islets is pulsatile with similar properties as in single β cells (117). Interestingly, Ca^{2+} spikes resulting from intracellular Ca^{2+} mobilization can be synchronized among isolated cells lacking physical contact (118). These oscillations are sensitive to purinergic P2 receptor antagonists, indicating that β cells communicate via release of ATP (115). Since a distinct $[Ca^{2+}]_i$ spike results in exocytotic release of ATP co-stored with insulin, intercellular diffusion of the nucleotide can result in propagation of $[Ca^{2+}]_i$ spikes between cells lacking physical contact (43,115,119). The implication of this intercellular communication for oscillatory control of insulin secretion is that the $[Ca^{2+}]_i$ spikes can synchronize the slow glucose-induced Ca^{2+} oscillations by resetting their phase relationship (120). This mechanism may serve as a complement to synchronization by gap junctional coupling within the islet but is probably more important for the entrainment of different islets to a common rhythm. The synchronizing spiking among islets can be initiated by ATP released from intrapancreatic neurons (105). As mentioned above, ATP released together with insulin has strong autocrine effects on β cells by mechanisms briefly addressed already, which feedbacks on the glucose-induced $[Ca^{2+}]_i$ oscillations that generate pulsatile insulin release. In the same way, released ATP can synchronize glucose-induced $[Ca^{2+}]_i$ oscillations among closely located β cells and islets lacking direct contact (115,121).

(iii) Control of exocytotic process

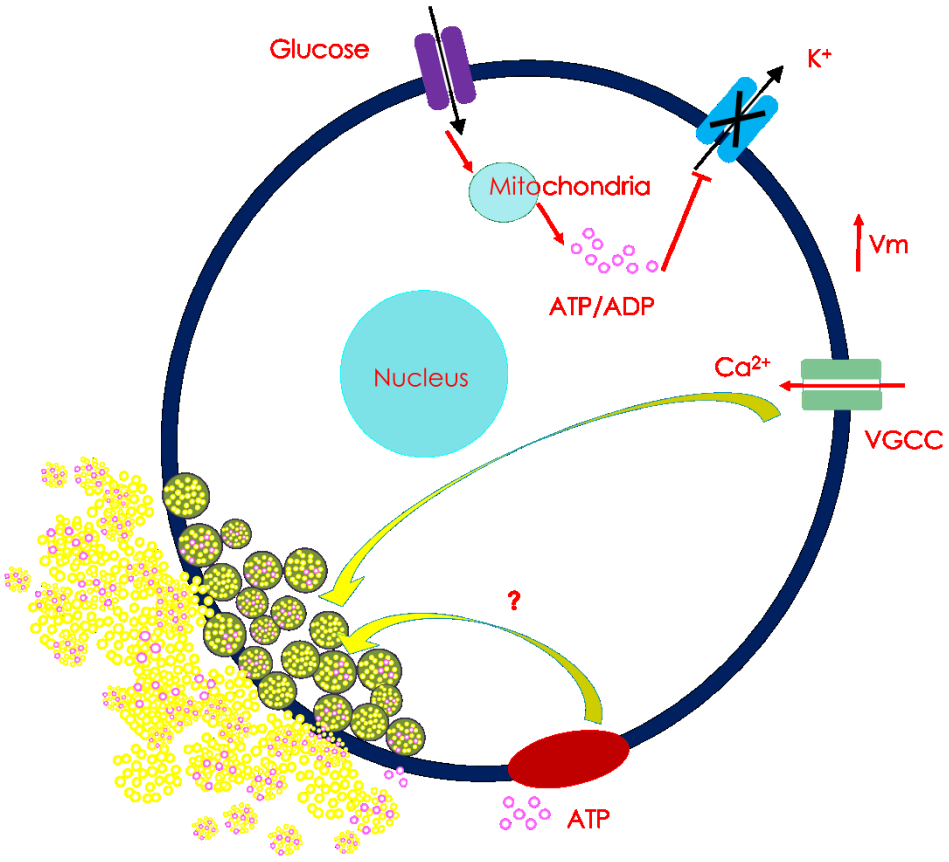
ATP can be secreted by “kiss- and-run” exocytosis while insulin is retained in the granule, suggesting that ATP release precedes that of insulin (122,123). Thus, although

present in the same granule, ATP and insulin may be released under different circumstances. It has been shown that at increasing cytosolic Ca^{2+} concentrations full fusion events are progressively favoured over kiss-and-run events (124). Therefore, Braun et al speculates that release of ATP during kiss-and-run exocytosis can lead, via autocrine signalling, to an additional increase in $[Ca^{2+}]_i$, which in turn promotes full granule fusion and insulin release (75). This helps to regulate the speed and amplitude of the insulin response making the insulin secretion fast and robust.

Figure 3: Autocrine regulation of insulin secretion

Glucose uptake via glucose transporters leads to accelerated mitochondrial glucose metabolism, increased ATP production, closure of the K_{ATP} channels, membrane depolarization and Ca^{2+} influx leading pancreatic β cells to secrete granules containing insulin and ATP. ATP feedback activates purinergic P2 receptors (red) on the same cell, resulting in potentiation of insulin secretory response.

Figure 3



1.3 Purinergic Receptors in Islets

1.3a Pharmacology of purinergic P2 receptors

Pancreatic β cells express different subtypes of membrane-spanning P2 purinergic receptors which, upon binding of ATP or structural analogues, induce or modulate the release of insulin. Both ligand gated non-selective cation channels (P2X) and G-protein coupled receptors (P2Y) have been characterized on these cells by different experimental approaches exploring receptor function, expression and stimulus-secretion coupling mechanisms.

1.3a.i Purinergic P2X receptors

Seven different P2X subunits ($P2X_{1-7}$) have been found in mammals, which assemble together in a homomeric and/or heteromeric fashion to form trimeric ion channel structures (125–130). Each subunit has several unifying features: C- and N-termini are located intracellularly, linked by two transmembrane-spanning segments (TM1 and TM2) and a large extracellular domain (ectodomain), where the ATP-binding sites are nestled (131–133) (Figure 4A). In response to the binding of extracellular ATP, these receptors switch between different conformational states (131,134–136). Initial agonist binding causes the receptor to progress from a closed, resting state to an open, conducting state, allowing mainly the flow of Na^+, K^+ and Ca^{2+} across the membrane (137–139). Prolonged exposure to ATP leads to receptor desensitization, a temporary inactivation that terminates ion flux despite the fact that ATP remains bound to the receptor. The gating properties of the ion channel by agonist vary markedly with receptor subtype, with the $P2X_2$, $P2X_4$ and $P2X_5$ homomeric channels showing slow desensitization and the $P2X_1$ and $P2X_3$ channels exhibiting rapid desensitization. In contrast, $P2X_7$ receptors exhibit no apparent desensitization (140). Dissociation of ATP from the desensitized state, in turn, reverts the channel to the initial closed, resting state, in which it is able to be activated once again. This process is known as resensitization. A second conducting state is also found for selected P2X subtypes, whereby a dilation of the pore occurs. Several studies suggest that P2X receptors, especially after prolonged ATP application, are permeable to large organic cations and dyes such as N-methyl-D-glucamine (NMDG) (141,142).

1.3a.ii Purinergic P2Y receptors

All P2Y receptors belong to the class A of G-protein coupled receptors (GPCRs) (143,144). As of today, there are eight accepted human P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (145). The missing numbers represent either non-mammalian orthologs or receptors having some sequence homology to P2Y receptors but for which there is no functional evidence of responsiveness to nucleotides. Published crystal structures of the $P2Y_1$ and more recently of $P2Y_{12}$ receptors confirm typical features known for GPCRs including seven hydrophobic TMs connected by three extracellular loops (ELs) and three intracellular loops (146–148). All P2Y receptors possess at their extracellular domains four cysteine residues which (as shown for the $P2Y_1$, $P2Y_2$, and $P2Y_{12}$ receptors) form two disulfide bridges: the first one between the N-terminal domain and EL3 and the second bridge between EL1 and EL2 (149–152) (Figure 4B). The receptors of the P2Y receptor family show a relatively high diversity in the amino acid composition. Sequence alignments, phylogenetic analysis, and effector coupling of the P2YRs have distinguished two P2YR subfamilies (153,154). Most P2Y receptors couple to Gq/G11 proteins and thus activate PLC-β, except for $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ that couple to Gi proteins and inhibit adenylate cyclase, and $P2Y_{11}$ couples to Gs and Gq (155,156). The sequence identity between the two subfamilies is quite low, with only 20% identity between $P2Y_1$ receptor and $P2Y_{12}$ receptor, while the sequence identity is higher between the members within the same subfamily, for example, with a 45% identity between $P2Y_{12}$ receptor and $P2Y_{14}$ receptor. P2Y receptors form homo- and heterodimers as known for other GPCRs (157,158). One example is a dimer composed of the $P2Y_1$ receptor and the adenosine A1 receptor (159,160). These dimers showed marked differences in their pharmacological properties when compared with the respective monomers. Agonist activation of the $P2Y_1$ receptor has been proposed to induce homodimerization followed by receptor internalization (161). There is also evidence for homodimerization of $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors (162–164).

1.3a.iii Control of purinergic signalling

The autocrine action of ATP has been extensively covered in the previous section: "Overview of autocrine regulators". Extracellular ATP for stimulating β cells can come from two potential sources: ATP co-released with transmitters from nerve terminals, and ATP released from

insulin-containing granules (95–97,119). Another possible source of ATP is from the transmembrane channel, pannexin-1 (98). As mentioned earlier, ATP released from insulin-containing granules can reach local concentrations in micromolar range (95–97,119). However, it appears that release of small molecules like ATP precedes release of peptide hormone, and in up to 70% of the cases exocytosis does not result in significant release of peptide hormone (75,123). Accumulation of ATP within vesicles is thought to occur via the vesicular nucleotide transporter, VNUT/SLC17A9, and knockdown of VNUT leads to diminished glucose-responsive ATP release, though described effects on insulin release are disparate (101,165). The lifetime of ATP is closely regulated by ectonucleotidases that have their catalytic site on the outer side of the plasma membrane (166). Of the four types of ectonucleotidases, ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) are responsible for catalytic hydrolysis of nucleoside tri- and di-phosphate only, mono phosphates are not hydrolyzed by these enzymes (167). E-NTPDases can either directly convert ATP to AMP, or ATP is first converted to ADP which further gets converted to AMP (168).

1.3a.iv Agonists and Antagonists

A summary of all agonists and antagonists of the purinergic receptor subtypes is beyond the scope of this thesis. Therefore, important non-selective synthetic analogs and selective agonists and antagonists with respect to one example from the P2X group, the $P2X_3$ receptor, and another from the P2Y group, the $P2Y_1$ receptor, will be discussed in this section as they have been studied in human pancreatic β cells.

The P2X receptors were originally defined by the potency profile of the agonists α,β-methyleneATP (α,β-meATP), 2-methylthioATP (2-meSATP), and ATP. Since the cloning of P2X subunits, ATP was found to be an agonist at all P2X receptor subtypes with widely varying potency (169,170). A complicating factor in characterizing agonist activity is that both ATP and 2-meSATP but not α,β-meATP undergo degradation by E-NTPDases (89,168,171). Thus, the relative agonist potency profile of the P2X receptor was defined as α,β-meATP > 2-meSATP = ATP (172). 2',3'-O-(4-benzoylbenzoyl)ATP (BzATP) is the most potent P2X agonist currently available and acts at all P2X subtypes studied to date (169). BzATP is commonly described as a selective agonist at $P2X_7$ receptors, but in fact its affinity is highest for the $P2X_1$ subtype. The mimicry of ATP by α,β-meATP makes homomeric $P2X_3$ receptors and $P2X_1$ and distinct from the other homomeric

forms. 2-methylthio-ATP (2-meSATP) is as potent as or more potent than ATP at $P2X_3$ receptors (89,128,171).

Suramin and PPADS antagonize P2X receptors but with low potency and little subtype selectivity. Suramin antagonizes most homomeric P2X subtypes at low micromolar concentrations, except for the $P2X_7$ receptor, where hundreds of micromolar suramin are required, and the $P2X_4$ receptor, which is insensitive (154,169,170). PPADS tends to have a similar potency or be slightly more potent than suramin, but again the $P2X_7$ receptor is much less sensitive and the $P2X_4$ receptor insensitive (165,166). The antagonistic actions of PPADS tend to develop and reverse slowly and antagonism is generally non-competitive. Its ability to inhibit ATP breakdown by ectonucleotidases is less than suramin. TNP-ATP is a highly potent competitive antagonist at the $P2X_3$ receptor, with IC_{50} values in the low nanomolar range, but has less than 10-fold selectivity over the $P2X_1$ receptor (169,170). (Table: Pharmacological profile of $P2X_3$)

For the P2Y receptors, some receptor subtypes are still lacking potent and selective synthetic agonists and antagonists. ADP is the endogenous agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors, and it generally interacts with greater affinity than ATP (89,155,171). At $P2Y_{11}$ receptors, ATP is the preferred native ligand (173). The $P2Y_2$ receptor is activated nearly equipotently by UTP and ATP (89,174). The $P2Y_4$ receptor is primarily activated by UTP (175), the $P2Y_6$ by UDP (176) and the $P2Y_{14}$ by UDP-glucose (177). As outlined above, ADP is the physiological agonist of the $P2Y_1$ receptor. Its analog 2-methylthio-ADP has a higher potency at the human $P2Y_1$ receptor than ADP (178). The Northern (N) conformation of the ribose is preferred, and the (N)-methanocarba analog of 2-methylthio-ADP (MRS2365) is much more potent (EC_{50} 0.4 nmol/l) than 2-methylthio-ADP and displays strict selectivity for the $P2Y_1$ receptor over the ADP-activated $P2Y_{12}$ and $P2Y_{13}$ receptors (179). 2-Methylthio-ATP and ATPγS act as agonists at the $P2Y_1$ receptor with potencies similar to that of ADP. ATP itself is a relatively weak partial agonist (178).

The non-selective suramin and PPADS are also antagonists at some of the P2Y receptor subtypes (154). The human $P2Y_1$ receptor is also blocked by suramin and PPADS. Bisphosphate antagonists with higher affinity and selectivity for the $P2Y_1$ receptor include MRS2179 (20-deoxy-N6-methyladenosine-30,50-bisphosphate) (180). Replacement of the ribose with a cyclopentane fused in the N-conformation with a propane bridge resulted in non-nucleotide bisphosphate

analogs that retained high affinity for the $P2Y_1$ receptor. For example, the chloro- and iodo-analogs MRS2279 (2-chloro-N6-methyl-(N)-methanocarba-20-deoxyadenosine 30,50-bisphosphate) and MRS2500 (2-iodo-N6-methyl-(N)-methanocarba-20-deoxyadenosine 30,50-bisphosphate) exhibit affinity constants of about 4 and 1 nmol, respectively (181,182). (Table: Pharmacological profile of $P2Y_1$)

1.3b Role of purinergic P2 receptors in pancreatic β cells

It was first reported in 1963 that ATP causes an increase in insulin secretion in the β cells of rabbit pancreas (183). Purinergic P2 receptors have since been studied extensively in both in vivo and in vitro preparations of rat and mouse pancreas, as well as on islet preparations of these, and also on the human pancreas, other species and several insulinoma cell lines.

1.3b.i P2X receptor studies performed in insulin secreting cell lines, rodents and other species

Evidence of $P2X_1$ and $P2X_3$ receptors in dispersed mouse β cells, intact mouse islets, porcine β cells and human β cells by electrophysiology and immunocytochemistry was reported by Silva et al about a decade ago (184). They hypothesized that the P2X receptor subtypes investigated in the study, once activated by locally released ATP, may facilitate glucose- and/or acetylcholine-induced insulin secretion (184).

Selective nucleotide (such as ATP) release from large dense-core granules in INS-1 cells transfected with $P2X_2$ receptors was studied by Obermüller et al. by employing capacitance measurements, electrophysiological detection of ATP release and single-granule imaging. The authors reported that up to two-thirds of exocytotic events were not associated with detectable peptide release but nevertheless resulted in the release of low-molecular-weight granule constituent, ATP (123). In 2009, Karanauskaite et al. using rat pancreatic β cells infected with $P2X_2$ receptors and employing electrophysiological, amperometric and photolysis of caged Ca^{2+} techniques showed that the granules inside the pancreatic cells contain not only insulin but also ATP and ADP (185). Moreover, other molecules as 5-hydroxytryptamine, gamma-aminobutyric acid, glutamate and zinc are released together with ATP and may affect the autocrine secretion of insulin (185). The role of the receptor subtype $P2X_3$ with respect to positive autocrine activation in human β cells will be discussed in detail in the next sub-section.

Regulation of insulin secretion in pancreatic mouse islets, proliferation and survival of Beta-TC6 cells was investigated by Ohtani et al. with respect to P2X receptors, with a primary focus on $P2X_4$ receptor; they also studied the $P2Y_1$ receptor (186). By RT-PCR, Northern blot and immunohistochemical studies, they confirmed the presence of $P2X_4$ (186). Although the ATP analogue ATPγS stimulated insulin secretion, the lack of specific $P2X_4$ agonist and antagonist rendered the role of $P2X_4$ inconclusive. MRS2179, a specific $P2Y_1$ antagonist, partially blocked the stimulatory effect of ATP (186). The inhibitory effect of ATP on Beta-TC6 cell proliferation was partially reduced in the cells treated with $P2X_4$ siRNA. DNA fragmentation was not detectable in Beta-TC6 cells treated with ATP when examined by gel electrophoresis and TUNEL assay. This suggested that activation of $P2Y_1$ and P2X receptors was not involved in apoptosis of Beta-TC6 cells (186). Rat pancreatic islets or perfused pancreases were subjected to insulin radioimmunoassay or rubidium efflux assay by Petit et al. to provide evidence that activation of P2X receptors present on pancreatic β-cells results in a transient increase in insulin secretion regardless of low glucose concentration and that activation of P2Y receptors occurs at stimulating glucose concentration to potentiate insulin secretion (187). Although the authors didn't study any specific receptor subtype, they hinted at the expression $P2X_4$ in insulin-secreting cell lines RINm5F and HIT-T15 (187).

While examining the co-secretion of extracellular ATP and zinc with insulin in pancreatic rat islets and rodent β cell lines (INS-1 cells, clone 832/13 and βTC3 cells), Richards-Williams et al. observed that the presence of an extracellular ATP scavenger, a zinc chelator, and P2 receptor antagonists attenuated GSIS. Furthermore, mRNA and protein were expressed in β cell lines and primary islets for $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_6$ (188). Based on these results, the authors proposed that P2XR channel subtypes, $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_6$, are each gated by extracellular ATP and modulated positively by extracellular zinc to potentiate insulin secretion (188).

Lee et al. reported opposite effects of the receptors $P2X_7$ and $P2Y_{11}$ with activation of $P2X_7$ leading to inhibitory activity on glucose dependent insulin secretion, whereas activation of the $P2Y_{11}$ receptor leading to enhancing effect in HIT-T15 cells (189). They came to this conclusion by performing radioimmunoassay of insulin secretion in HIT-T15 (189). However, the $P2RX_7$ is a highly polymorphic gene with the hypofunctional $P2X_7$ receptor variants being related to impaired glucose homeostasis (190). The hyperfunctional variant 1068 G>A on the other hand has

been associated with an increase in both insulin sensitivity and secretion in one study (191) and an increase in IL-1 receptor (IL-1R) levels, insulin secretion and pancreatic β cell function in another (192). The latest study in $P2X_7$ receptor has been conducted this year by Tozzi et al. They reported that the $P2X_7$ receptor and pannexin-1 are involved in glucose-induced autocrine regulation of pancreatic β cells (98). They observed that glucose induces rapid release of ATP and significant fraction of release involves the $P2X_7$ receptor and pannexin-1, both expressed in INS-1E cells, rat and mouse β cells. Furthermore, they provided pharmacological evidence that extracellular ATP, via $P2X_7$ receptor, stimulates Ca^{2+} transients and cell proliferation in INS-1E cells and insulin secretion in INS-1E cells and rat islets (98). Hence, they concluded that the P2X7 receptor is expressed in INS-1E cells (and mouse and rat islets) and affects important cell functions such as ATP metabolism and release, Ca^{2+} oscillations, insulin secretion and cell proliferation (98).

1.3b.ii P2Y receptor studies performed in insulin secreting cell lines, rodents and other species

The identity of purinergic receptor subtypes on pancreatic β cells was deduced from studies using pharmacological tools and monitoring effects on, for example, insulin release and intracellular Ca^{2+} signals. The findings indicated the presence of the $P2Y_1$ receptor, an ADP-preferring receptor (154). The molecular evidence for the $P2Y_1$ receptor was provided by cloning of the receptor from rat and mouse insulinoma cells, i.e. RINm5F and MIN6, respectively (193). The receptor $P2Y_1$ has been widely studied. Although all studies regarding $P2Y_1$ has been found to amplify glucose stimulated insulin secretion in both rodent and human studies; one in vivo study however demonstrated a negative effect where the authors detected enhanced glucose-induced insulin secretion in $P2Y_1$-knockout mice (106). In 2002, Verspohl et al. published the effect on insulin secretion of purinergic agonists and antagonists from the activation of the receptor subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2X_3$, in INS-1 cells and rat pancreatic islets (194). They reported that ATP at low concentrations is effective via $P2Y_1$ receptor but not via $P2X_3$, $P2Y_2$ or $P2Y_4$ receptors (194). Activation of $P2Y_1$ and $P2Y_6$ receptors in MIN6 cells was studied by Balasubramanian et al. (195). They employed radioligand binding, insulin secretion, calcium immobilization, flow cytometry and inositol phosphate assays to show that the activation of $P2Y_1$ and $P2Y_6$ receptors by their selective agonists leads to increased insulin secretion through G protein signalling (195). Role of $P2Y_1$ receptor in the modulation of insulin secretion, proliferation and cell viability in pancreatic mouse islets and Beta-TC6 cells was discussed in the immediate

previous paragraph and the role of $P2Y_1$ receptor in ATP autocrine feedback resulting in activation of PLC and spatially restricted production of DAG (known to promote recruitment and activation of protein kinase C) in single MIN6 cells, primary mouse and human β cells will be discussed in the immediate next sub-section. The same laboratory who studied positive autocrine feedback of ATP through $P2Y_1$ receptor also investigated recruitment of both conventional and novel PKCs to the plasma membrane and the implication of the mentioned PKCs in autocrine regulation of MIN6 insulinoma cells by using total internal reflection microscopy, fluorescent protein-tagged PKCs, and signalling biosensors (196). Wuttke et al. drew the conclusion that autocrine feedback activation of $P2Y_1$ receptor induces transient DAG microdomains that rapidly recruit both conventional and novel PKCs to the β cell plasma membrane and therefore are involved in the autocrine regulation of β cell function (196). Using $[Ca^{2+}]i$ measurements, insulin secretion and insulin enzyme immunosorbent assays in mouse islets and Beta-TC6 cells, Ohtani et al. provided evidence that $P2Y_1$ or $P2Y_6$ receptor subtypes respond to the purinergic agonists by increasing $[Ca^{2+}]i$ via intracellular Ca^{2+} mobilization and modulation of insulin secretion and that they may play a role as autocrine regulators of insulin secretion (197).

Parandeh et al. also studied $P2Y_6$ receptors and published that $P2Y_6$ is expressed in isolated mouse pancreatic islets and β cells (198). By performing insulin secretion assay, they reported that the extracellular pyrimidines, uridine triphosphate (UTP) and uridine diphosphate (UDP), stimulates insulin secretion by activation of $P2Y_6$ receptor (198). Sassman et al. identified that $P2Y_6$ receptors mediate their autocrine potentiation of insulin secretion in mice through the Gq/G11-mediated signalling pathway (199). Another study involving $P2Y_6$ receptor using insulin secretion assay, RNA interference studies and western blot analyses in MIN6 cells showed that $P2Y_6$ receptor activation increases calcium signalling pathways in MIN6 cells to increase phosphorylation of AMPK, which then phosphorylates acetylcoenzyme A carboxylase (200).

Activation of the $P2Y_{11}$ receptor leading to enhanced insulin secretion in HIT-T15 cells has already been mention in the previous paragraph. As for $P2Y_{13}$ receptor, contrary to the other receptor subtypes it has been reported to have an inhibitory effect on insulin secretion. The results showed that co-incubation with the $P2Y_1$ antagonist MRS2179 inhibited insulin secretion, while co-incubation with the $P2Y_{13}$ antagonist MRS2211 stimulated insulin secretion, indicating that ADP acting via $P2Y_1$ stimulates insulin secretion, while signalling via $P2Y_{13}$ inhibits the secretion

of insulin. $P2Y_{13}$ antagonism through MRS2211 increased the secretion of insulin and administration of MRS2211 during glucose injection in vivo resulted in increased secretion of insulin. Therefore, Amisten et al. arrived at the conclusion that ADP mediates inhibition of insulin secretion by activation of $P2Y_{13}$ receptors in mouse islets, β cells and MIN6c4 cells (201).

Meister et al. examined the UDP sensitive $P2Y_{14}$ receptor by using RNA sequencing method and insulin secretion assay in $P2Y_{14}$-deficient mouse islets and published that $P2Y_{14}$ deficiency significantly changed expression of components (e.g. GLUT2) involved in insulin secretion and also had reduced insulin secretion (202).

1.3b.iii Controversy regarding the predominant receptor subtype present in human β cells

Only few studies have investigated purinergic signalling in human islets. These suggest a stimulatory effect of ATP on insulin secretion, but considerable controversy exists regarding the receptor subtypes and mechanisms involved. Some reported that the P2X receptors are more important for regulation of insulin secretion than P2Y receptors in humans. The human β cells express $P2X_3$, $P2X_5$ and $P2X_7$ receptors (100). Silva et al. employed in situ hybridization, RT-PCR, immunohistochemistry, western blotting as well as recordings of cytoplasmic-free Ca^{2+} concentration, $[Ca^{2+}]_i$, and hormone release in isolated human and rat islets and showed that the β cell secretes ATP along with insulin when the glucose concentration increases. Released ATP then activates $P2X_3$ receptors in the β cell plasma membrane. Activation of $P2X_3$ receptors leads to membrane depolarization and subsequent opening of voltage-gated Ca^{2+} channels. This results in increased $[Ca^{2+}]_i$ and enhanced insulin secretion (100). In contrast, others reported that P2Y receptors are of particular importance to humans. Wuttke et al used single MIN6 cells, primary mouse β cells, and human β cells within intact islets transfected with translocation biosensors for DAG, PKC activity, or insulin secretion and then imaged them with total internal reflection fluorescence microscopy (107). They showed that glucose stimulation of β cells triggers exocytosis of granules containing insulin and ATP which positively feedbacks and activates purinergic $P2Y_1$ receptors, resulting in activation of PLC and spatially restricted production of DAG. This feedback loop translates into transient PKC activity and stimulation of insulin secretion (107). (Figure 5: Proposed mechanisms of action for the positive autocrine feedback loop mediated by ATP in human β cells) Therefore, the predominant receptor subtypes present in human β cells and the mechanisms involved definitely merits further investigation. This thesis aims to carefully analyse

the effects of ATP on membrane potential, membrane currents, Ca^{2+} signalling, exocytosis and insulin secretion in isolated human islets and dispersed pancreatic human β cells. Chapter 2 of this thesis includes the published report on the resolution of this issue.

Figure 4: Structure of P2X and P2Y receptor.

Figure 4 (a): Schematic presentation of general features of P2X receptor subunit. The seven cloned P2X receptors ($P2X_{1-7}$) are ATP-gated ion channels forming cation-permeable pores with rapid activation kinetics. Each putative subunit comprises of two hydrophobic transmembrane spanning regions (TM1 and TM2), a large extracellular loop and two intracellularly located terminal tails (amino (NH_2)-and carboxy(COOH)-tail). TM2 is believed to line the pore of the channel. Positively charged amino-acid residues on the extracellular loop contribute to ATP binding and receptor activation.

Figure 4 (b): P2Y receptors consist of seven transmembrane spanning segments (TM1-TM7) connected by three extracellular loops (EL) and three intracellular loops. The NH_2-tail is located outside the cell, while the COOH-tail is located in the cytoplasm. Two disulphide bridges exist between cysteine residues: the first one between the N-terminal domain and EL3 and the second bridge between EL1 and EL2.

Figure 4 a.

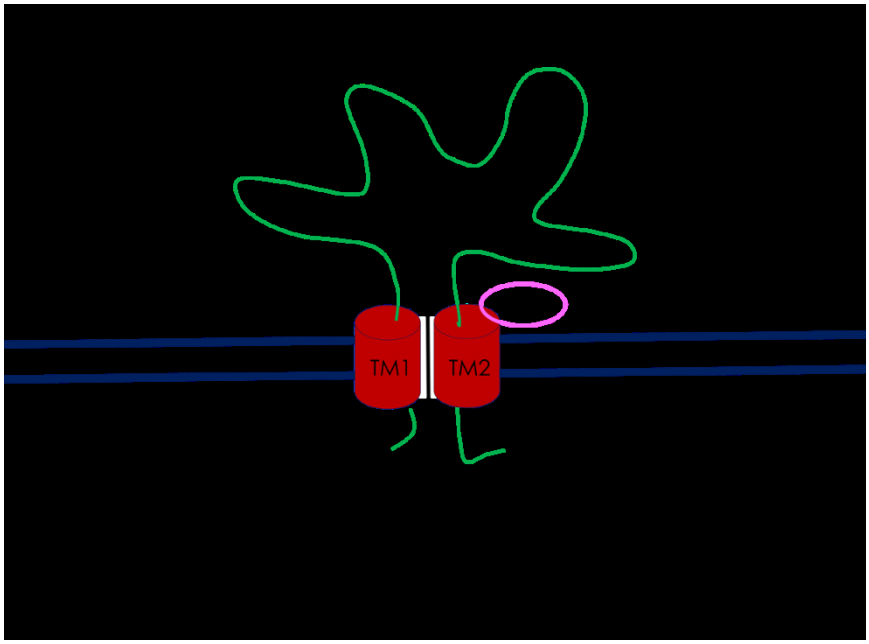


Figure 4 b.

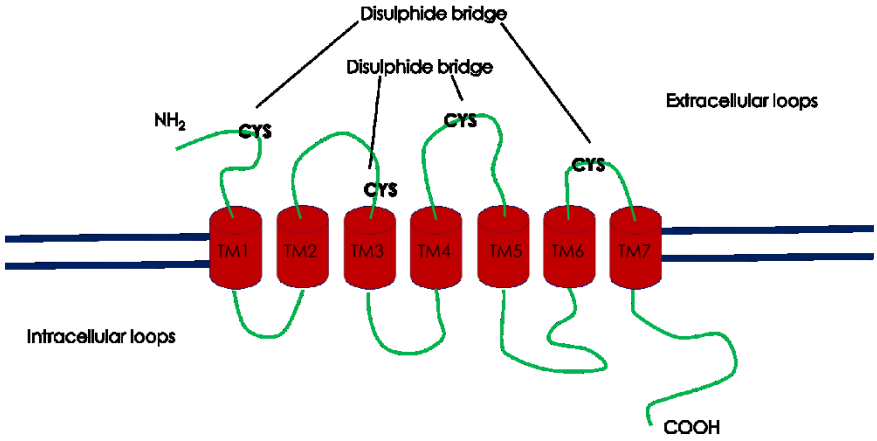


Figure 5: Proposed mechanisms of action for the positive autocrine feedback loop mediated by ATP in human β cells.

Figure 5 (a): Glucose stimulation of β cells triggers exocytosis of granules containing insulin and ATP. ATP, coreleased with insulin, activates ionotropic $P2X_3$ receptors in the β cell plasma membrane. This opens the cation selective $P2X_3$ channel pore to let Na^+ and Ca^{2+} flow into the cell. The resultant membrane depolarization increases Ca^{2+} flux through high voltage-gated Ca^{2+} channels. Increased $[Ca^{2+}]_i$ stimulates insulin secretion.

Figure 5 (b): Glucose stimulation of β cells triggers exocytosis of granules containing insulin and ATP. ATP, coreleased with insulin, activates purinergic $P2Y_1$ receptors, resulting in activation of PLC and spatially restricted production of DAG. The elevated plasma membrane DAG concentration locally and transiently activates PKC and/or other effectors to potentiate the insulin secretory response.

Figure 5 a.

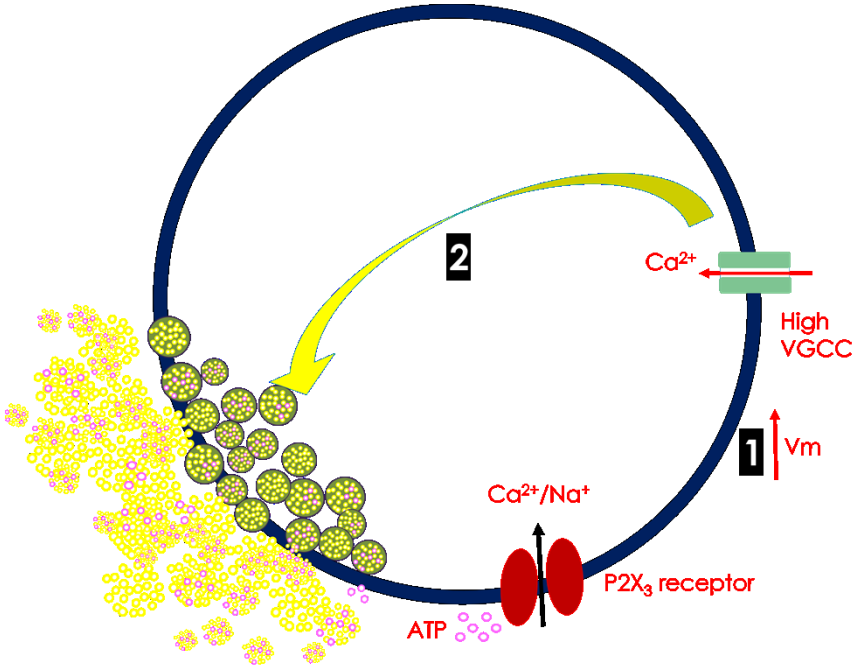


Figure 5 b.

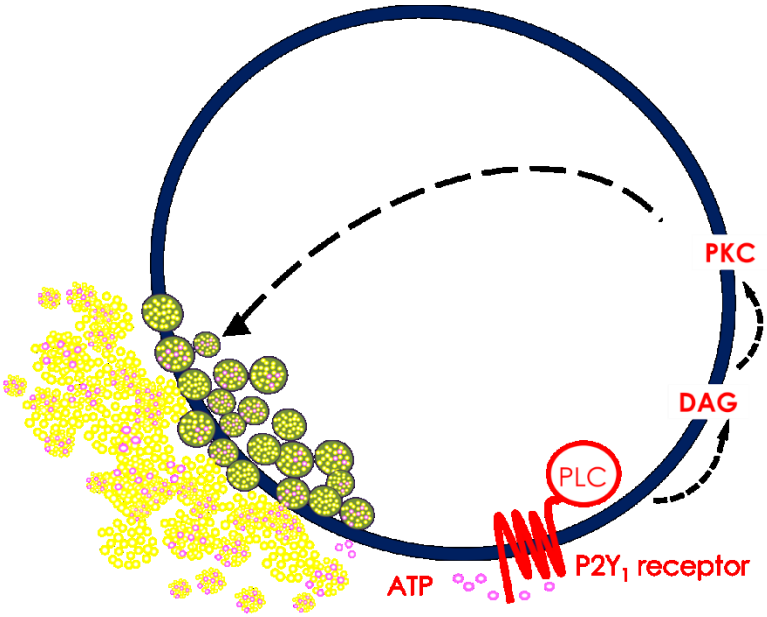


Table 1: Pharmacological profile of $P2X_3$. EC_{50}/IC_{50} values expressed in micromolar unless otherwise specified.

COMPOUND	EC_{50}/IC_{50}
Full agonists	
ATP	1
αβ-meATP	1–2
2-meSATP	0.3
Ap6A	1.5
Ap5A	1
Ap4A	1
Partial agonists	
ATPγS	10
BzATP	Not determined
βγ-meATP	>300
Antagonists	
Suramin	3
PPADS	1.5
TNP-ATP	1 nM
A-317491	20 nM
NF023	8.5
NF279	2
NF449	3
RO-85	30 nM
Ip4I	1
MRS2159	150 nM
MRS2257	30 nM
AF-353	10 nM
RO-4	13 nM
RO-51	10 nM

Table 2: Pharmacological profile of $P2Y_1$. EC_{50}/IC_{50} values expressed in nanomolar unless otherwise specified.

Compound	EC_{50}/IC_{50}
Full agonists	
MRS2365	0.4
2-MeSADP	8.29
ADP	643
ATP	777
ATPγS	3.2 μM
Antagonists	K_i
Suramin	4.9 μM
PPDAS	6.2 μM
MRS2179	84
MRS2279	13
MRS2500	0.78
MRS2298	29.6
MRS2496	76
NF340	19.5

1.4 Protein Kinase D1 and insulin secretion

1.4a Background

A large number of external signals involved in intercellular communication, including hormones, neurotransmitters, growth and developmental factors, cytokines, and bioactive lipids, bind to receptors, such as $P2Y_1$, GPR40, M3 etc., that promote the stimulation of isoforms of the phospholipase C (PLC) family and catalyze the hydrolysis of phosphatidylinositol 4,5-biphosphate to produce two second messengers: Ins $(1,4,5)P_3$, which triggers the release of Ca^{2+} from internal stores, and diacylglycerol (DAG), which elicits cellular responses through Protein Kinase C (PKC) (203).

Wuttke et al. showed that glucose has a minor carbachol-induced, dose-dependent rise in plasma membrane DAG which was confirmed using live-cell imaging and a fluorescent DAG-binding C1aC1b domain from PKC (99). Their results showed that elevation of the glucose concentration resulted in a more complex pattern, consisting of brief, irregular DAG increases of varying amplitude, which could be observed in MIN6, primary mouse β cells and in β cells within intact human islets (99). The glucose-induced DAG spiking was found to be caused by an autocrine feedback loop, which involves release of ATP from insulin-containing vesicles and positive feedback activation of Gq protein-coupled $P2Y_1$ receptors, which resulted in PLC mediated generation of DAG (99). Plasma membrane DAG generation often occurred as bursts of spikes, consistent with the idea that ATP is co-released with insulin in a pulsatile manner. The short duration of each spike is most likely due to transient activation of the purinergic receptor since the released ATP will be either rapidly diluted or degraded by ectonucleotidases as seen in pancreatic β cells and astrocytes (204,205).

It has been suggested that the $P2Y_1$ feedback loop underlying DAG spiking is important to sustain full-amplitude insulin secretion, but it could not be clarified which specific DAG effector mediates this effect, nor could it be excluded that other agents downstream of $P2Y_1$ receptor activation are involved (99). Signal transduction from DAG is generally thought to be mediated via interaction with C1 domain containing proteins. Conventional and novel PKCs were the first DAG targets identified. Other C1 domain-containing proteins include DAG kinases, allowing a quick feedback on DAG concentrations, Munc13-1 and protein kinase D (206,207). Protein kinase

D (PKD) was recently suggested to be important in glucose and muscarinic stimulation of insulin secretion (208–211). PKD, the founding member of a family of serine/threonine protein kinases occupies a unique position in the signal transduction pathways initiated by DAG and PKC. PKD not only is a direct DAG target but also lies downstream of PKCs in a novel signal transduction pathway implicated in the regulation of multiple fundamental biological processes.

1.4b Protein Kinase D

The protein kinase D (PKD) family of serine/threonine kinases belongs to the Calmodulin-dependent protein kinases (CaMKs) superfamily and comprises three isoforms in mammals, PKD1, PKD2, and PKD3. PKD1 was the first member identified in human and mouse in 1994 (212,213). In 1999 and 2001, PKD3 and PKD2, respectively, complemented the family (214,215). PKDs are activated downstream of novel PKCs by phosphorylation of serines within the activation loop of the kinase domain (216,217). PKDs are receptors for diacylglycerol (DAG), which binds to the cysteine-rich domain of the kinases and is crucial for kinase activation. Thus, the local amount of DAG determines the localization and activation state of PKD, thereby restricting PKD responses to specific organelles such as the Golgi complex, the nucleus, and the plasma membrane. It is thus not surprising that PKD is implicated in several intracellular processes and signalling pathways such as cell proliferation, differentiation, apoptosis, immune regulation, cardiac contraction, cardiac hypertrophy, angiogenesis, cancer, vesicle trafficking, survival responses, cell motility and secretion.

1.4b.i Structure of Protein Kinase D

Complementary DNA clones encoding human PKD (initially called atypical PKCμ) and PKD from mouse were identified by two different laboratories in 1994 (212,213). Subsequently, two additional mammalian protein kinases have been identified that share extensive overall homology with PKD, termed PKD2 and PKCν/PKD3 (214,215). The N-terminal regulatory portion of PKD contains a tandem repeat of zinc finger-like cysteine-rich motifs (the cysteine-rich domain termed C1; it is the DAG-binding domain that contains C1a and C1b domains) highly homologous to domains found in DAG sensitive PKCs (218). As described below, the cysteine rich C1 domain plays a critical role in mediating PKD translocation to the plasma membrane and

nucleus in cells challenged with a variety of stimuli and also represses the catalytic activity of the enzyme (219).

Interposed between the C1 domain and the catalytic domain, PKD also contains a pleckstrin homology (PH) domain (220). Found in many signal transduction proteins, PH domains bind to membrane lipids as well as to other proteins. PH domains have also been determined to play an autoregulatory role in some protein kinases, including PKD. Thus, PKD mutants with deletions or with single amino acid substitutions within the PH domain are fully active (220,221), indicating that the PH domain, like the C1 domain, helps to maintain PKD in an inactive catalytic state (Figure 6).

The initial description of PKD as an atypical isoform of PKC (213) and the inclusion of PKD/PKCμ in reviews concerning the PKC family, which belongs to the AGC group (named for PKA, PKG, and PKC) (222,223), contributed to a perception that PKD belongs to the PKC family. However, it was noted from the outset that the catalytic domain of PKD has highest sequence homology with myosin light chain kinase and Ca^{2+}/calmodulin-dependent kinases (CaMKs) (212). Indeed, the three isoforms of PKD are now classified as a new protein kinase family within the CaMK group, separate from the AGC group (224). This scheme reflects the notion that the evolutionary relationship between protein kinases is most appropriately linked to their respective catalytic domain structures. Full-length PKD isolated from multiple cell types or tissues exhibits very low catalytic activity that can be stimulated by phosphatidylserine micelles and either DAG or phorbol esters (225–227). These early studies demonstrated that PKD is a phospholipid/DAG-stimulated serine/threonine protein kinase and implied that PKD represents a novel component of the signal transduction initiated by DAG production in their target cells (228).

1.4b.ii Activation of Protein Kinase D

DAG regulates PKD through dual pathways: (i) DAG regulates PKD localization by binding to its C1 domain; and (ii) DAG induces the activation of PKD by PKC dependent phosphorylation. A milestone in the elucidation of the mechanism of PKD signalling was the discovery that PKC activates PKD (228) (Figure 7).

Studies by Rozengurt and colleagues showed that PKC directly interacts with the PH domain of PKD and transphosphorylates its activation loop at serine 744 and serine 748, leading

to PKD activation (229). Non-phosphorylated PKDs have minimal catalytic activity; activation loop phosphorylation induces a conformational change that maximizes kinase activity. PKC family of enzymes is subdivided into conventional, novel, and atypical isozymes. Conventional PKCs (cPKCs), PKCα, βI, βII, and γ, are activated by DAG and Ca^{2+}. Novel PKCs (nPKCs), PKC δ, ε, η, and θ, respond to DAG but not to Ca^{2+}. The atypical isoforms (aPKCs), PKC ζ and 1/λ, are activated independent of both DAG and Ca^{2+} (230). Pancreatic β cells express members of all three PKC families of isozymes. There is evidence that PKCα, βII, δ, ε, ζ, and 1/λ are expressed whereas the γ isoform is not (231–238). Conflicting results have been reported regarding expression of the βI, η, and θ isoforms (231–233,235–238), which may be a reflection of differences in species, cell lines, and methodology to examine expression of the different isozymes.

Studies by Cantrell and colleagues identified the C-terminal serine 916 of the catalytic domain of PKD as an in vivo phosphorylation site (239). Phosphorylation of the serine 916 site correlated extremely well with PKD catalytic activity. Studies of serine 916 phosphorylation in a set of constitutively active or kinase-dead mutants identified serine 916 as an autophosphorylation site for PKD (239). The nomenclatures discussed so far were according to the amino acid sequence of murine PKD1.

In humans, PKD1 activation is generally attributed to DAG accumulation which colocalize PKD1 at lipid membranes with allosterically activated nPKC isoforms, and promote nPKC-dependent trans-phosphorylation of PKD1 at two highly conserved serine residues in the activation loop, serine 738 and serine 742 (221). The activated form of PKD1 then autophosphorylates at serine 910, a serine at the extreme C terminal of the catalytic domain that resides in a consensus PKD1 phosphorylation motif (240)

1.4b.iii Pharmacological modulators

There are no known specific activators or agonists of PKD. Different agonists such as norepinephrine, endothelin-1, phorbol myristate acetate etc. have been used to activate PKD in the cardiac system (241). But the norepinephrine also activates thrombin and endothelin-1 and phorbol myristate both activate PKD2 (241). However, there are four known specific inhibitors of PKD1: cell permeable PKD inhibitor CID 2011756 (242), selective PKD inhibitor CID 755673 (243), selective PKD inhibitor; analog of CID 755673 called kb NB 142-70 and finally, a potent PKD

inhibitor CRT 0066101 (244). Previously, the non-selective rottlerin, which is a PKCδ inhibitor, has also been used in the pancreatic β cells to inhibit PKD1 (211).

1.4c Role of Protein Kinase D1 in glucose homeostasis

This section will discuss the role of PKD with respect to glucose homeostasis and insulin secretion.

1.4c.i Involvement of PKD1 in glucose transport

PKD has been found to be present in the heart (245). In the study conducted by Luiken et al., they explored whether PKD is activated by contraction, and whether this is linked to glucose uptake in cardiac myocytes via the glucose transport protein GLUT4. They determined that electrically-induced contraction and oligomycin treatment of cardiac myocytes stimulated PKD translocation and phosphorylation at serine 916 (246). They linked the contraction-induced PKD activation to contraction-induced glucose uptake using pharmacological agents and concluded that PKD is responsible for contraction-induced GLUT4-mediated glucose uptake independent of the AMPK pathway (246). However, the pharmacological agents used to inhibit PKD additionally inhibited PKC members and a variety of other kinases (246). Hence, the role of PKD1 in contraction-induced GLUT4 translocation needed more direct evidence. So Dirkx et al. investigated both GLUT4 and CD36 (main cardiac transporter of long chain fatty acids). They observed that silencing of PKD1 abolishes contraction-induced GLUT4 but not CD36 translocation in cardiomyocytes and this was again independent of the AMPK pathway (247). Another study from the same lab by Steinbusch et al. investigated the overexpression of PKD and AMPK to prevent loss of insulin-stimulated glucose uptake in cardiomyocytes incubated under insulin resistance-inducing conditions (248). They induced lipid loading and insulin resistance by exposure of cardiomyocytes to high insulin or high palmitate medium and observed that overexpression of both AMPK and PKD prevented loss of insulin stimulated glucose uptake in cardiomyocytes but through different mechanisms (248). Thus PKD1 plays an important role in glucose transport within cells. This makes it easy to speculate that PKD1 might have a role in potential homeostasis.

1.4c.ii Involvement of PKD1 in insulin granule formation

Baron and Malhotra published that diacylglycerol specifically recruited PKD1 to trans-Golgi network (TGN) and increased its activity of fission of cell surface destined transport carriers from the TGN in mammalian cells (249). In INS-1 cells, Gehart et al. reported that PKD1 phosphorylates Arfaptin-1, which promotes the maintenance of the neck (pronounced curvature of the membrane) between the TGN and the growing secretory granule precursor (250). The regulation of the interaction of Arfaptin-1 with TGN by PKD1 is particularly important because if Arfaptin-1 is not phosphorylated by PKD1, it remains attached to TGN and the insulin secretory granule cannot be cleaved (250). However, in the complete absence of the interaction of Arfaptin-1 with TGN, the secretory granules are separated prematurely and do not possess the membrane components necessary to fuse with the cytoplasmic membrane (250). Therefore, PKD-mediated Arfaptin-1 phosphorylation (once the granule precursor has been completely loaded) is essential to ensure the biogenesis of functional transport carriers at the TGN in regulated secretion. PKD1 can phosphorylate Arfaptin-1 at serine 100 and serine 132. Phosphorylation of Arfaptin-1 at serine 132 inhibits its interaction with ADP ribosylation factors (ARFs) which allows it to remain attached to the TGN and phosphorylation of Arfaptin-1 at serine 100 decreases its binding with the phosphatidylinositols 4-phosphates (PI(4)P) present in the TGN membrane (250,251). Interestingly, these (PI(4)P) are produced at the TGN membrane by phosphatidylinositol-4 kinase III (PI4KIIIβ) and are required for the secretion of the granules synthesized at the TGN (251,252). Since Malhotra et al observed that the kinase activity of PI4KIIIβ is increased when phosphorylated by PKD1 in HEK293 and COS7 cells (251), it is safe to say that PKD1 possibly influences the formation of insulin granules through multiple mechanisms and hence, plays an important role in the formation and final cleavage of insulin granules from the TGN.

1.4c.iii Involvement of PKD in pancreatic β cells and insulin secretion

Sumara et al. reported that autophosphorylation of PKD1 at serine 916 is significantly increased in MIN6 cells or in the pancreas of MAPK p38δ deficient mice compared to control MIN6 cells or the pancreas of wild-type mice (210). An increase in PKD1 activity is thus potentially responsible for the hyperinsulinemia observed in MAPK p38δ deficient mice (210). This increase in insulin secretion appears to because of enhanced activity of PKD1 at the TGN

(membrane fission) and because of PKD1's regulation of the proximal and distal steps of exocytosis (independent of the concentration of calcium) resulting in an enhanced glucose stimulated insulin secretion (210). They also observed that in the wild-type mice, MAPK p38δ negatively regulates PKD1 by inhibiting its action to increase glucose induced insulin secretion (210). Thus, Sumara et al. were the first to identify PKD1 as a pivotal regulator of glucose stimulated insulin exocytosis.

Kong et al investigated the effect on insulin secretion from mouse islets containing a phosphorylation deficient mutant form of M3-muscarinic receptor gene in response to an agonist. This mutation of the muscarinic receptor in the islets of Langerhans reduced the recruitment of β arrestins ex vivo and the potentiation of insulin secretion in response to Methacholine (209). The authors also found that activation of the muscarinic receptor in vitro and ex vivo caused an increase in phosphorylation of PKD1 even in the presence of PLC inhibitor or calcium chelator (209). Phosphorylation of PKD1 was, however, strongly reduced in the islets as a result of the muscarinic receptor mutation which reduced β arrestin recruitment and in a cell line where β arrestin 1 expression was significantly decreased (209). In addition, a decrease in the expression of PKD1 in the islets reduced the secretion of insulin in response to a muscarinic receptor agonist (209). Hence they concluded that the decrease in insulin secretion following a muscarinic receptor mutation is due to a lower recruitment of β arrestin that results in lower activation of PKD1 (209).

Ferdaoussi et al. provided evidence that PKD is rapidly activated at serine 744/748 and at serine 916, in a GPR40-dependent manner, in response to the fatty acid, oleate. They showed that pharmacological inhibition or deletion of the gene encoding PKD1 abrogates the potentiation of GSIS by oleate without significantly affecting GSIS itself (211). These results therefore demonstrated a key role for PKD1 in GPR40 receptor signalling in response to fatty acids. They also proposed a mechanism for PKD1 to promote second-phase insulin secretion in response to fatty acids based on the fact that cortical actin depolymerisation in response to oleate requires PKD. And demonstrated that deletion of the gene encoding PKD1 in islets prevented oleate-induced F-actin depolymerisation (211). In summary, Ferdaoussi et al. found that PKD1 is important for the oleate-induced F-actin depolymerisation and also for potentiation of GSIS as they are lost upon pharmacological inhibition of PKD1 or deletion of PKD1 gene.

Iglesias et al. published that autophosphorylation at serine 916 of PKD1 is significantly higher in pancreatic β cells deficient for PPARβ/δ (253). Ex vivo, islets deficient for PPARβ/δ have a significantly higher second phase of glucose-induced insulin secretion (253). This increase in the second phase of insulin secretion is due the number of membrane granule fusions, measured by fluctuations in membrane area, that is significantly increased in depolarized PPARβ/δ deficient pancreatic β cells (253). This increase in insulin vesicle fusion appears to be the result of increased depolymerization of actin filaments in PPARβ/δ deficient pancreatic β cells (253). The Golgi marker giantin and the TGN marker TGN38 showed an extended and diffused distribution in the absence of PPARβ/δ in isolated β cells, but distribution of the marker returned to normal when PKD1 was inhibited with a synthetic agent (253) which led the authors to believe that PPARβ/δ participates in mechanisms regulating membrane fission at the TGN through the regulation of PKD1 activity. This study once again highlighted the importance of PKD1 in enhanced insulin release during the second phase of glucose stimulated insulin secretion.

Bergeron et al. conducted the latest study this year where they examined the role of PKD1 in β cell-specific, inducible PKD1 knockout mice (βPKD1KO) under chow fed and high-fat diet fed conditions. Under basal conditions, the authors observed no significant differences in βPKD1KO and the MIP-CreERT mice in terms of the results of oral glucose tolerance test, hyperglycemic clamps and GSIS experimets (208). This raises the intriguing question whether the isoforms, PKD2 and PKD3, compensate for PKD1 under basal conditions or not. In contrast, under high-fat diet fed conditions, the βPKD1KO mice were hyperglycemic, had elevated levels of insulin i.e. they were hyperinsulinemic and were much more glucose intolerant compared to the MIP-CreERT mice (208). So far, all the evidence related to the role of PKD1 in regulating GSIS was based on ex vivo studies. Bergeron et al. provided the first in vivo evidence that deletion of PKD1 in pancreatic β cells was accompanied by impaired insulin secretion in hyperglycemic clamp studies (despite the mice being hyperinsulinemic) (208). Glucose stimulated insulin secretion in isolated islets from high-fat diet fed βPKD1KO mice at high glucose also resulted in significantly reduced insulin secretion. This defect in insulin secretion was not due to any observed changes in islet mass of the groups studied (208). And through these experiments, Bergeron et al. demonstrated an essential role for PKD1 in the compensatory increase in glucose stimulated insulin secretion in response to high-fat feeding in mice.

Zhang et al. also published this year about PKD where phosphatidylinositol-4-kinase IIα (PI4KIIα) negatively regulated PKD activity via protein - protein interaction. They generated PI4KIIα transgenic mice and observed that the mice have abnormal glucose tolerance and higher serum glucose levels (208). Furthermore, they showed that glucose stimulated insulin secretion was significantly reduced in both PI4KIIα transgenic mice and PI4KIIα - overexpressing pancreatic β cell lines (208). They identified PKD as the protein that interacted with PI4KIIα by employing a proximity - based biotin labeling technique, BioID. They also studied co-localization of PI4KIIα, PKD, and insulin, interaction between PKD and PI4KIIα and regulation of PKD activity by PI4KIIα with respect to insulin secretion. The reduction effect of PI4KIIα on insulin secretion was completely rescued by treatment with PKD agonist, TPA, whereas the PKD inhibitor, CID755673, blocked the PI4KIIa siRNA-mediated increasing of insulin secretion (208). Together, these results by Zhang et al. indicated that the negative regulation of insulin secretion by PI4KIIα is dependent on PKD activity.

This PKD1 appears to play a pivotal role in multiple steps of insulin secretion and actively contributes to the potentiation of glucose induced insulin secretion as attributed by the handful of studies outlined above. Evidence also suggests that there is an autocrine $P2Y_1$ feedback loop in pancreatic β cells where DAG recruits conventional and novel PKCs and it is quite well known that PKD1 is a direct target of both DAG and PKCs. This raises the question whether PKD1 is involved in the positive autocrine feedback loop of the purinergic $P2Y_1$ receptor and whether it plays any role in the regulation of insulin secretion in pancreatic β cells in mouse and humans. Therefore, this thesis investigates the activation of PKD1, its role in exocytotic response and regulation of insulin secretion in mouse and human islets. Chapter 3 of this thesis includes to be published report on autocrine activation of PKD1.

Figure 6: Schematic structure of PKD1

Domain structure and regulatory phosphorylation sites in PKD1. Mammalian PKD1 has highly conserved DAG-binding (C1a, C1b) cysteine-rich zinc finger domain, Pleckstrin Homology (PH) and catalytic domain. Serine residues within the activation loop of PKD become phosphorylated via novel PKCs.

Figure 6.

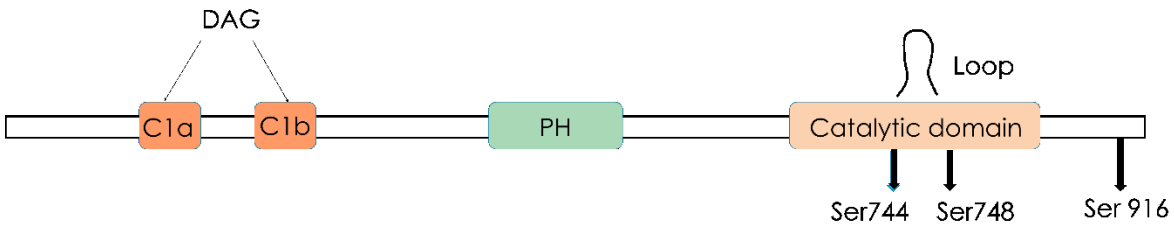
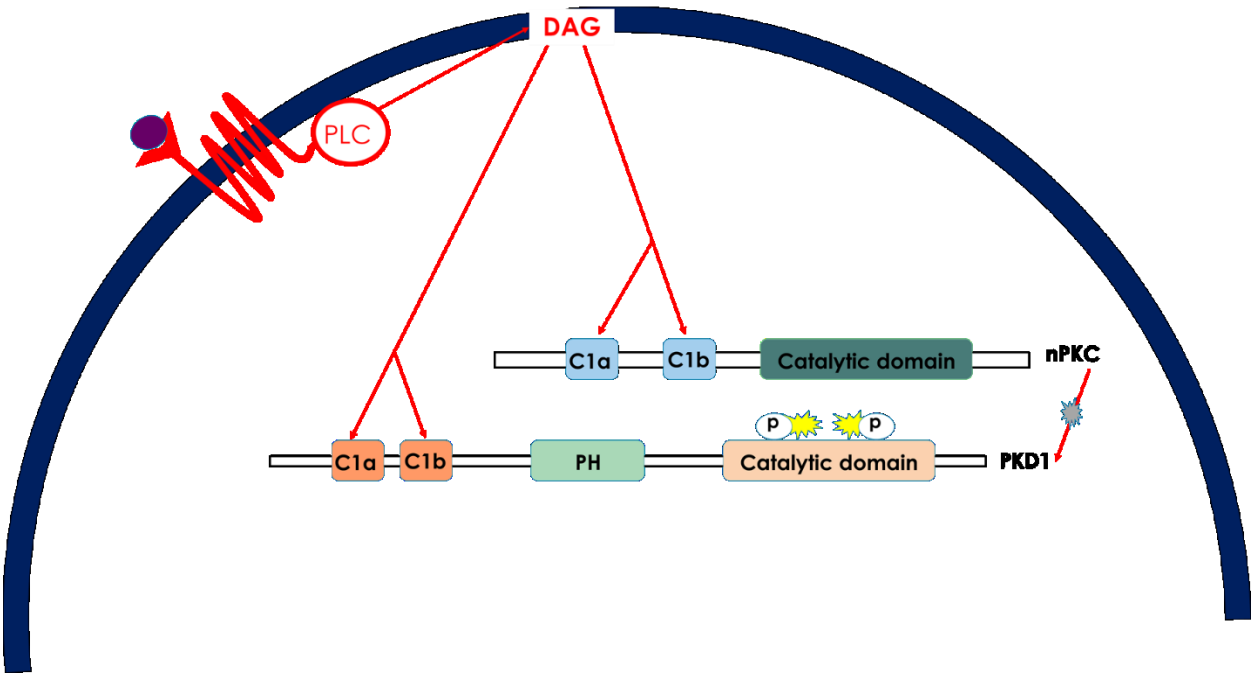


Figure 7: Activation of PKD1

The binding of seven transmembrane G-protein coupled receptor agonists to their respective receptors cause the activation of PLC, and subsequent DAG production. DAG, on the one hand, targets PKD to different subcellular compartments by binding to the C1a/C1b domain of PKD and, on the other hand, activates novel PKC (nPKC) isoforms that, in turn, phosphorylate PKD at the activation loop and cause its activation. Serine residues within the activation loop of PKD that become phosphorylated via nPKCs are indicated by phosphorylation (p). The activated PKD contributes to a range of cellular responses

Figure 7.


DAG
PLC
C1a
C1b
Catalytic domain
nPKC
P
P
C1a
C1b
PH
Catalytic domain
PKD1

1.5 General Hypothesis

The present thesis hypothesizes that $P2Y_1$ is the predominant purinergic receptor subtype in pancreatic human β cells. ATP is released by the pancreatic β cells upon glucose stimulated exocytosis and activates $P2Y_1$ receptors. Autocrine purinergic signalling modulates the amplitude and kinetics of insulin secretion by regulating membrane excitability and Ca^{2+} signals by activating protein kinase D1 in pancreatic β cells. Both of these pathways contribute to the potentiation of glucose stimulated insulin secretion in mouse and humans.

1.6 Specific aims

1.6a **AIM 1: Characterizing the role of $P2Y_1$ receptors in glucose-induced Ca^{2+} signalling in human pancreatic β cells**

There is strong evidence that ATP and ADP serve as autocrine messengers in pancreatic β cells, but the functional effects and detailed mechanisms of action are under debate. Therefore, the receptor subtype(s) and mechanism(s) mediating the effects of ATP on isolated human β cells were investigated. The effects of purinergic agonists and antagonists on membrane potential, membrane currents, intracellular Ca^{2+} ($[Ca^{2+}]_i$) and insulin secretion in human β cells were examined.

1.6b **AIM 2: Evaluating the role of $P2Y_1$ autocrine signalling via the activation of protein kinase D1 and insulin secretion in mouse and human islets.**

$P2Y_1$ activation activates DAG, which has been shown to activate PKD1. Therefore, western blotting was performed to study agonist-induced, depolarization-induced and antagonist-inhibited activation of PKD1 in response to KCl in INS 832/13 insulinoma cells and in mouse islets. Insulin secretion was measured from intact PKD1 knockout islets. Capacitance measurements of exocytosis were employed in single mouse β cells. Expression of PKD1 mRNA was analysed by quantitative RT-PCR in human islets. Correlation between the insulinotropic capacity of PKD1 activation and donor characteristics was examined in human islets.

CHAPTER 2: AUTOCRINE ACTIVATION OF P2Y1 RECEPTORS COUPLES CA^{2+} INFLUX TO CA^{2+} RELEASE IN HUMAN PANCREATIC B CELLS

The following chapter is published in Diabetologia, and is reprinted with the permission of the European Association for the Study of Diabetes:

Shara Khan, Richard Yan-Do, Eric Duong, Xichen Wu, Austin Bautista, Stephen Cheley, Patrick E. MacDonald. Autocrine activation of P2Y1 receptors couples Ca^{2+} influx to Ca^{2+} release in human pancreatic β cells. Diabetologia, 2014 Dec; 57(12):2535-45

Contributions by co-authors to the figures presented are stated in the figure legends, accompanied by the appropriate co-authors' initials.

2.1 Abstract

AIMS/HYPOTHESIS:

There is evidence that ATP acts as an autocrine signal in β cells but the receptors and pathways involved are incompletely understood. Here we investigate the receptor subtype(s) and mechanism(s) mediating the effects of ATP on human β cells.

METHODS:

We examined the effects of purinergic agonists and antagonists on membrane potential, membrane currents, intracellular $Ca(^{2+})$ ($[Ca(^{2+})]_i$) and insulin secretion in human β cells.

RESULTS:

Extracellular application of ATP evoked small inward currents (3.4 ± 0.7 pA) accompanied by depolarisation of the membrane potential (by 14.4 ± 2.4 mV) and stimulation of electrical activity at 6 mmol/l glucose. ATP increased $[Ca(^{2+})]_i$ by stimulating $Ca(^{2+})$ influx and evoking $Ca(^{2+})$ release via InsP3-receptors in the endoplasmic reticulum (ER). ATP-evoked $Ca(^{2+})$ release was sufficient to trigger exocytosis in cells voltage-clamped at -70 mV. All effects of ATP were mimicked by the $P2Y_{(1/12/13)}$ agonist ADP and the $P2Y_1$ agonist MRS-2365, whereas the $P2X_{(1/3)}$ agonist α,β-methyleneadenosine-5-triphosphate only had a small effect. The $P2Y_1$ antagonists MRS-2279 and MRS-2500 hyperpolarised glucose-stimulated β cells and lowered $[Ca(^{2+})]_i$ in the absence of exogenously added ATP and inhibited glucose-induced insulin secretion by 35%. In voltage-clamped cells subjected to action potential-like stimulation, MRS-2279 decreased $[Ca(^{2+})]_i$ and exocytosis without affecting $Ca(^{2+})$ influx.

CONCLUSIONS/INTERPRETATION:

These data demonstrate that ATP acts as a positive autocrine signal in human β cells by activating $P2Y_1$ receptors, stimulating electrical activity and coupling $Ca(^{2+})$ influx to $Ca(^{2+})$ release from ER stores.

2.2 Introduction

In addition to serving as an energy carrier and intracellular signal, ATP has an important role as an extracellular signal and neurotransmitter. After its release from cells by exocytosis or via non-vesicular pathways, ATP activates two types of purinergic P2 receptors in the plasma membrane. P2X receptors are ligand-gated non-selective cation channels, while P2Y receptors are G-protein coupled. In humans, the P2X and P2Y families comprise 7 and 11 isoforms, respectively (154,170).

ATP is present at millimolar concentrations in insulin granules (65,97) and is released from β cells upon glucose stimulation (96,119,123). There is evidence for the expression of both P2X and P2Y receptors in rat and mouse β cells, suggesting that ATP acts as an autocrine signal in islets, although it is debatable whether purinergic signalling stimulates or inhibits insulin secretion (75,102). Overall, the few studies that have been conducted in human islets suggest a stimulatory role for ATP (100,107,108). However, controversy exists regarding the receptor subtypes and signal transduction pathways involved. While one study proposed that ATP acts principally via $P2X_3$ receptors, membrane depolarisation and increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (100), a more recent study suggested a prominent role for $P2Y_1$ receptors and activation of protein kinase C (107). Involvement of $P2X_7$ has also been proposed (254). An ATP-evoked, P2X-mediated membrane current in human β cells has been suggested (184), although the effect of ATP on glucose-induced electrical activity has not been investigated.

We sought to characterise the effects of extracellular ATP on membrane currents and membrane potential in human β cells. We found that the effects of ATP were mimicked by the P2Y agonist ADP and demonstrated that autocrine activation of $P2Y_1$ receptors plays a significant role in the regulation of electrical activity, $[Ca^{2+}]_i$ and insulin secretion in human β cells. Autocrine signalling via $P2Y_1$ represents a novel link between Ca^{2+} influx and Ca^{2+} release from intracellular stores.

2.3 Methods

2.3a Materials

MRS-2279, MRS-2365, MRS-2500, α,β-methyleneadenosine-5-triphosphate (α,β-meATP), 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), heparin, thapsigargin and bafilomycin A1 were from R&D Systems (Minneapolis, MN, USA). Fura-2AM and Fura-2 Na^+-salt were from Life Technologies (Burlington, ON, Canada). Nucleotides and other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

2.3b Islet isolation, culture and transfection

Human islets were from the Clinical Islet Laboratory at the University of Alberta or the Alberta Diabetes Institute IsletCore [15, 16]. The study was approved by the local Human Research Ethics Board. Islets were dispersed in Ca^{2+}-free buffer and then plated onto plastic or glass-bottom Petri-dishes (In Vitro Scientific, Sunnyville, CA, USA) and incubated in RPMI-1640 medium containing 7.5 mmol/l glucose for at least 24 h before experiments. For measuring ATP release, cells were infected with an adenovirus encoding a $P2X_2$–green fluorescent protein fusion protein (Ad$P2X_2$-GFP) for 24–48 h [5]. All experiments, except for the assessment of insulin secretion, were carried out using dispersed β cells.

2.3c Immunohistochemistry

Paraffin-embedded tissue sections were heated in 10 mmol/l Na^+-citrate (pH 6) for 10 min. Sections were blocked using 20% goat serum and incubated with anti-$P2Y_1$ (1:50 dilution; P6487; Sigma-Aldrich) and anti-insulin antibodies for 1 h, followed by fluorescently labelled secondary antibodies. Images were captured using a Zeiss Apotome inverted microscope (Carl Zeiss Canada, Toronto, ON, Canada). Identification of β cells by immunocytochemistry after patch-clamp and Ca^{2+} imaging was as described previously (255).

2.3d Ca^{2+} imaging

Cells were pre-incubated with Fura-2AM (1 μmol/l) for 15 min. Glass-bottom Petri dishes were mounted onto an inverted microscope (Zeiss Axioobserver, Carl Zeiss Canada Ltd.) equipped with an ICCD-camera and a rapid-switching light source (Oligochrome; Till Photonics, Grafelfing,

Germany). Fluorophore, excited at 340 and 380 nm (intensity ratio 10:4) and emission detected at 510 nm, was imaged at 0.5 Hz using Life Acquisition software (Till Photonics). β cells were identified by immunostaining and fluorescence ratios were calculated using ImageJ (v1.46r; http://imagej.nih.gov.login.ezproxy.library.ualberta.ca/ij/).

2.3e Insulin secretion

Fifteen size-matched islets (in triplicates) were pre-incubated in 0.5 ml KRB buffer containing 1 mmol/l glucose and 0.1% BSA for 1 h, followed by a 1 h test incubation in KRB with the indicated glucose concentrations and test substances. The supernatant fraction was removed and the insulin concentration was determined using the MSD human insulin kit (Meso-Scale Discovery, Rockville, MD, USA).

2.3f Electrophysiology

Patch-clamp was performed using an EPC-10 amplifier and Patchmaster software (Heka Electronics, Lambrecht, Germany). Patch-pipettes were pulled from borosilicate glass (resistance 3–8 MΩ; Sutter Instruments, Novato, CA, USA). Solutions for whole-cell and perforated-patch recording are detailed in the electronic supplementary materials (ESM) Methods. Cells were continuously superfused (~1 ml/min) with extracellular solution at ~32°C. Rapid application of ATP was performed using a Fast-Step system (Warner Instruments, Hamden, CT, USA). B cells were identified by immunostaining or based on cell size (12.5 ± 0.3 pF; n = 189) (255).

2.3g PCR analysis

Expression of P2Y receptors ($P2RY_{1–14}$) was analysed by RT-PCR in RNA purified from isolated human islets, using a previously described protocol (256). Primer sequences are detailed in ESM Methods.

2.3h Data analysis

Data are presented as means ± SEM. The n values represent the number of cells, unless indicated otherwise. Statistical significance was evaluated using Student's t test, or by multiple-comparison ANOVA and Bonferroni post test when comparing multiple groups.

2.4 Results

2.4a Membrane currents evoked by purinergic receptor agonists

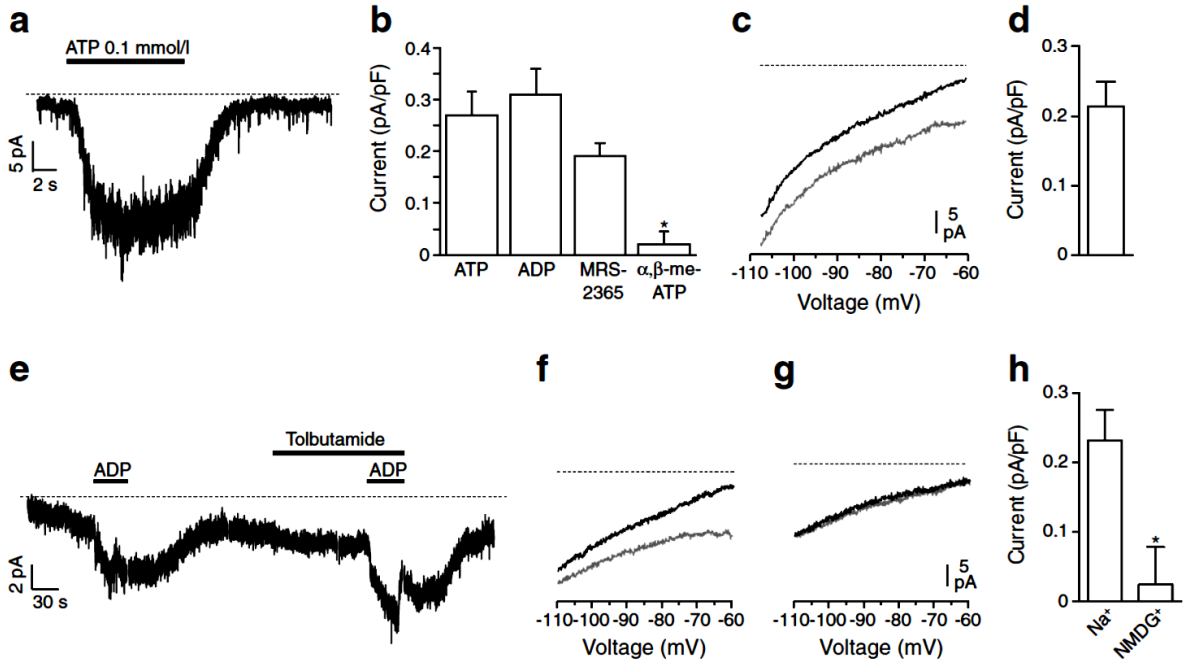
Using the whole-cell configuration in cells held at −70 mV in 6 mmol/l glucose, extracellular ATP application evoked a detectable inward current in 25% of human β cells (15 out of 60). The maximal current amplitude was 24 pA (Fig. 8a). In responding cells, the ATP-activated current averaged 6.7 ± 1.7 pA and was reduced 65 ± 17% by the P2 receptor blocker suramin (100 μmol/l; $p < 0.05$, $n = 5$).

In perforated-patch whole-cell recordings, ATP-evoked an inward current in all β cells (1.2–8.6 pA) and averaged 3.4 ± 0.7 pA (Fig. 8b). Similar responses were obtained with the $P2Y_{(1/12/13)}$ agonist ADP (4.2 ± 0.6 pA; 0.4–11.1 pA) and the $P2Y_1$ agonist MRS-2365 (2.4 ± 0.3 pA), but not the $P2X_{(1/3)}$ agonist α,β-meATP (Fig. 8b). The ATP- or ADP-evoked current was inhibited 78 ± 13% by the $P2Y_1$ antagonist MRS-2279 (2–3 μmol/l, $p < 0.05$, $n = 4$). The ADP-evoked current was inward during voltage ramps from −110 to −60 mV (Fig. 8c, d) and was not inhibited by tolbutamide (4.2 ± 1.1 pA, $n = 4$; Fig. 8e). The rapid upstroke in Fig. 8e following application of tolbutamide and ADP is likely to be an artefact. Instead, the current was attenuated (by 90 ± 25%; $p < 0.05$, $n = 5$) when Na^+ was replaced by the membrane-impermeable cation N-methyl-d-glucamine ($NMDG^+$) (Fig. 8f–h).

Figure 8: Effect of purinergic agonists on resting membrane currents.

(a) Whole-cell membrane current evoked by extracellular application of 0.1 mmol/l ATP, data collection by ED (b) Average amplitudes (normalised to cell size) of inward currents evoked by ATP (10 µmol/l, n=15 cells), ADP (2 µmol/l, n=40 cells), MRS-2365 (0.1 µmol/l, n=5 cells, two donors) and α,β-meATP (10 µmol/l, n=4 cells). (c) Membrane currents evoked by voltage ramps under control conditions (black trace) and after application of 2 µmol/l ADP (grey trace), data collection by ED. (d) Average ADP-activated inward current measured at −70 mV (n=21). (e) Membrane current evoked by 2 µmol/l ADP in the absence and presence of 0.2 mmol/l tolbutamide, data collection by MB. (f, g) Control (black traces) and ADP (2 µmol/l) evoked membrane currents (grey traces) during voltage ramps under control conditions (f) and after replacement of extracellular Na^+ with $NMDG^+$ (g), data collection by MB. (h) The ADP-activated currents were quantified at −70 mV (n=5, two donors). Data is from three to seven donors unless indicated otherwise. *$p<0.05$ compared with ATP or Na^+

Figure 8.


a
ATP 0.1 mmol/l
5 pA
2 s
b
Current (pA/pF)
0.4
0.3
0.2
0.1
0
ATP
ADP
MRS-2365
α,β-me-ATP
*
c
5 pA
-110 -100 -90 -80 -70 -60
Voltage (mV)
d
Current (pA/pF)
0.3
0.2
0.1
0
e
Tolbutamide
ADP
ADP
2 pA
30 s
f
-110 -100 -90 -80 -70 -60
Voltage (mV)
g
5 pA
-110 -100 -90 -80 -70 -60
Voltage (mV)
h
Current (pA/pF)
0.3
0.2
0.1
0
*
Na+
NMDG+

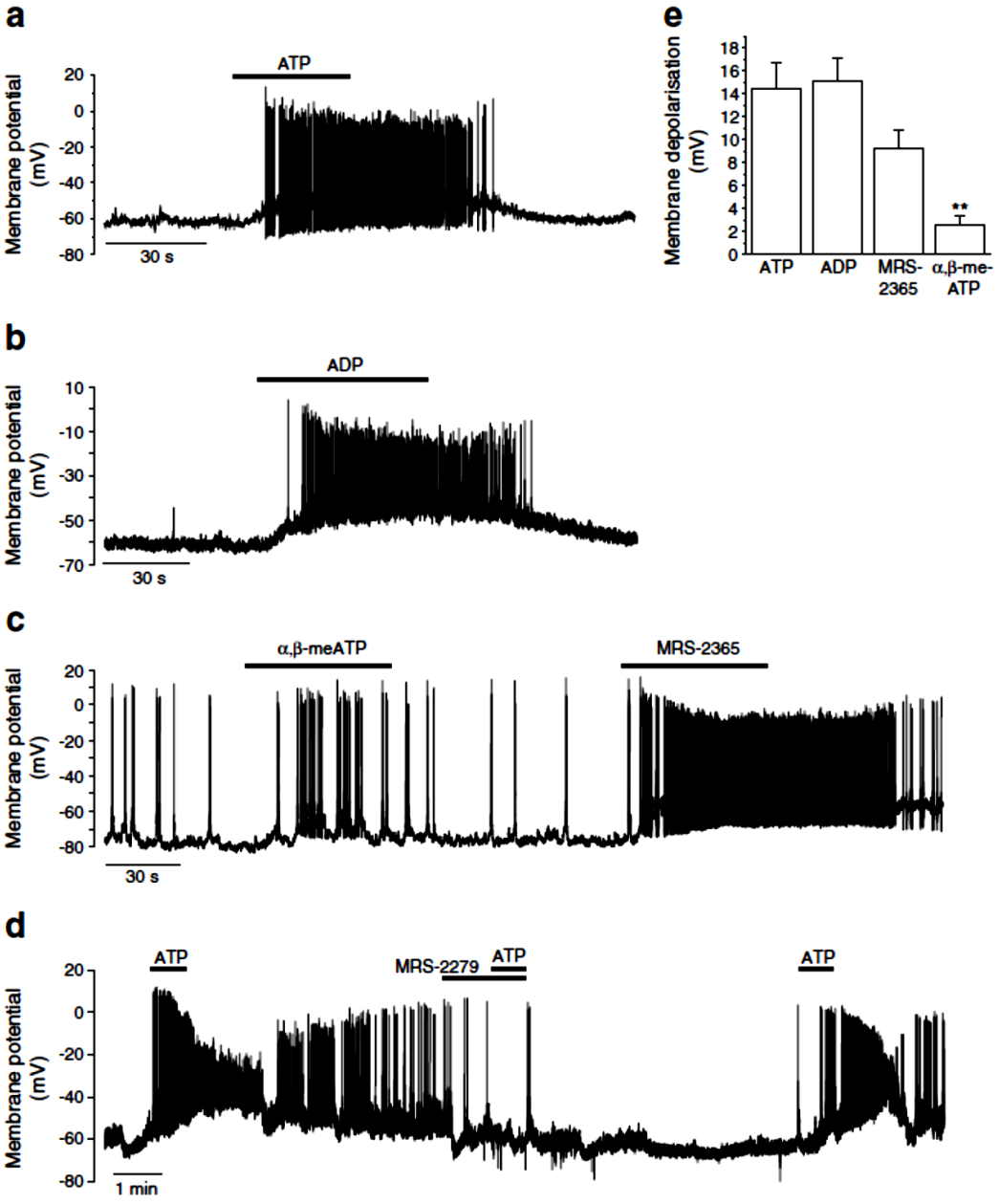
2.4b Effect of purinergic agonists on the membrane potential

At 6 mmol/l glucose, isolated β cells exhibited varying degrees of action potential firing (Fig. 9). Application of ATP (10 μmol/l) depolarised cells (from −53.7 ± 2.2 to −39.3 ± 2.9 mV, Fig. 9a, e) and stimulated or augmented action potential firing. This effect was mimicked by ADP, which depolarised human β cells from −53.6 ± 2.1 to −38.6 ± 2 mV (Fig. 9b, e). UTP, an agonist at $P2Y_{(2/6/8)}$ receptors, had no effect (data not shown). The $P2Y_1$ agonist MRS-2365 also potently depolarised the cells (from −53.3 ± 4 to −44.2 ± 2.9 mV, Fig. 9c, e), while the $P2X_{(1/3)}$ agonist α,β-meATP depolarised the membrane potential only slightly (from −59.7 ± 4.8 to −57.2 ± 4.1 mV, Fig. 9c, e). The effect of ATP was prevented in four out of five cells by the $P2Y_1$ antagonist MRS-2279 (Fig. 9d). ATP did not directly modulate voltage-gated Ca^{2+} or K^+ currents (Supplemental data Fig. 1).

Figure 9: Effect of purinergic agonists on the membrane potential. Membrane potential recordings from human β cells by perforated patch.

(a) ATP (10 μmol/l) was applied as indicated by the bar. (b) ADP (1 μmol/l) was added as indicated. (c) Effect of α,β-meATP (10 μmol/l) and the agonist MRS-2365 (0.1 μmol/l) in the same cell. (d) ATP (10 μmol/l) was applied in the absence or presence of the antagonist MRS-2279 (1 μmol/l) as indicated. (e) Average depolarisation evoked by ATP (10 μmol/l, n=16), ADP (1–2 μmol/l, n=11), MRS-2365 (0.1 μmol/l, n=11) and α,β-meATP (10 μmol/l, n=7), all the data collected by MB. Data are from four to six donors in each experiment. **$p<0.01$ compared with ATP

Figure 9.


a
ATP
Membrane potential (mV)
20
0
-20
-40
-60
-80
30 s
e
Membrane depolarisation (mV)
18
16
14
12
10
8
6
4
2
0
**
ATP
ADP
MRS-2365
α,β-me-ATP
b
ADP
Membrane potential (mV)
10
-10
-30
-50
-70
30 s
c
α,β-meATP
MRS-2365
Membrane potential (mV)
20
0
-20
-40
-60
-80
30 s
d
ATP
MRS-2279
ATP
ATP
Membrane potential (mV)
20
0
-20
-40
-60
-80
1 min

2.4c Effect of purinergic agonists on $[Ca^{2+}]_i$

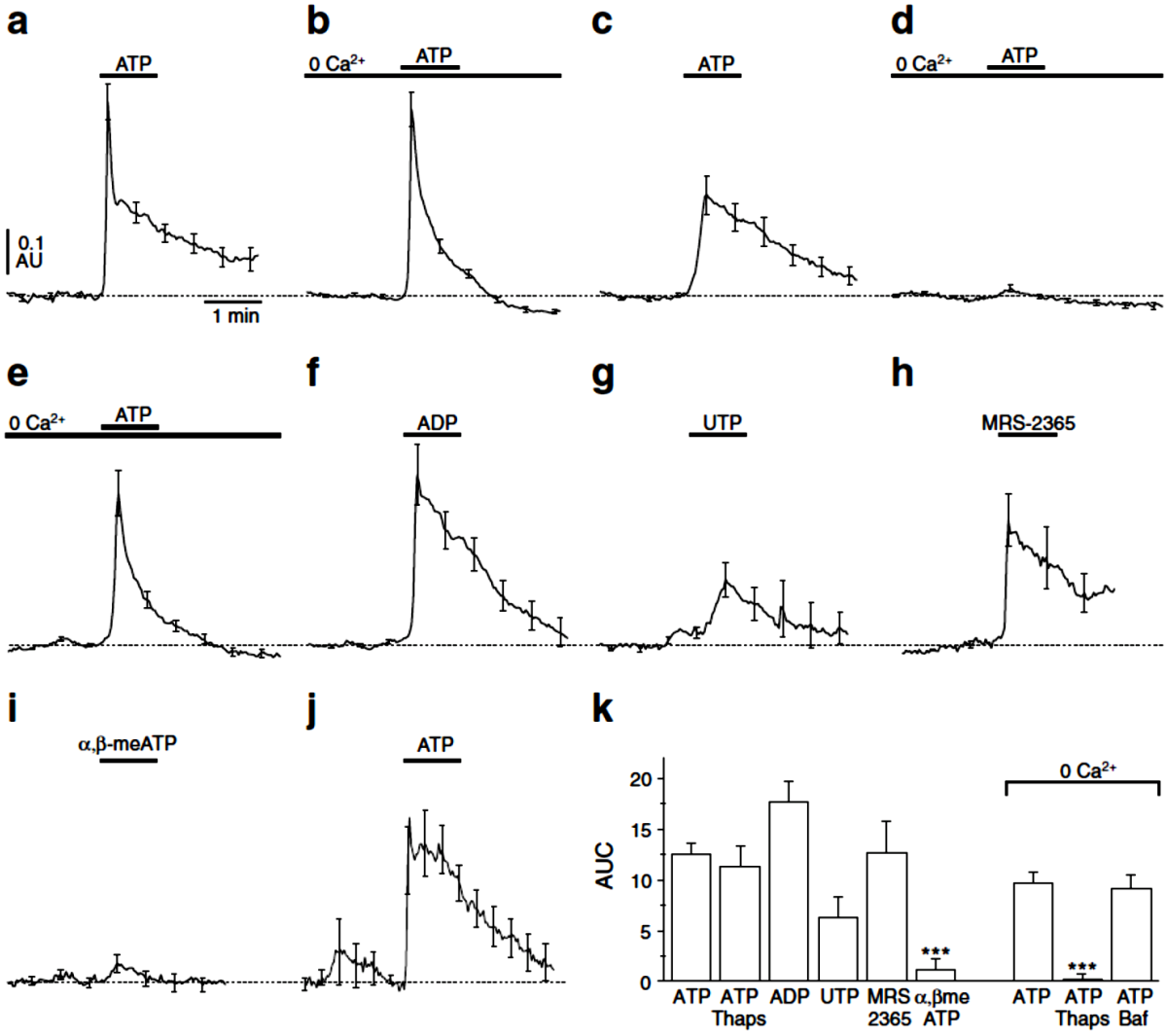
At 6 mmol/l glucose, ATP induced a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial rapid spike followed by a plateau (Fig. 10a). In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ spike was largely unchanged, whereas the plateau was reduced (Fig. 10b). Pretreatment of cells with thapsigargin removed the $[Ca^{2+}]$i spike without affecting the plateau when extracellular Ca^{2+} was present, and completely suppressed the $[Ca^{2+}]_i$ increase under Ca^{2+}-free conditions (Fig. 10c, d, k). In contrast bafilomycin A1, which depletes acidic Ca^{2+} stores, had little effect on the $[Ca^{2+}]_i$ signal (Fig. 10e, k). The effect of ATP on $[Ca^{2+}]_i$ was mimicked by ADP and MRS-2365 (Fig. 10f, h, k). UTP and α,β-meATP evoked only small responses (Fig. 10g, i, k). B cells from a donor with type 2 diabetes were observed to have an ATP-sensitive Ca^{2+} response that appeared smaller than the response of healthy β cells, but did not attain statistical significance and lacked the initial rapid spike (Fig. 10j).

To identify the intracellular Ca^{2+} channel underlying ATP-induced Ca^{2+} release, $[Ca^{2+}]_i$ was measured in cells voltage-clamped at −70 mV and infused with the $InsP_3$ receptor blocker heparin. The $[Ca^{2+}]_i$ increase evoked by extracellular ATP was completely suppressed by heparin (Fig. 11a, b), suggesting that the ATP-evoked $[Ca^{2+}]_i$ spike results from $InsP_3$-dependent endoplasmic reticulum (ER) Ca^{2+} release while the plateau is due to Ca^{2+} influx through plasma membrane channels.

Figure 10: Effect of purinergic agonists on $[Ca^{2+}]_i$.

(a–j) Averaged $[Ca^{2+}]_i$ responses shown to the same scales (AU, arbitrary units) at 6 mmol/l glucose ($[Ca^{2+}]_i$ spikes of individual β cells are not apparent in these averaged traces), data collection by RY, MB and SK. ATP (10 µmol/l) was applied under the following conditions: (a) under control conditions (n=68); (b) in the absence of extracellular Ca^{2+} (n=63); (c) to cells pretreated with thapsigargin (10 µmol/l, 10 min, n=27); (d) to cells pretreated with thapsigargin in the absence of extracellular free Ca^{2+} (n=21, two donors) and (e) to cells pretreated with bafilomycin A1 (0.1–2 µmol/l, 10 min) in the absence of extracellular Ca^{2+} (n=27). (f) ADP (2 µmol/l) was applied (n=18). (g) Effect of UTP (10 µmol/l, n=13). (h) Effect of MRS-2365 (0.1 µmol/l, n=9). (i) α,β-meATP (10 µmol/l) was added (n=20). (j) ATP (10 µmol/l) was added to β cells from a donor with type 2 diabetes (n=7). (k) Bar graphs showing average AUC (baseline-subtracted) during agonist application (Baf, bafilomycin A1; Thaps, thapsigargin). Data are from three to seven donors unless stated otherwise. ***$p<0.001$ compared with ATP

Figure 10.


a
ATP
0.1
AU
1 min
b
0 Ca2+
ATP
c
ATP
d
0 Ca2+
ATP
e
0 Ca2+
ATP
f
ADP
g
UTP
h
MRS-2365
i
α,β-meATP
j
ATP
k
AUC
20
15
10
5
0
0 Ca2+
***
***
ATP
ATP Thaps
ADP
UTP
MRS 2365
α,βme ATP
ATP
ATP Thaps
ATP Baf

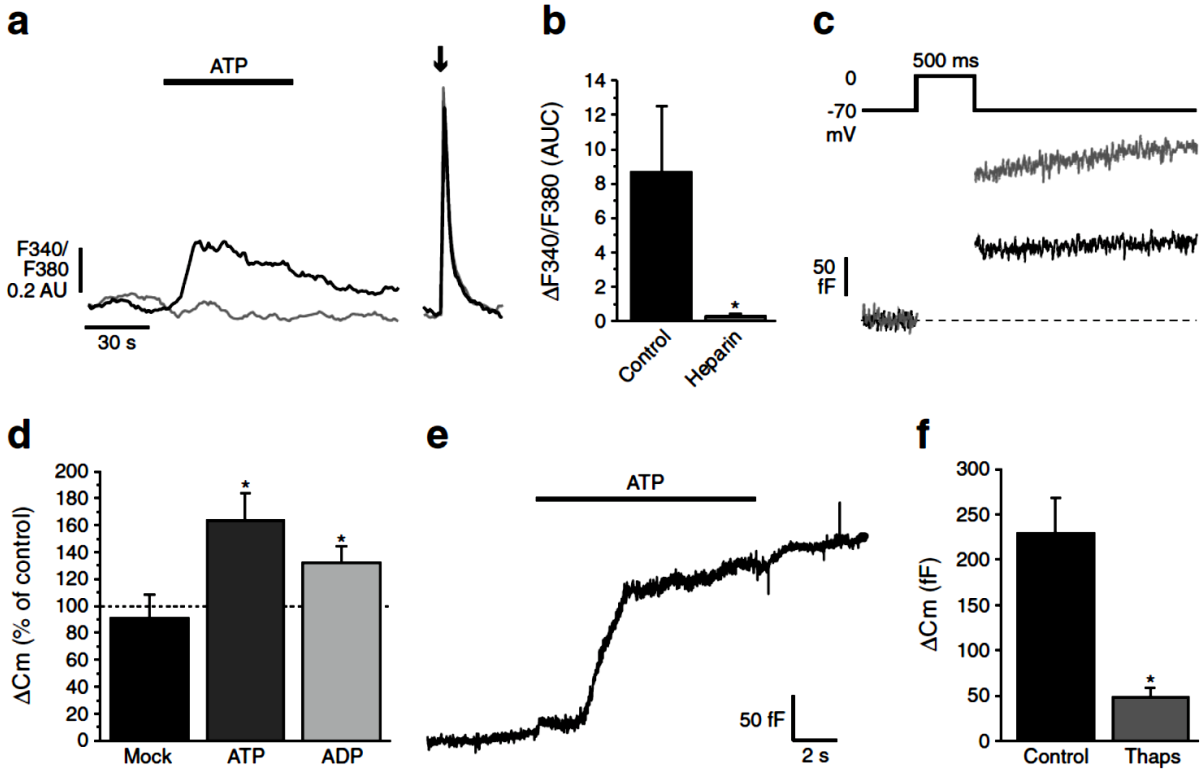
2.4d Effect of ATP on exocytosis

Exocytosis was elicited by voltage-clamp depolarisations from −70 to 0 mV, which triggers Ca^{2+} influx through voltage-gated Ca^{2+} channels. Exocytosis was potentiated 64% and 32% by extracellular application of ATP and ADP, respectively (Fig. 11c, d). We next monitored the membrane capacitance in cells clamped at −70 mV to prevent opening of voltage-gated Ca^{2+} channels, and in the presence of 5 mmol/l glucose. ATP application alone was sufficient to evoke a clear exocytotic response under these conditions (Fig. 11e). This response was strongly inhibited in cells pretreated with thapsigargin (Fig. 11f).

Figure 11: Effect of ATP on Ca^{2+} release and exocytosis**.**

(a) $[Ca^{2+}]_i$ was monitored in voltage-clamped cells, without (control, black trace) or with addition of 0.2 mg/ml heparin (grey trace). ATP was added as indicated. The $[Ca^{2+}]_i$ signal evoked by a 500 ms depolarisation from −70 to 0 mV (arrow) was used as a control, data collection by MB. (b) Average integrated $[Ca^{2+}]_i$ responses in experiments as described in (a) (n=7 and 4). (c) Representative traces showing capacitance responses before (black trace) and after addition of ATP (10 µmol/l, grey trace) in the same cell, data collection by MB. (d) Average changes in exocytotic responses (ΔCm) after mock application (n=5) or application of ATP (10 µmol/l, n=15) or ADP (1 µmol/l, n=9), normalised to control values in the same cells. (e) Capacitance response evoked by application of ATP (100 µmol/l) in a cell clamped at −70 mV, data collection by AB. (f) Average exocytotic responses (ΔCm) evoked by ATP under control conditions (n=18) and in cells pretreated with thapsigargin (Thaps; 10 µmol/l, 10 min; n=6), calculated as the change in average Cm from the 5 s immediately before and immediately after ATP application. Data are from three to four donors. *$p<0.05$ compared with control

Figure 11.


a
ATP
F340/
F380
0.2 AU
30 s
b
ΔF340/F380 (AUC)
14
12
10
8
6
4
2
0
Control
Heparin
c
500 ms
0
-70
mV
50
fF
d
ΔCm (% of control)
200
180
160
140
120
100
80
60
40
20
0
Mock
ATP
ADP
e
ATP
50 fF
2 s
f
ΔCm (fF)
300
250
200
150
100
50
0
Control
Thaps

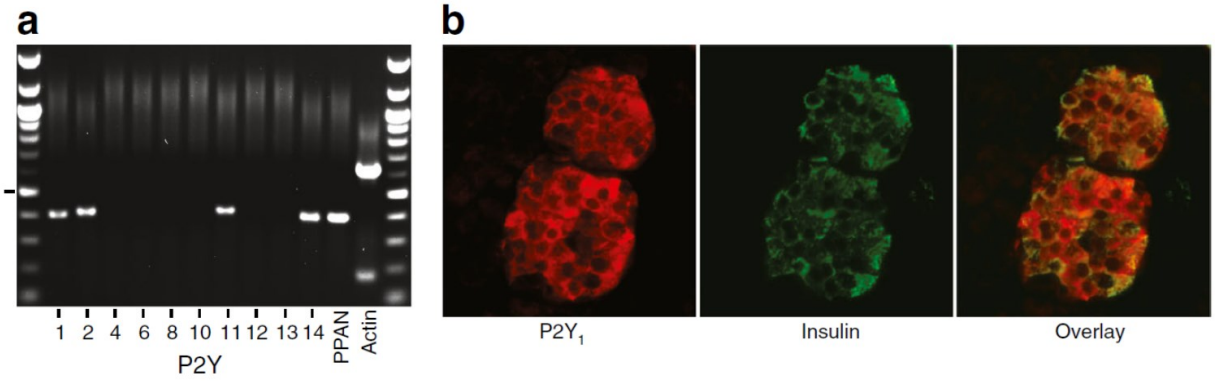
2.4e Expression of P2Y receptors in human β cells

Expression of P2Y receptor isoforms in human islets was analysed by RT-PCR. Transcripts were identified for $P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{14}$ (Fig. 12a). The expression of $P2Y_1$ in β cells was confirmed by co-immunostaining of human pancreatic tissue sections with anti-$P2Y_1$ and anti-insulin (Fig. 12b).

Figure 12: Expression of P2Y receptors in human islets.

(a) Expression of P2Y receptor isoforms and the peter pan homologue-P2YR11 transcript (PPAN) were analysed by RT-PCR. The horizontal line indicates the 500 base pair marker, data collection by SC. (b) A human pancreatic tissue section was co-immunostained with antibodies against the $P2Y_1$ receptor and insulin, data collection by MB. Data are representative of results from two donors

Figure 12.



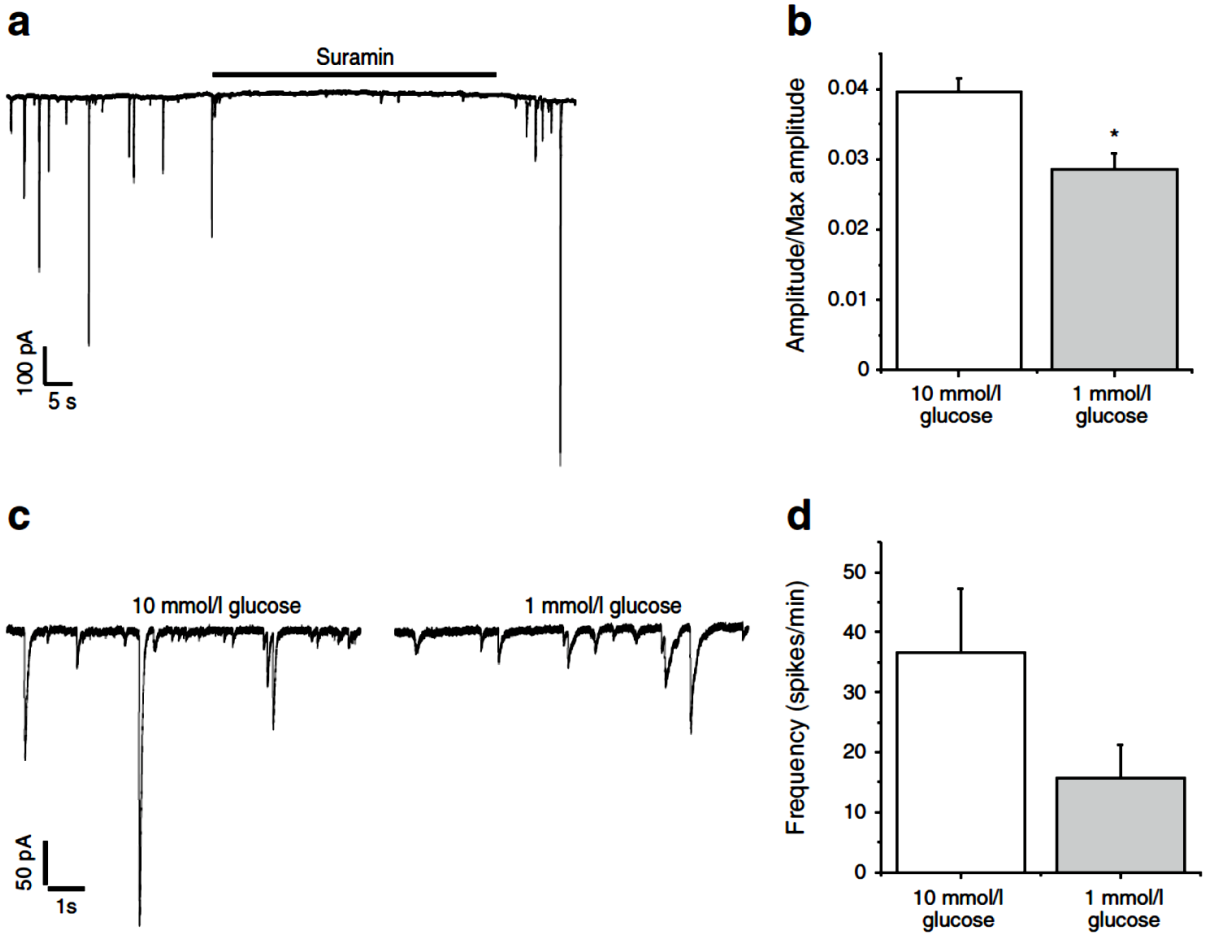
2.4f Exocytotic release of ATP

Rat and mouse β cells release ATP by Ca^{2+}-dependent exocytosis of insulin granules (96,119,122,123,185). To examine whether ATP is secreted from human β cells we overexpressed $P2X_2$ receptors (96,123) and stimulated exocytosis by infusion of Ca^{2+} (2 μmol/l) via the patch pipette. Resultant transient inward currents were blocked by the P2 antagonist suramin (Fig. 13a; $n = 4$). These events reflect release of ATP from single insulin granules (119,123). B cells were then incubated in 1 or 10 mmol/l glucose for 1 h with 100 μmol/l diazoxide to prevent K_{ATP}-mediated depolarisation. To avoid artefacts resulting from $P2X_2$ receptor overexpression, transient inward currents were compared with the current produced by 300 μmol/l ATP. Upon infusion of Ca^{2+} (2 μmol/l) the ATP release events were larger (Fig. 13c, $p < 0.05$) and tended to be more frequent (Fig. 13d) following glucose stimulation.

Figure 13: Exocytotic release of ATP from human β cells.

(a) A β cell infected with AdP2X_2-GFP was clamped at −70 mVand infused with solution containing 2 μmol/l free Ca^{2+}. Suramin (100 μmol/l) was added as indicated (n=4), data collection by MB. (b) Sample trace of ATP transient inward current in human cells in 10 and 1 mmol/l glucose for 1 h (n=11), data collection by MB. (c) The normalised amplitudes of transient inward currents (n=11). (d) The frequency of transient inward currents (n=11). Data are from four donors. *$p<0.05$ compared with 10 mmol/l glucose. Max, maximum

Figure 13.


a
Suramin
100 pA
5 s
b
Amplitude/Max amplitude
0.04
0.03
0.02
0.01
0
*
10 mmol/l glucose
1 mmol/l glucose
c
10 mmol/l glucose
1 mmol/l glucose
50 pA
1s
d
Frequency (spikes/min)
50
40
30
20
10
0
10 mmol/l glucose
1 mmol/l glucose

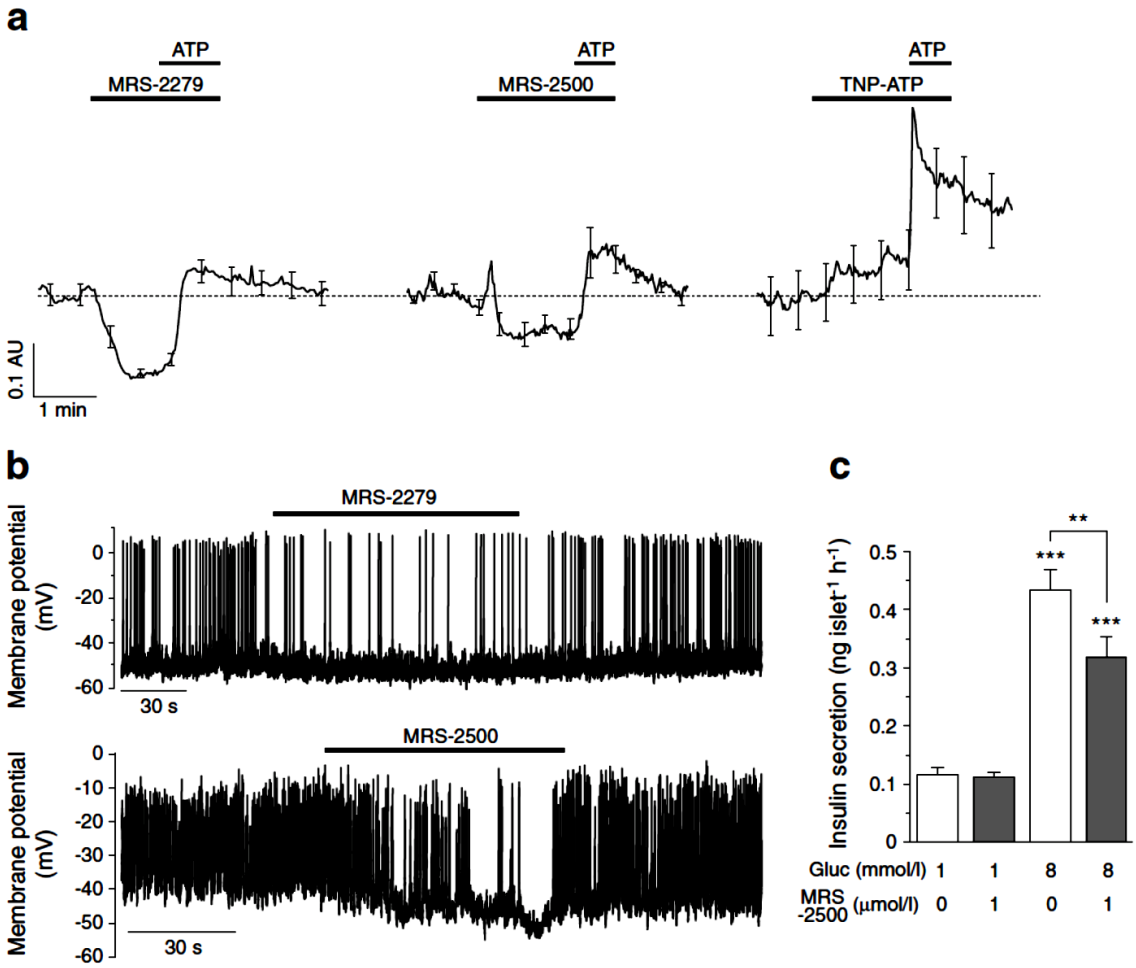
2.4g Autocrine activation of $P2Y_1$ receptors potentiates glucose-induced $[Ca^{2+}]_i$ signals, electrical activity and insulin secretion

We applied P2 receptor antagonists in the absence of exogenous nucleotides. The $P2Y_1$ antagonists MRS-2279 and MRS-2500, but not the $P2X_{(1/3)}$ blocker TNP-ATP, reduced $[Ca^{2+}]_i$ in β cells stimulated with 6 mmol/l glucose (Fig. 14a). Both MRS-2279 and MRS-2500 reversibly hyperpolarised the β cells in the absence of exogenous ATP and inhibited glucose-induced electrical activity (Fig. 14b). The $P2Y_1$ receptor antagonists decreased the membrane potential by 3.5 ± 1.4 mV (from -47.4 ± 2.8 to -50.8 ± 2.2 mV, $p < 0.05$; Fig. 14b). In control experiments, the $P2Y_1$ antagonists had no direct effects on voltage-gated Ca^{2+}, Na^+ or K^+ currents or on K_{ATP} current in human β cells (Supplemental data Fig. 1). In islets from four donors, MRS-2500 reduced the secretory response to glucose by 35% (Fig. 14c).

Figure 14: Effect of $P2Y_1$ antagonists on electrical activity, $[Ca^{2+}]_i$ and insulin secretion.

(a) Effect of antagonists MRS-2279 (1 µmol/l, n=22), MRS-2500 (1 µmol/l, n=18) and TNP-ATP (1 µmol/l, n=41) on $[Ca^{2+}]_i$ (at 6 mmol/l glucose), data collection by SK. (b) Effect of MRS-2279 and MRS-2500 (both at 1 µmol/l) on glucose (6 mmol/l)-induced electrical activity (n=8), data collection by MB. (c) Insulin secretion was measured from isolated human islets at 1 and 8 mmol/l glucose (Gluc) in the absence and presence of MRS-2500 (n=4 donors), data collection by SK. Data are from three to six donors. **$p<0.01$ and ***$p<0.001$ compared with 1 mmol/l glucose or as indicated

Figure 14.


a
ATP
MRS-2279
ATP
MRS-2500
ATP
TNP-ATP
0.1 AU
1 min
b
MRS-2279
Membrane potential (mV)
0
-20
-40
-60
30 s
MRS-2500
Membrane potential (mV)
0
-10
-20
-30
-40
-50
-60
30 s
c
Insulin secretion (ng islet-1 h-1)
0.5
0.4
0.3
0.2
0.1
0
**
***
***
Gluc (mmol/l) 1 1 8 8
MRS -2500 (µmol/l) 0 1 0 1

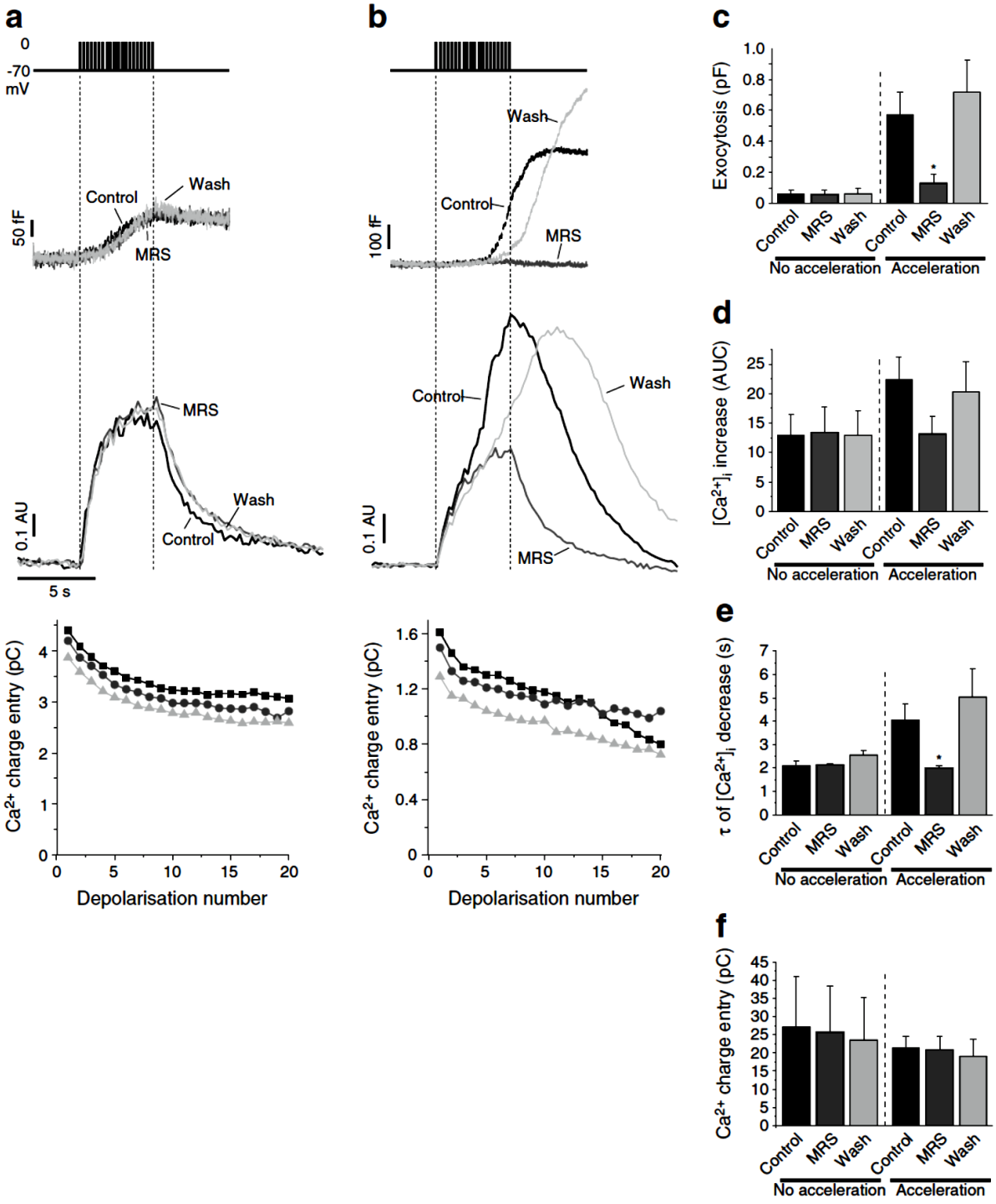
2.4h $P2Y_1$ receptors couple Ca^{2+} influx to Ca^{2+} release from stores

In voltage-clamped cells $[Ca^{2+}]_i$ was monitored during a series of short depolarisations mimicking glucose-induced electrical activity. In four of nine cells, the capacitance increase was nearly linear and ceased immediately after the end of the stimulation (Fig. 15a) and the $[Ca^{2+}]_i$ signal plateaued after a steep initial rise, returning to baseline with a time constant (τ) of ~2 s. In these cells neither $[Ca^{2+}]_i$ signal nor exocytosis and Ca^{2+} influx were affected by MRS-2279 (Fig. 15a, c–f). In five of nine cells, both exocytotic response and $[Ca^{2+}]_i$ signal displayed a secondary acceleration during the second half of the stimulation (Fig. 15b); also, the $[Ca^{2+}]_i$ signal returned to baseline with a significantly slower τ (~4 s). In these cells MRS-2279 strongly and reversibly inhibited exocytosis, the $[Ca^{2+}]_i$ response and the τ of $[Ca^{2+}]_i$ decline to levels observed in cells lacking secondary acceleration (Fig. 15b–f). The Ca^{2+} influx evoked by the depolarisations was not different between groups of cells (and unaffected by MRS-2279; Fig. 15f).

Figure 15: Contribution of Ca^{2+} release to the autocrine $P2Y_1$-mediated Ca^{2+} signal and exocytosis.

(a, b) B cells were stimulated by depolarisations from −70 to 0 mV (20 ms each, at 4 Hz) andmembrane capacitance, Ca^{2+} currents and $[Ca^{2+}]_i$ were monitored simultaneously. Tetrodotoxin (0.1 µmol/l) was included to block voltage-gated Na^+ currents. The stimulation was performed under control conditions (black traces, black squares), with 1 µmol/l MRS-2279 (MRS, dark-grey traces, dark-grey circles) and after wash-out of the $P2Y_1$ antagonist (light-grey traces, lightgrey triangles) in the same cells, data collection by MB. Cells that did not (a) or did (b) show a secondary acceleration of exocytosis and $[Ca^{2+}]_i$ increase are shown. (c) Average exocytotic responses under control conditions, with 1 µmol/l MRS-2279 and after wash-out in cells without (No acceleration, n=4) or with (Acceleration, n=5) secondary acceleration of exocytosis and $[Ca^{2+}]_i$ increase during the stimulation protocol. (d) As for (c), showing the integrated $[Ca^{2+}]_i$ increase over baseline (AUC). (e) As for (c), showing the time constant (τ) of the $[Ca^{2+}]_i$ signal return to baseline after the depolarisation series. (f) As for (c), showing the integrated Ca^{2+} current evoked by the depolarisations. Data are from two donors. *$p<0.05$ compared with the ‘acceleration’ control

Figure 15.


a
0
-70
mV
Control
Wash
MRS
50 fF
MRS
Wash
Control
0.1 AU
5 s
Ca2+ charge entry (pC)
Depolarisation number
b
Wash
Control
MRS
100 fF
Control
Wash
MRS
0.1 AU
Ca2+ charge entry (pC)
Depolarisation number
c
Exocytosis (pF)
Control
MRS
Wash
Control
MRS
Wash
No acceleration
Acceleration
d
[Ca2+]i increase (AUC)
Control
MRS
Wash
Control
MRS
Wash
No acceleration
Acceleration
e
τ of [Ca2+]i decrease (s)
Control
MRS
Wash
Control
MRS
Wash
No acceleration
Acceleration
f
Ca2+ charge entry (pC)
Control
MRS
Wash
Control
MRS
Wash
No acceleration
Acceleration

2.5 Discussion

This study provides evidence that ATP acts as a positive autocrine feedback signal in human β cells, by amplifying glucose-induced $[Ca^{2+}]_i$ responses. Several findings support a central role for $P2Y_1$ in this: (1) The effects of ATP were mimicked by ADP (at five- to tenfold lower concentrations), which selectively activates $P2Y_{(1/12/13)}$ (only $P2Y_1$ was detected in human islets) (170); (2) the selective $P2Y_1$ agonist MRS-2365 increased electrical activity and $[Ca^{2+}]_i$, while the $P2X_{(1/3)}$ agonist α,β-meATP did not; (3) $P2Y_1$ inhibition blocked the ATP-evoked membrane depolarisation; (4) MRS-2500 reduced insulin secretion to a similar extent as the non-specific P2 antagonist suramin (100). While in agreement with the findings of a recent study (107), our findings vary from those of another study suggesting a dominant role for $P2X_3$ (100). However, the latter study employed pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid (iso-PPADS) and oxidised ATP at concentrations that also strongly inhibit $P2Y_1$ (257,258). The more selective $P2X_{(1/3)}$ blocker TNP-ATP (259) does not affect $[Ca^{2+}]_i$ (Fig. 14a). While others have also suggested a role for $P2X_7$ (254), human $P2X_7$ has a very low affinity for ATP (half maximal effective concentration [ED_{50}] 0.78 mmol/l) and is insensitive to ADP and AMP (260), making a role for $P2X_7$ unlikely here.

$P2Y_1$ has a ~20-fold lower affinity for ATP than $P2X_3$ (154,170). However, it has been reported that insulin granules contain similar concentrations of ATP and ADP (97), suggesting that both nucleotides play an important role. We demonstrated that human β cells secrete ATP in response to increased $[Ca^{2+}]_i$, and the magnitude of ATP-release events was increased by glucose. This could result from intragranular ATP accumulation via granule-resident vesicular nucleotide transporter (101). It should be noted, however, that in intact rodent islets extracellular ATP plays an important role in synchronising the electrical and Ca^{2+} responses among β cells within and between islets through the induction of Ca^{2+} release from $InsP_3$-sensitive stores (116,120,121), in addition to stimulating exocytosis.

Some studies conducted in rodents, particularly mice, have found that ATP inhibits insulin secretion (102) and that insulin secretion in islets from mice lacking $P2Y_1$ is elevated (106). This was attributed to direct inhibitory effects of ATP on exocytosis (103) or voltage-gated Ca^{2+} currents (261). It has been reported that adenosine, acting on P1 receptors, inhibits insulin secretion

from INS-1 cells (262), but this was not confirmed in human islets (100). We show here that ATP stimulates depolarisation-evoked exocytosis without affecting Ca^{2+} currents in human β cells. We found no evidence for a negative role of ATP in insulin secretion from human islets, consistent with the potentiation of insulin secretion from human islets following block of extracellular ATP degradation (100,204).

ATP increased $[Ca^{2+}]_i$ in a biphasic manner, with an initial peak reflecting Ca^{2+} release from stores and a plateau reflecting Ca^{2+} influx. Similar to rat β cells (263), Ca^{2+} was released via heparin-sensitive $InsP_3$ receptors, but was from thapsigargin-sensitive (ER) rather than bafilomycin-sensitive (acidic) compartments. Our findings differ from those of Jacques-Silva et al, who concluded that Ca^{2+} stores contribute little to the ATP-evoked Ca^{2+} signal in human β cells (100), but this may be explained by experimental differences: the Ca^{2+} response under Ca^{2+}-free conditions is transient and will appear small (as AUC) when compared with prolonged agonist application in the presence of extracellular Ca^{2+}. Although a recent study suggests that autocrine activation of $P2Y_1$ stimulates diacylglycerol production in rodent and human β cells (107), we were unable to determine a role for phospholipase C as the inhibitor U-73122 (5–10 μmol/l) also suppressed KCl-evoked Ca^{2+} responses (data not shown).

In mice, $P2Y_1$ receptors depolarise β cells via inhibition of K_{ATP} channels (103). In contrast, the ADP-evoked membrane current in human β cells did not reverse at the K^+ equilibrium potential and was insensitive to tolbutamide. Instead, the current was abolished by removal or replacement of Na^+, indicating a Na^+- or non-selective cation conductance similar to $P2Y_1$-activated currents in neurons (264–266). This effect was insensitive to thapsigargin (arguing against a store-operated channel) and to Gd^{3+}, a blocker of the Na^+ leak channel NALCN (267). While the molecular identity of the $P2Y_1$-activated leak channel remains unclear, candidates include members of the transient receptor potential channel family (268).

We now show that blocking $P2Y_1$ receptors also inhibits electrical activity, $[Ca^{2+}]_i$ signalling and insulin secretion in human β cells in the absence of exogenous ATP (Fig. 14). While $P2Y_1$ blockade inhibits spontaneous $[Ca^{2+}]_i$ transients in mouse β cells (115), this was in the presence of a Ca^{2+} channel blocker and thus not likely caused by electrical activity and Ca^{2+} influx. We obtained similar results with two different, selective $P2Y_1$ antagonists (181,182) that lacked non-specific effects on a number of human β cell ion channels (Supplemental data Fig. 1).

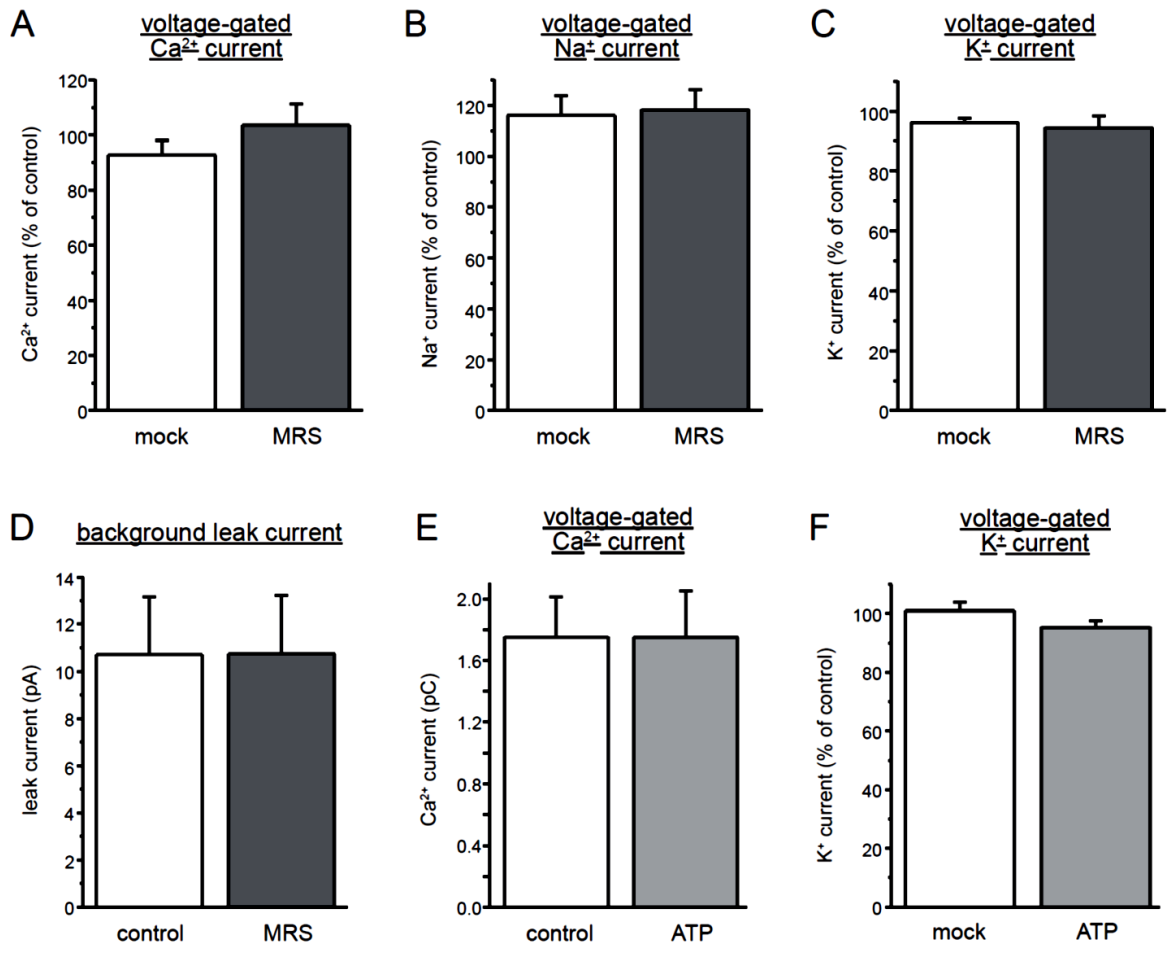
In cells stimulated with action potential-like depolarisations, $P2Y_1$ blockade reduces $[Ca^{2+}]_i$ and exocytosis without affecting Ca^{2+} currents (Fig. 15). Thus the MRS-2279-sensitive component of the [Ca2+]i increase reflects Ca^{2+} release from stores triggered by autocrine activation of $P2Y_1$ receptors. This, in combination with activation of diacylglycerol and protein kinase C (DAG/PKC) (107), potentiates exocytosis. The Ca^{2+} signal required for insulin secretion is largely generated by Ca^{2+} influx through voltage-gated Ca^{2+} channels (269,270); our results are compatible with this because membrane depolarisation and Ca^{2+} influx are necessary to evoke ATP release and initiate the feedback loop. The secondary acceleration of depolarisation-evoked exocytosis, which we suggest reflects ER Ca^{2+} release, is observed in human (255,271) but not mouse (272) β cells.

Our data indicate that the contribution of Ca^{2+} release to the glucose-induced Ca^{2+} signal may have been underestimated. We present some limited data suggesting that release of Ca^{2+} from thapsigargin-sensitive stores during ATP stimulation is absent in a donor with type 2 diabetes; this could contribute to impaired secretion. There is evidence that ER stress is involved in the pathogenesis of type 2 diabetes (273) and is associated with reduced SERCA2b (274,275), the main ER Ca^{2+} pump in β cells (276). The resulting lowering of ER Ca^{2+} levels may not only promote apoptosis but also impair β cell stimulus–secretion coupling.

Supplemental data Figure 1: Effects of $P2Y_1$ antagonists and ATP on membrane currents in human β cells

A)-C) The indicated currents were measured before (control) and 2 minutes application of 1 µmol/l MRS-2500, data collection by MB. Values are expressed in % of control. To account for spontaneous changes during the experiments (e.g. run-down), mock applications were used for statistical comparison. No significant differences were observed. All experiments were performed using the standard whole-cell configuration. A) + B) K^+ currents were blocked by including TEA (20 mmol/l) in the extracellular solution and replacing K^+ with Cs^+ in the pipette solution. Peak Na^+ current were measured during depolarizations from -70 to 0 mV (n=5). Integrated Ca^{2+} currents were measured during 50 ms depolarizations from -70 to 0 mV (n=5). C) Voltage-gated K^+ currents were measured during depolarizations from -70 to +20 mV (n=7-8). D) The background leak current was measured at -70 mV before and after addition of $P2Y_1$ antagonists in the same cells, data collection by MB. Experiments were performed at 6 mmol/l glucose using the perforated-patch configuration. Data for MRS-2279 (1 µM, n=5) and MRS-2500 (1 µmol/l, n=2) were combined. E) Integrated voltage-gated Ca^{2+} current evoked by 50 ms depolarizations from -70 to 0 mV before and after addition of ATP (10 µmol/l) in the same cells (standard whole-cell, n=5), data collection by MB. F) Effect of ATP (10 µmol/l) on the voltage-gated K^+ current evoked by depolarizations from -70 to +20 mV (n=11) compared to mock application (n=8), data collection by MB.

Supplemental data Figure 1.



CHAPTER 3: ACTIVATION OF PKD1 BY AUTOCRINE ATP SIGNALLING IN PANCREATIC B CELLS

Shara Khan*, Mourad Ferdauossi, Valérie Bergeron, Nancy Smith, Austin Bautista, Vincent Poitout, Patrick E. Macdonald. Activation of PKD1 by Autocrine ATP Signalling in Pancreatic β Cells.

Contributions by co-authors to the figures presented are stated in the figure legends, accompanied by the appropriate co-authors' initials.

3.1 Abstract

BACKGROUND AND AIMS:

β cells co-secrete the neurotransmitter ATP along with insulin. ATP acts as a positive autocrine signal in β cells by activating $P2Y_1$ receptors to activate phospholipase C and increase production of DAG. However, the downstream signalling that couples $P2Y_1$ activation to insulin secretion remains to be fully elucidated. Since DAG is known to activate protein kinase D1 (PKD1) to potentiate glucose stimulated insulin secretion, it is hypothesized that autocrine ATP signalling activates downstream PKD1 to regulate insulin secretion.

METHODS:

Western blotting was performed to study agonist-induced, depolarization-induced and antagonist-inhibited activation of PKD1 in INS 832/13 insulinoma cells and in mouse islets. Capacitance measurements of exocytosis were employed in single mouse β cells following PKD1 knockdown. Expression of PKD1 mRNA was analysed by RT-PCR in human islets. Insulin secretion was measured from intact $PKD1^{-/-}$ islets, and from human islets following inhibition of $P2Y_1$ or PKD1 signalling

RESULTS:

The $P2Y_1$ receptor agonists, MRS2365 and ATP, induce PKD1 phosphorylation at serine 916 in mouse islets and INS 832/13 cells. Similarly, direct depolarization with KCl causes activation of PKD1, which is reduced upon antagonism of $P2Y_1$. The potentiation of insulin secretion by $P2Y_1$ activation was lost from $PKD1^{-/-}$ mouse islets, and knockdown of PKD1 reduced the ability of $P2Y_1$ activation to facilitate β cell exocytosis. Finally, qPCR analysis confirmed expression of PKD1 in human islets and inhibition of either $P2Y_1$ or PKD1 impaired glucose-stimulated insulin secretion. This pathway was, interestingly, more important in islets from overweight and obese donors, as the inhibition of insulin secretion by $P2Y_1$ or PKD1 antagonists was correlated with donor BMI.

CONCLUSIONS/INTERPRETATION:

A $P2Y_1$ receptor-dependent activation of PKD1 by autocrine ATP signalling increases insulin secretion. This pathway is more important in supporting insulin secretion from islets of overweight or obese donors than in from islets of lean donors.

3.2 Introduction

Diabetes results from impaired or insufficient insulin secretion. While plasma glucose is a key regulator of insulin secretion, insulin release is modulated by other nutrients, circulating hormones, the autonomic nervous system, and local paracrine and autocrine signals. The neurotransmitter ATP is released from β cells and acts as a positive autocrine signal by activating $P2Y_1$ receptors (107,277). While it has been shown that ATP feedback activates purinergic $P2Y_1$ receptors, resulting in activation of PLC and spatially restricted production of diacylglycerol (DAG) (107), the downstream signalling that couples $P2Y_1$ activation to enhance insulin secretion remains to be fully elucidated. The elevated plasma membrane DAG concentration locally and transiently activates PKCs and/or other effectors (possibly PKD1) to potentiate the insulin secretory response (107,196). It has also been demonstrated that generation of DAG leads to activation of PKD1, F-actin depolymerisation, and potentiation of glucose stimulated insulin secretion (211). The first evidence of a role for PKD1 in pancreatic β-cells was provided by Sumara et al. who showed that PKD1 promoted both insulin secretion and β-cell survival (210). PKD1 is also involved in the augmentation of insulin secretion via muscarinic M3 receptor signalling (209) and affects the second phase of glucose stimulated insulin secretion through the PPARβ/δ pathway (253). In regulated insulin secretion, PKD1 and its substrate Arfaptin-1 promotes insulin vesicle fission at the trans-Golgi network (250). Recent work on pancreatic β cell specific PKD1 deletion showed, however, that while insulin secretion was not impaired in chow-fed mice, an insulin secretory deficit was evident following high-fat feeding (278). This raises the possibility that PKD1 signalling within β cells may be particularly important in maintaining secretory function under metabolic stress.

PKD1 is a serine/threonine kinase that belongs to the Ca^{2+}/calmodulin-dependent kinases (CaMKs) superfamily (212). Its activation is dependent on the phosphorylation of two activation loop sites, serine 744 and serine 748, via a PKC-dependent signalling pathway (229). In addition, serine 916 has been identified as an autophosphorylation site for PKD (239). However, in humans the autophosphorylation site for PKD1 is serine 910 (240).

In the previous Chapter, the focus was on the PLC/$InsP_3$ pathway upon autocrine signalling via the $P2Y_1$ receptor. It was demonstrated that ATP acts as a positive autocrine signal in human

β cells by activating $P2Y_1$ receptors, stimulating electrical activity and increasing $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx and evoking Ca^{2+} release via $InsP_3$-receptors in the endoplasmic reticulum; thereby resulting in potentiation of glucose stimulated insulin secretion (277). Here the other arm of the PLC pathway, the PLC/DAG pathway is being investigated, upon autocrine activation of the $P2Y_1$ receptor. It is hypothesized that autocrine signalling via ATP activates PKD1 downstream to contribute to the potentiation of insulin secretion.

3.3 Methods

3.3a Cells and cell culture.

Islets from male C57Bl/6 were isolated by collagenase digestion and cultured in RPMI 1640 containing 11.1 mmol/l glucose with 10% FBS and 100 U/ml of penicillin/streptomycin. βPKD1KO and MIP-CreERT mouse islets were a kind gift from Dr. Vincent Poitout (University of Montreal) (278). Human islets were isolated from donor pancreata at the Alberta Diabetes Institute IsletCore (http://www.bcell.org/IsletCore.html) at the University of Alberta (Edmonton, Alberta, Canada) or the Clinical Islet Laboratory at the University of Alberta and were cultured in low-glucose (5.5 mmol/l) DMEM with L-glutamine, 110 mg/l sodium pyruvate, 10% FBS, and 100 units/ml penicillin/streptomycin. In total, islets from 16 human donors were examined in this study. For single cell experiments, mouse islets were dispersed by shaking in cell dissociation buffer (Gibco, Thermo Scientific) and plated in 35-mm culture dishes. Islets, and dispersed cells were cultured at 37°C and 5% CO_2. INS 832/13 cells (from C. Newgard, Duke University, Durham, North Carolina, USA) were cultured in RPMI 1640 containing 11.1 mmol/l glucose with 10% FBS, 10 mmol/l HEPES, 0.29 mg/ml L-glutamine, 1 mmol/l sodium pyruvate, 50 µl β-mercaptoethanol, and 100 U/ml of penicillin/streptomycin. Islets, INS 832/13 cells, or dispersed cells were cultured at 37°C and 5% CO_2.

3.3b Immunoblotting.

Mouse islets were preincubated for 1 hour in 1 mmol/l glucose KRB and subsequently treated for 20 minutes with $P2Y_1$ agonists, ATP (10µmol/l) and MRS2365 (100nmol/l; EC_{50} 0.4nmol) or 30mmol KCl ± $P2Y_1$ antagonist, MRS2500 (1µmol/l; EC_{50} 0.78nmol). Following treatment, cells were lysed in buffer containing (in mmol/l): 20 Tris-HCl (pH = 7.5); 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 EDTA, 1 b-glycero-phosphate, 25 N-ethylmaleimide, 1% Triton X-100, and 1X protease inhibitor cocktail. Protein lysates were separated using SDS-PAGE, transferred to nitrocellulose membranes, incubated overnight at 4°C with primary antibodies (phospho-PKD/PKCmu (serine 916) Rabbit A [Cell signalling] and PKD [Cell signalling]) and visualised with horseradish peroxidase-labelled anti-rabbit IgG as secondary antibodies (Amersham, Baie d'Urfe, PQ). Images were acquired using a ChemiDoc MP System (Bio-Rad) and analyzed using Image Lab Software 5.2.1 (Bio-Rad).

3.3c Static incubation insulin secretion.

For static incubations, measurements were performed at 37°C in Krebs-Ringer Bicarbonate (KRB) solution containing (in mmol/l): 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 0.1% fatty-acid free BSA (pH 7.4). Intact mouse or human islets in batches of 15 islets each were preincubated for 2 hours at 1.0 mmol/l glucose-KRB. Islets were transferred to fresh KRB solution containing 1.0 mmol/l glucose for 1 hour, followed by incubation for 1 hour in 10.0 mmol/l glucose-KRB. Each condition was run in triplicate. Supernatant fractions were collected, and islets were lysed in buffer containing 1.5% concentrated hydrochloric acid, 23.5% acetic acid, and 75% ethanol for assay of insulin content. Samples were stored at –20°C and assayed for insulin via insulin detection kits (Meso Scale Discovery).

3.3d Electrophysiology.

Dispersed mouse islets were plated in 35-mm culture dishes. Prior to electrophysiological recordings, mouse pancreatic β cells were transfected with scrambled or PKD1 siRNA for 48 hours. Transfected cells were identified by labeling with Alexa Fluor 488 dye. Solutions used for capacitance measurements have been previously described (277). The $P2Y_1$ agonist, MRS2365 (100nmol/l), was added to the extracellular bath solution for the duration of the recordings. One group of cells were patched following a 15 minute pre-treatment with thapsigargin (10μmol/l). The standard whole cell technique with the sine+DC lockin function of an EPC10 amplifier and Patchmaster software (HEKA Electronics, Lambrecht/Pfalz) was used. Experiments were performed at 32°C–35°C. Quantification of the average cumulative increase of the capacitance of 500 ms depolarizations from –70 to 0 mV was calculated.

3.3e siRNA constructs and quantitative PCR.

PKD1 and scrambled siRNA constructs were from Origene. These were transfected in dissociated mouse islet cells using DharmaFECT 1 (GE Healthcare, Mississauga, ON). For quantitative PCR, RNA from dispersed mouse islets was extracted 48-hrs post transfection using TRIzol Reagent (Life Technologies, Burlington, ON), and cDNA was synthesized using Super Script II and oligodT (Life Technologies) according to the manufacturer's protocol. Real-time PCR to detect PKD1 was performed as previously described (211). Primers were as follows: left:

TAGCCAAGGGTGACTCAAGG and right: CTGGACATGTGGTCTGTTGG. Quantitative PCR was also used to measure PKD1 mRNA expression in Human Embryonic Kidney cells and in isolated human islets from healthy donor (n=3 replicates from 1 donor). Primers were as follows: left: TTCTCCCACCTCAGGTCATC and right: CCAAATCCCTGGAAGGAAAT.

3.3f Statistical analyses.

Data analysis was performed using GraphPad Prism (v7.0c). Comparison of multiple groups was by one-or two-way ANOVA followed by Bonferroni or Tukey post-test. Data are expressed as means ±SEM, where $P < 0.05$ is considered significant.

3.4 Results

3.4a Autocrine activation of $P2Y_1$ receptor induces protein kinase D1 phosphorylation on serine 916 in mouse islets.

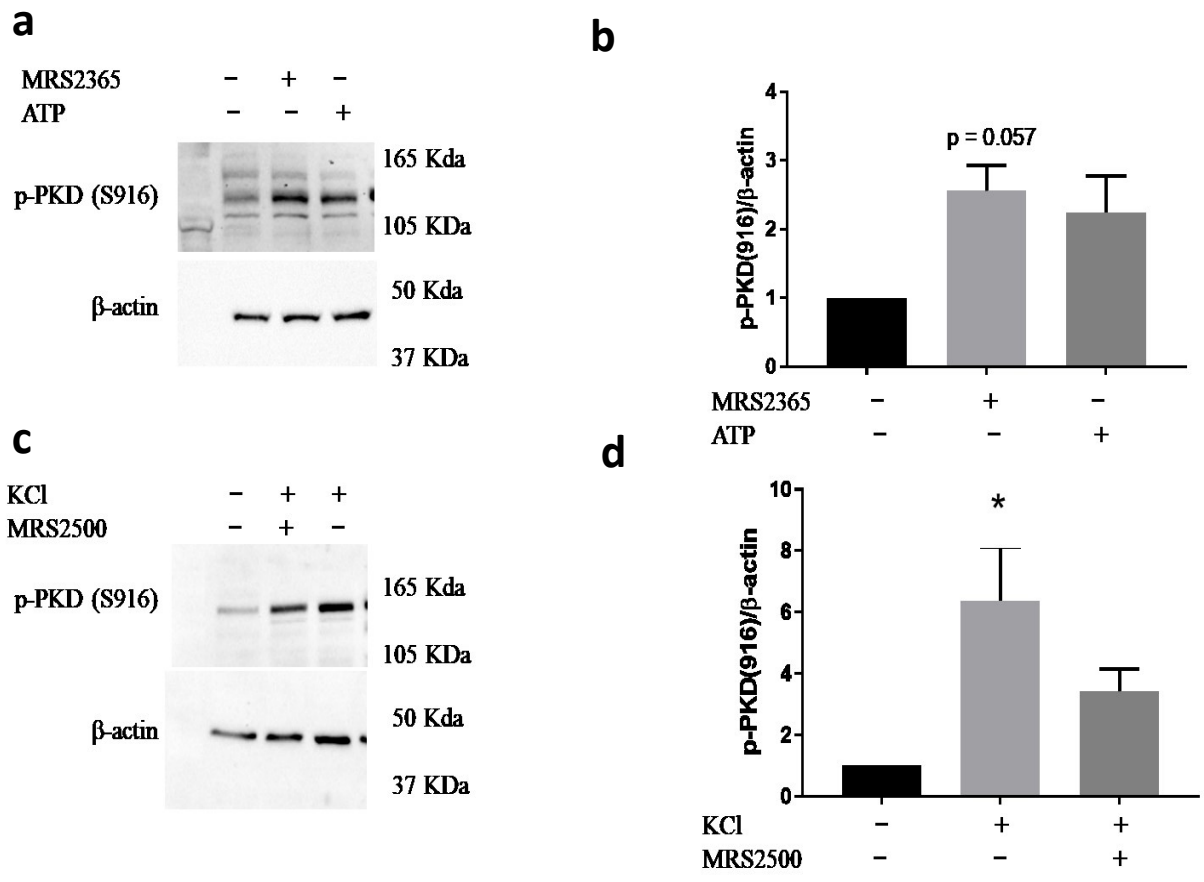
First, agonist-induced, glucose induced, depolarization-induced, and antagonist-induced activation of PKD1 in INS 832/13 insulinoma cells was investigated. The $P2Y_1$ receptor agonist, MRS2365, promotes phosphorylation at the serine 916 site of PKD1 in INS 832/13 cells at a concentration as low as 10nmol (Supplemental data, Fig 2A). An increasing concentration of glucose led to an increased level of phosphorylation of PKD1 at serine 916 (Supplemental data, Fig 2B). Also, there was a robust autocrine activation of PKD1 after depolarization with 30mmol KCl at low glucose (Supplemental data Fig 2C) in these cells which was reduced by a $P2Y_1$ antagonist, MRS 2279 (Supplemental data, Fig 2D). However, no phosphorylation was observed at the activation loop site, serine 744 and serine 748 (data not shown).

In mouse islets, antibodies against total PKD1 protein were found unreliable, and therefore, in these experiments phospho-PKD1 (serine 916) levels were normalized to β-actin. Following a 20 min exposure to $P2Y_1$ receptor agonists, MRS2365 and ATP, PKD1 phosphorylation was induced at serine 916 in mouse islets (Fig 16a and b). Similarly, direct depolarization with KCl caused significant induction of PKD1 and the effect was lost upon application of the $P2Y_1$ antagonist, MRS 2500 (Fig 16c and d). Together, these data suggest that PKD1 phosphorylation on serine 916, indicative of PKD1 activation (239), is stimulated by $P2Y_1$ receptor activation and that autocrine activation of the $P2Y_1$ receptor induces this PKD1 phosphorylation in mouse islets.

Figure 16: Effect of purinergic agonists and KCl-induced depolarization on PKD1 activation.

Representative immunoblots of protein extracts from mouse islets were stimulated for 20 min with 1 mmol glucose with (a) $P2Y_1$ agonists, MRS2365 (100 nmol/l) and ATP (10 µmol/l), data collection by MF and (c) with KCl (30mmol/l) ± $P2Y_1$ antagonist, MRS2500 (1µmol/l), data collection by SK and were analyzed by Western blot for phospho-PKD1 (serine 916) and β-actin. (b and d) Quantification of phospho-PKD1 (serine 916) normalized to β-actin. Data are mean ± SEM for 3 independent experiments, $p < 0.05$ compared to 1 mmol/l of glucose

Figure 16.


a
MRS2365 − + −
ATP − − +
p-PKD (S916)
165 Kda
105 KDa
β-actin
50 Kda
37 KDa
b
p-PKD(916)/β-actin
p = 0.057
MRS2365 − + −
ATP − − +
c
KCl − + +
MRS2500 − + −
p-PKD (S916)
165 Kda
105 KDa
β-actin
50 Kda
37 KDa
d
p-PKD(916)/β-actin
*
KCl − + +
MRS2500 − − +

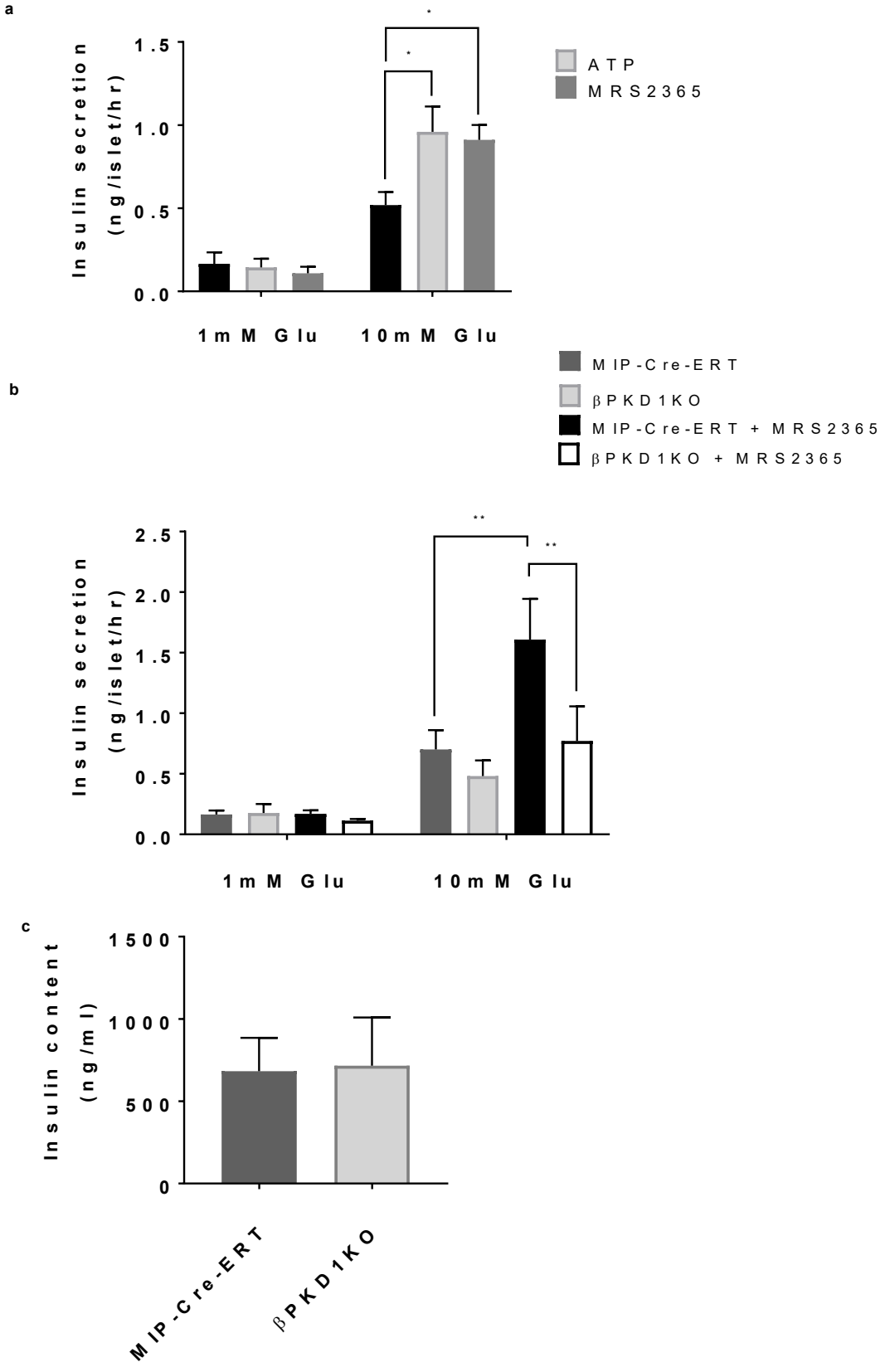
3.4b $P2Y_1$ dependent potentiation of insulin secretion in mouse islets is mediated by protein kinase D1

To examine the functional impact of PKD1 phosphorylation in response to $P2Y_1$ agonists, its role in insulin secretion in C57Bl/6 mouse islets and βPKD1KO mouse islets and MIP-Cre-ERT controls was investigated. There was significant potentiation of insulin secretion upon treatment with both $P2Y_1$ agonists, ATP and MRS 2365, at high glucose compared to the high glucose group alone in C57Bl/6 mouse islets (Fig 17a). For the βPKD1KO and MIP-Cre-ERT mouse islets, no difference in insulin secretion was observed at high glucose between groups. However, upon addition of MRS 2365 at high glucose the MIP-Cre-ERT group had a dramatic potentiation of insulin secretion which the βPKD1KO group failed to mimic (Fig. 17b). This difference in secretion was not due to changes in insulin content (Fig 17c). These results indicate that activation of $P2Y_1$ potentiates insulin secretion from mouse islets in a PKD1-dependent manner.

Figure 17: Potentiation of insulin secretion via activation of $P2Y_1$ is PKD1 dependent

(a) Insulin secretion was measured from C57Bl/6 mouse islets at 1 and 10 mmol/l glucose in the presence of ATP (10µmol/l) and MRS2365 (100nmol/l), data collection by SK and NS. Insulin secretion in response to 1 and 10 mmol/l glucose in the presence of (b) MRS2365 (100nmol/l) and (c) insulin content was assessed in 1-h static incubations in MIP-Cre-ERT and βPKD1KO mouse islets, data collection by SK. Data are mean ± SEM for 3-5 independent experiments, $p < 0$ .05 compared to 10 mmol/l glucose.

Figure 17.


a
Insulin secretion (ng/islet/hr)
1.5
1.0
0.5
0.0
1mM Glu
10mM Glu
ATP
MRS2365
b
MIP-Cre-ERT
βPKD1KO
MIP-Cre-ERT + MRS2365
βPKD1KO + MRS2365
Insulin secretion (ng/islet/hr)
2.5
2.0
1.5
1.0
0.5
0.0
1mM Glu
10mM Glu
c
Insulin content (ng/ml)
1500
1000
500
0
MIP-Cre-ERT
βPKD1KO

3.4c $P2Y_1$ increases β cell exocytosis in a protein kinase D1-dependent manner

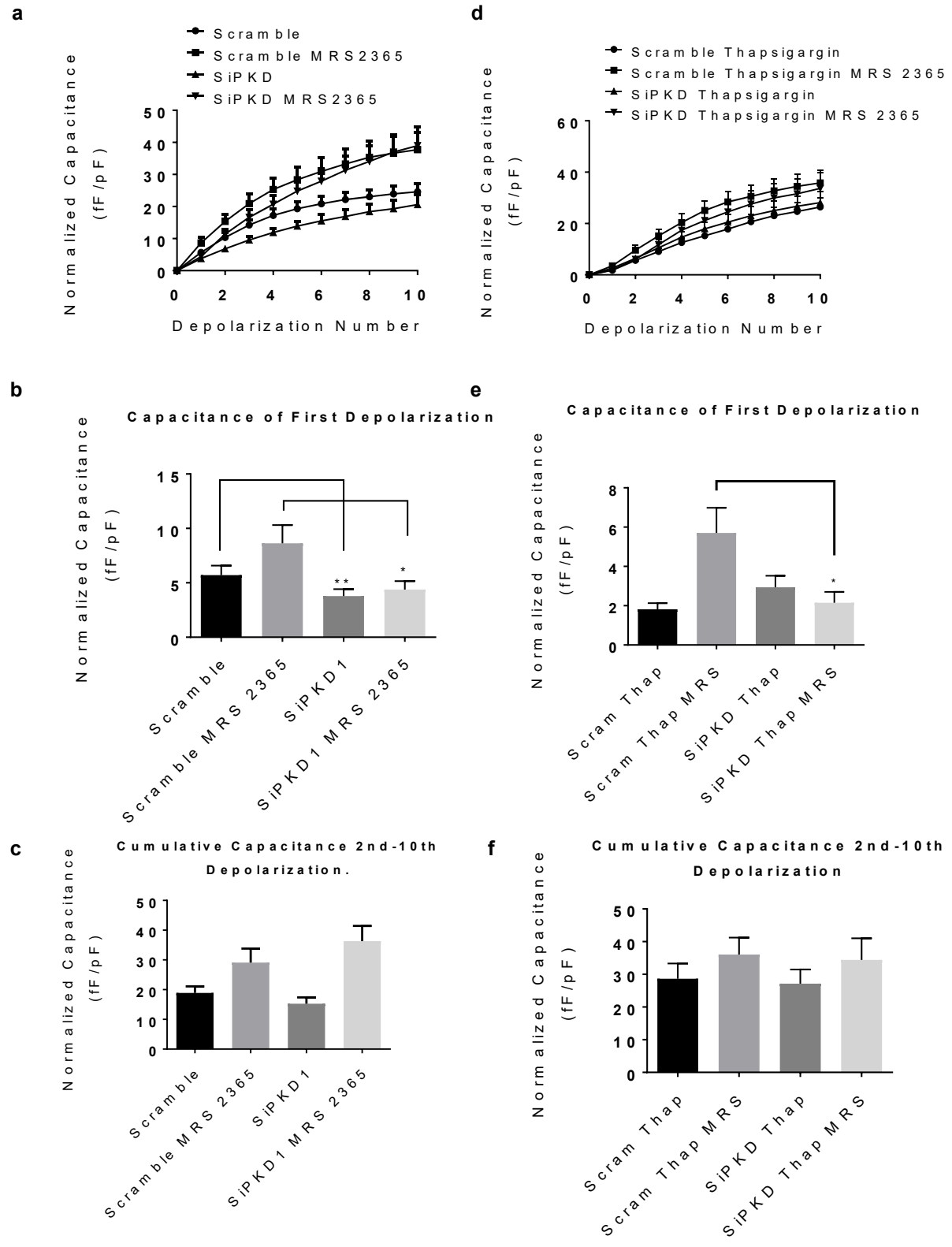
Next the role for PKD1 in the $P2Y_1$-dependent facilitation of β cell exocytosis was examined. For this, siRNA mediated PKD1 knockdown was employed in mouse β cells. After 48 hours, qPCR analyses of mouse β cells transfected with siRNA revealed a significant knockdown (87%) of PKD1 mRNA compared to control siRNA (Supplemental data, Fig 2). To address whether exocytosis is affected in siRNA-mediated PKD1 knockdown in β cells, capacitance measurements of exocytosis in single β cells was performed. A train of ten depolarization steps from -70mV to 0mv evoked larger responses in control β cells in the presence of MRS2365 compared to PKD1 knockdown cells, resulting in a significant increase in exocytotic response to the first depolarization (Fig 18a and b). However, this PKD1 dependent exocytotic effect was lost in second to tenth depolarizations (Fig 18c). This shows that PKD1 facilitates exocytosis at the very initial step of the first phase of glucose stimulated insulin secretion.

Positive feedback signalling by ATP via the $P2Y_1$ receptor leads to Ca^{2+} induced Ca^{2+} release from endoplasmic reticulum stores (277). To determine whether this PKD1 dependent decrease in exocytotic response to the first depolarization is affected by Ca^{2+} release via $InsP_3$-receptors or not, the mouse pancreatic β cells were preteated with thapsigargin (depletes $InsP_3$-sensitive endoplasmic reticulum Ca^{2+} stores). Although there was an apparent decrease observed in exocytosis to the first depolarization, it was not statistically significant (Fig 18d and e). Similar trend in exocytotic responses was observed in cells pretreated with thapsigargin compared to cells without thapsigargin pre-treatment in second to tenth depolarizations (Fig 18f). These results indicate that potentiation of insulin secretion by PKD1 is not caused by difference in Ca^{2+} release via $InsP_3$-receptors but by a direct effect on the exocytotic machinery via the PLC/DAG pathway.

Figure 18: Effect of $P2Y_1$ agonist on exocytosis and Ca^{2+} release via $InsP_3$-receptors in single mouse β cells following PKD1 knockdown

(a) Average cumulative capacitance response with or without application of MRS2365 (100 nmol/l) in mouse pancreatic β cells transfected with scrambled or PKD1 siRNA during each depolarization step is shown, data collection by AB. (d) shows the same as (a), but in cells pretreated with thapsigargin, data collection by AB. Quantification of normalised capacitance values of the (b and d) first depolarization and (c and e) second to tenth depolarisations are shown here.

Figure 18.


a
Scramble
Scramble MRS2365
SiPKD
SiPKD MRS2365
Normalized Capacitance (fF/pF)
Depolarization Number
d
Scramble Thapsigargin
Scramble Thapsigargin MRS 2365
SiPKD Thapsigargin
SiPKD Thapsigargin MRS 2365
Normalized Capacitance (fF/pF)
Depolarization Number
b
Capacitance of First Depolarization
Normalized Capacitance (fF/pF)
Scramble
Scramble MRS 2365
SiPKD1
SiPKD1 MRS 2365
e
Capacitance of First Depolarization
Normalized Capacitance (fF/pF)
Scram Thap
Scram Thap MRS
SiPKD Thap
SiPKD Thap MRS
c
Cumulative Capacitance 2nd-10th Depolarization.
Normalized Capacitance (fF/pF)
Scramble
Scramble MRS 2365
SiPKD1
SiPKD1 MRS 2365
f
Cumulative Capacitance 2nd-10th Depolarization
Normalized Capacitance (fF/pF)
Scram Thap
Scram Thap MRS
SiPKD Thap
SiPKD Thap MRS

3.4d Potentiation of insulin through PKD1 correlates with glucose stimulation index and BMI of humans

To date, role of PKD1 has not been investigated in human islets. Therefore, whether PKD1 is expressed in human pancreatic β cells or not and what might be the possible role of PKD1 in human islets was tested. Quantitative RT-PCR was used to measure PKD1 mRNA expression in human embryonic kidney (HEK) cells and in isolated human islets from healthy donors. Healthy donors expressed almost 2-fold PKD1 mRNA compared to control HEK cells. (Supplemental data, Fig 4). Western blotting was performed to detect PKD1 in human islets. However, although the antibody was expected to react with both HEK and human islet PKD1, it only detected PKD1 in HEK cells (data not shown).

The ability of $P2Y_1$ antagonist, MRS 2500, and PKD1 inhibitor, CRT0066101 (IC_{50} 1nmol), to affect glucose stimulated insulin secretion was studied in 16 human donors where MRS 2500 and CRT0066101 reduced the secretory response to glucose by 35% and 20% respectively (Fig 19a and 20a). The donor-donor variability (Non-responders who weren't sensitive to MRS 2500 or CRT0066101 inhibition and responders who showed inhibition) was plotted against the inhibition capacity observed for each donor (Fig 19b and 20b). The correlation between the inhibition capacity of the antagonist $P2Y_1$ and PKD1 inhibitor and anthropometric and metabolic variables of the donors were then investigated. The percent inhibition by $P2Y_1$ antagonist positively correlated with glucose stimulation index ($r=0.55$, $p=0.02$; Fig 19c) and with BMI ($r=0.51$, $p=0.03$, Fig 19h) in the responders. A significant difference in the percent inhibition of insulin secretion was also observed between lean and obese donors (Fig 19i). The BMI no longer correlated with percent inhibition of insulin secretion when non-responding donors were included in the analysis. Other parameters such as body weight ($r=0.01$; Fig 19d), HbA1c ($r=0.13$; Fig 19e), age ($r=0.02$; Fig 19f) and sex (Female $r=0.00$ and male $r=0.63$; Fig 19g) were not found to be correlated with the ability of $P2Y_1$ to affect glucose stimulated insulin secretion.

For the PKD1 inhibitor, the percent inhibition also positively correlated with glucose stimulation index ($r=0.51$, $p=0.03$; Fig 20c) and weakly with BMI ($r=0.42$, $p=0.08$, Fig 20h) for the responders. However, as with $P2Y_1$, a significant difference in the percent inhibition of insulin

secretion was also observed between lean and obese donors (Fig 20i). And as with $P2Y_1$, the correlation is lost when non-responding donors were included in the analysis. Other parameters such as body weight (r=0.02; Fig 20d), HbA1c (r=0.00; Fig 20e), age (r=0.17; Fig 20f) and sex (Female r=0.05 and male r=0.30; Fig 20g) were not found to be correlated with the ability of PKD1 to affect glucose stimulated insulin secretion.

Figure 19: Ability of $P2Y_1$ to potentiate insulin secretion positively correlates with BMI of humans

(a) Representative graph of insulin secretion in (ng/islet/hr) was assessed in 1-h static incubations in response to 1 and 10 mmol/l glucose in presence of $P2Y_1$ antagonist, MRS 2500 (1μmol/l), data collection by SK. (b) Comparison of donor-donor response according to percentage inhibition by P2Y1 antagonist. (c-h) The percentage inhibition of responders after 20 mins MRS 2500 treatment plotted against (c) Stimulation index (n=14 donors), (d) Body weight (n=14 donors), (e) glycated hemoglobin; HbA1c (n=14 donors), (f) Age (n=14 donors), (g) Sex (n=14 donors) and (h) BMI (kg/m2; n=14 donors) in nondiabetic human islet donors. (i) Comparison of percentage inhibition by MRS2500 according to BMI tertiles (lean < 25; overweight 25–30; and obese > 35 kg/m2) (n=5, 5, 4 donors).

Figure 19.

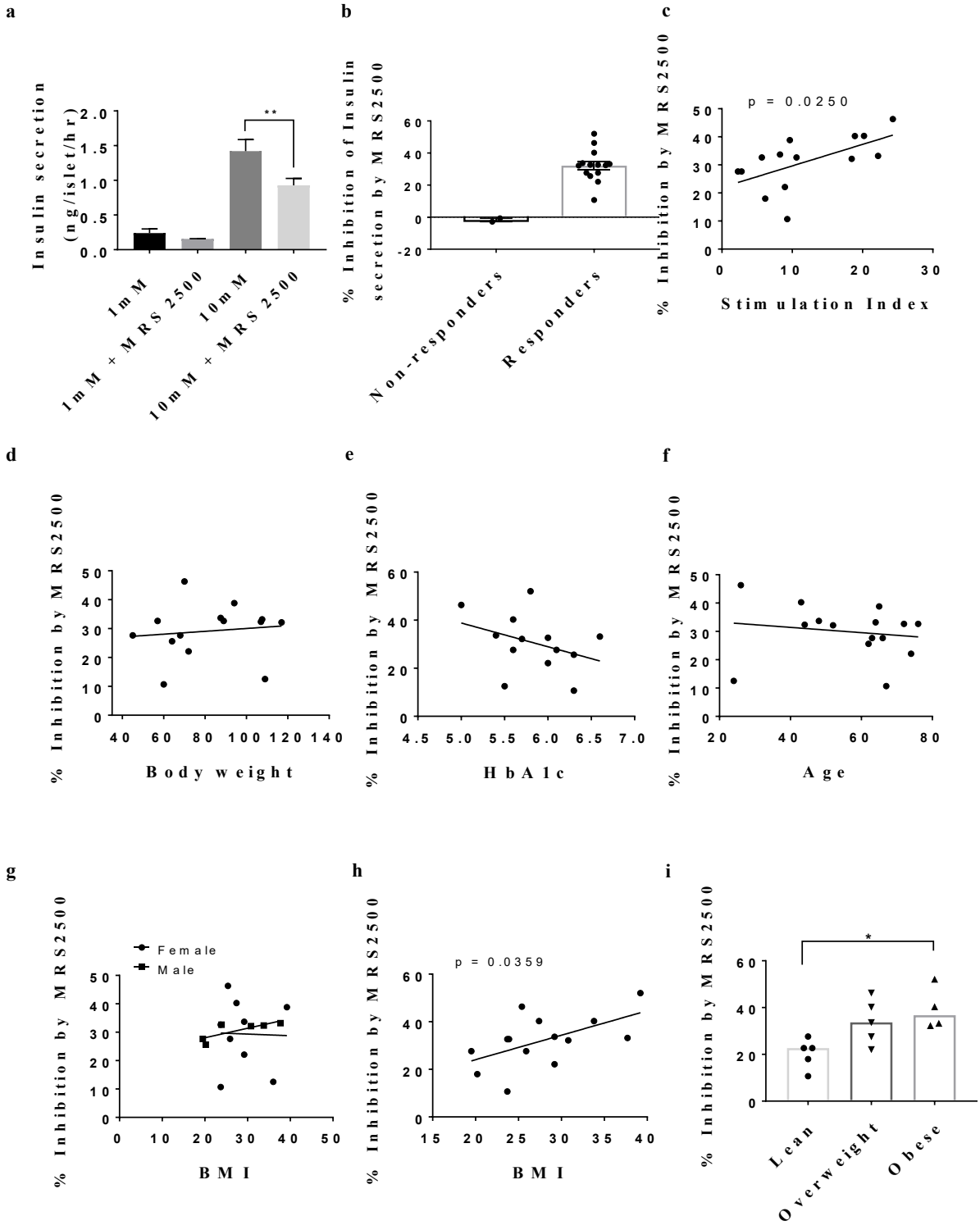
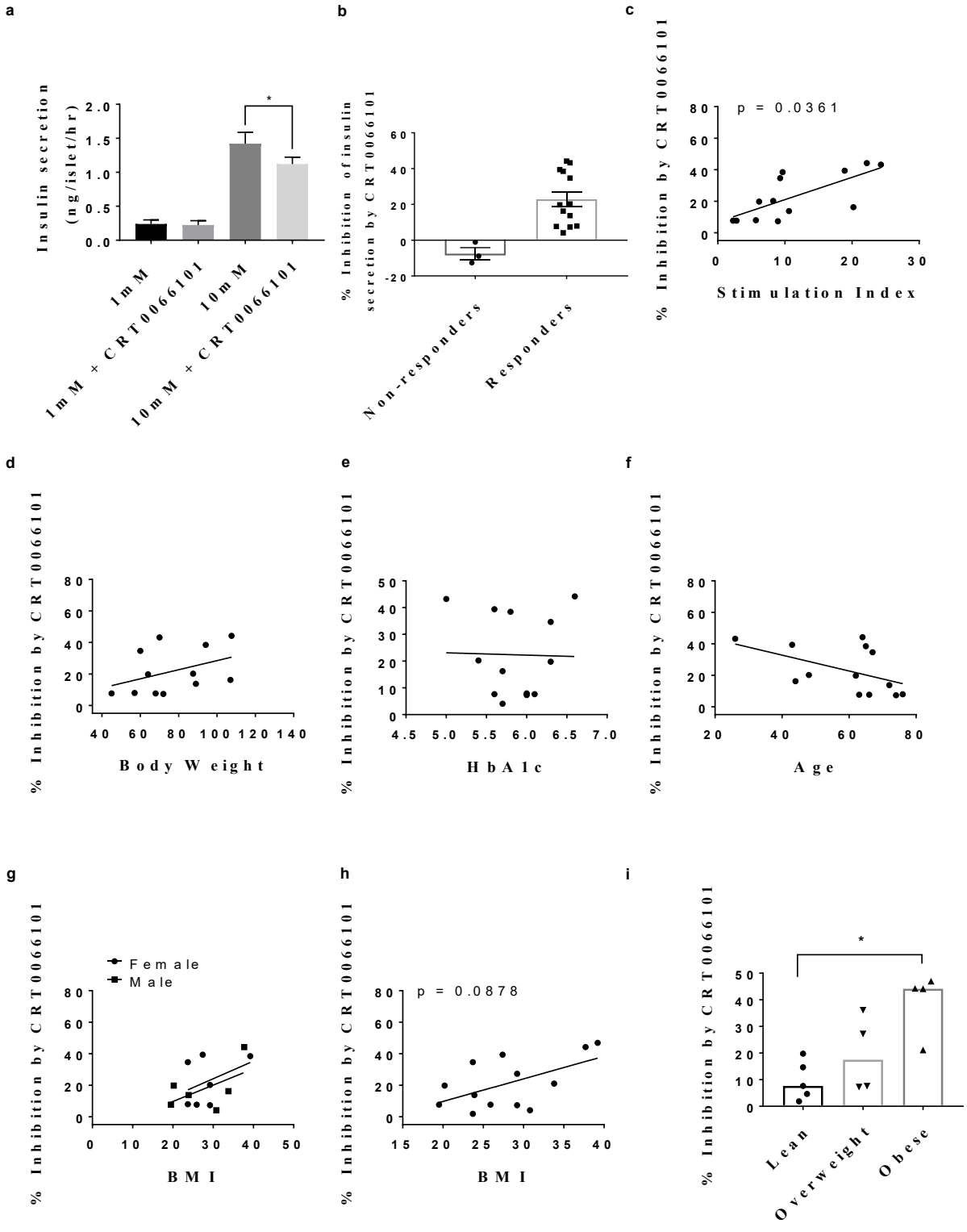

a
Insulin secretion (ng/islet/hr)
2.0
1.5
1.0
0.5
0.0
**
1mM
1mM + MRS 2500
10mM
10mM + MRS 2500
b
% Inhibition of Insulin secretion by MRS2500
60
40
20
0
-20
Non-responders
Responders
c
% Inhibition by MRS2500
p = 0.0250
50
40
30
20
10
0
0
10
20
30
Stimulation Index
d
% Inhibition by MRS2500
50
40
30
20
10
0
40
60
80
100
120
140
Body weight
e
% Inhibition by MRS2500
60
40
20
0
4.5
5.0
5.5
6.0
6.5
7.0
HbA1c
f
% Inhibition by MRS2500
50
40
30
20
10
0
20
40
60
80
Age
g
% Inhibition by MRS2500
Female
Male
50
40
30
20
10
0
0
10
20
30
40
50
BMI
h
% Inhibition by MRS2500
p = 0.0359
60
40
20
0
15
20
25
30
35
40
BMI
i
% Inhibition by MRS2500
*
60
40
20
0
Lean
Overweight
Obese

Figure 20: Ability of PKD1 to potentiate insulin secretion positively correlates with Stimulation index and BMI of humans

(a) Representative graph of insulin secretion in (ng/islet/hr) was assessed in 1-h static incubations in response to 1 and 10 mmol/l glucose in presence of PKD1 inhibitor, CRT0066101 (10 μmol/l), data collection by SK. (b) Comparison of donor-donor response according to percentage inhibition by PKD1 inhibitor. The percentage inhibition of responders after 20 mins CRT0066101 treatment plotted against (c) Stimulation index (n=13 donors), (d) Body weight (n=13 donors), (e) glycated hemoglobin; HbA1c (n=13 donors), (f) Age (n=13 donors), (g) Sex (n=13 donors) and (h) BMI (kg/m2; n=13 donors) in nondiabetic human islet donors. (i) Comparison of percentage inhibition by CRT0066101 according to BMI tertiles (lean < 25; overweight 25–30; and obese > 35 kg/m2) (n=5, 4, 4 donors).

Figure 20.


a
Insulin secretion (ng/islet/hr)
2.0
1.5
1.0
0.5
0.0
*
1mM
1mM + CRT0066101
10mM
10mM + CRT0066101
b
% Inhibition of insulin secretion by CRT0066101
60
40
20
0
-20
Non-responders
Responders
c
% Inhibition by CRT0066101
p = 0.0361
80
60
40
20
0
0
10
20
30
Stimulation Index
d
% Inhibition by CRT0066101
80
60
40
20
0
40
60
80
100
120
140
Body Weight
e
% Inhibition by CRT0066101
50
40
30
20
10
0
4.5
5.0
5.5
6.0
6.5
7.0
HbA1c
f
% Inhibition by CRT0066101
80
60
40
20
0
20
40
60
80
Age
g
% Inhibition by CRT0066101
Female
Male
80
60
40
20
0
0
10
20
30
40
50
BMI
h
% Inhibition by CRT0066101
p = 0.0878
80
60
40
20
0
15
20
25
30
35
40
BMI
i
% Inhibition by CRT0066101
*
50
40
30
20
10
0
Lean
Overweight
Obese

3.5 Discussion

This study demonstrates that signalling at the $P2Y_1$ receptor plays an important role in facilitating insulin release by activating PKD1 in pancreatic islets. This activity is essential for autocrine action as a dramatic increase in PKD1 phosphorylation is observed upon depolarization of the islets at low glucose which is inhibited in the presence of the $P2Y_1$ antagonist. Hence, this study supports the concept that potentiation of insulin secretion via $P2Y_1$ receptor is in part dependent on activation of PKD1. In addition, pharmacological modulation increases insulin secretion in response to purinergic ligands whereas, deletion of the gene encoding PKD1 abrogates the potentiation of exocytosis and glucose stimulated insulin secretion by MRS 2365. These results also confirm a key role for PKD1 in $P2Y_1$ signalling regulating glucose stimulated insulin secretion.

PKD is known to prime vesicles for efficient transport and fusion to promote secretion of neurotensin (279) and this PKD mediated secretion of neurotensin requires the target protein Kidins220 which has been proposed to regulate more peripheral steps of exocytosis (280). In line with this, another study observed two-fold increase in membrane capacitance in the first few depolarizations in p38δ deficient pancreatic β cells compared to control β cells and attributed this increase in capacitance to an enhanced activity of PKD at the peripheral step of exocytosis (281). The results obtained here are compatible with this. There is PKD1 dependent exocytosis to the first in a series of depolarizations.

PKD1 affects various steps of glucose homeostasis. It was reported that PKD is involved in the transcriptional regulation of the insulin receptor gene (282). Several studies corroborated its role in the trans-Golgi network where it plays a key role in the formation and final cleavage of insulin granules (249–251). And finally, PKD1 is important for glucose stimulated insulin secretion as shown here and by others. In particular, a study demonstrated that the free fatty acid, oleate, activates PKD1 through GPR40 which enhances insulin secretion (211). It is well known that plasma free fatty acids (FFA) levels are elevated in obesity (283). Elevated plasma FFA levels have been shown to account for insulin resistance in obese patients with type 2 diabetes mellitus (284). And hence, it is not surprising that the magnitude of PKD1 inhibition of glucose stimulated insulin secretion observed is positively correlated with human donor BMI, where islets from lean

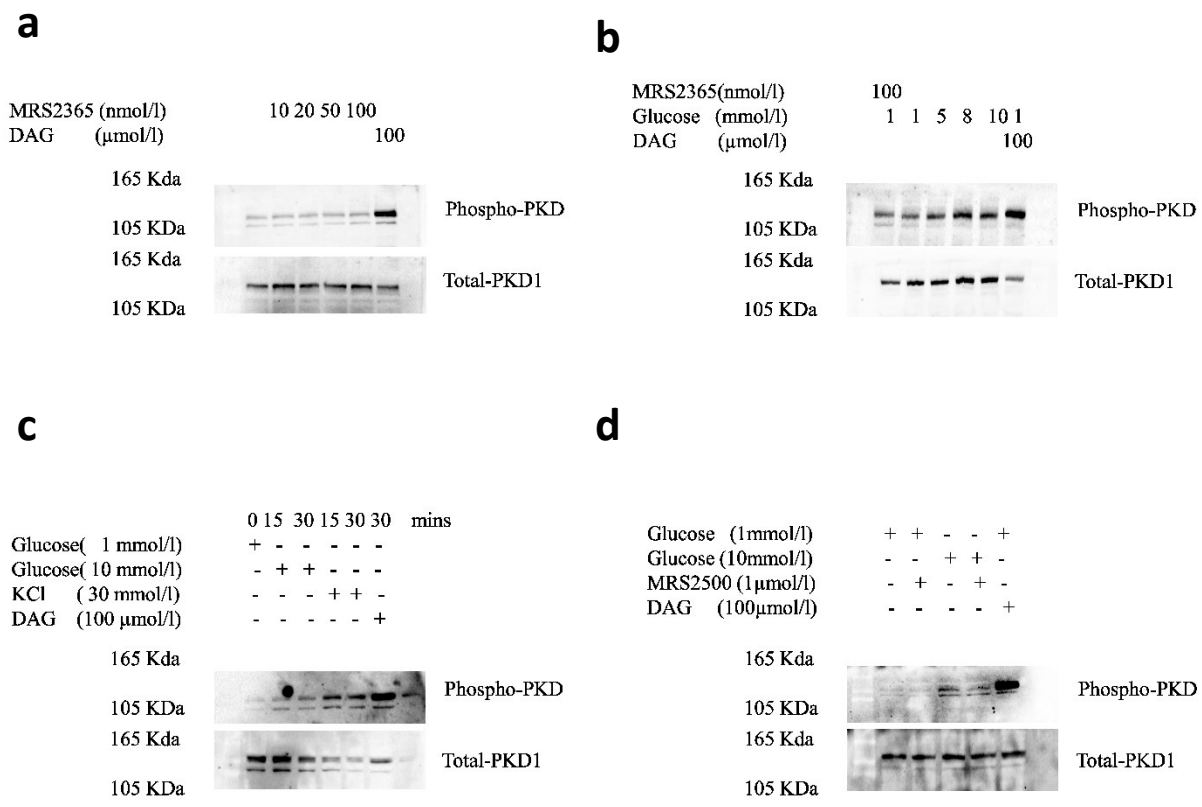
human donors have minimal responses to PKD1 and maximal response to obese donors. Interestingly, in an in vivo study of the βPKD1KO mice, the authors observed no significant differences in βPKD1KO and the control mice at basal condition but observed impaired glucose stimulated insulin secretion under high-fat diet condition in βPKD1KO mice versus the control mice both in vivo and ex vivo (208). The observations here are in complete agreement with these results and therefore, the role of PKD1 in obesity and diabetes warrants further investigation.

A specific function of the PKD1 isoform of the PKD family with respect to autocrine purinergic signalling has not been elucidated so far. This is the first evidence that $P2Y_1$/PKD1 pathway represents a key mechanism of potentiation of glucose stimulated insulin secretion. This work supports a positive regulatory role for both $P2Y_1$ and PKD1 in obese donors suggesting that lack of $P2Y_1$ and PKD1 function may lead to β cell dysfunction in obese and diabetic subjects.

Supplemental data Figure 2: Effect of purinergic agonists, antagonist, glucose and KCl on PKD1 activation in INS832/13 cells.

Representative immunoblots of phospho-serine 916 PKD and total PKD1 in protein extracts of INS832/13 cells after 20 minutes (unless otherwise indicated) exposure to (a) 1 mmol/l glucose + $P2Y_1$ agonist, MRS2365 at 10, 20, 50 and 100nmol/l, (b) 1, 5, 8 and 10 mmol/l glucose, (c) KCl (30mmol/l) applied for 15 and 20 minutes and (d) $P2Y_1$ antagonist, MRS 2500 (1µmol/l) at 1 and 10mmol glucose. DAG (10µmol/l) was used a positive control, data collection by SK.

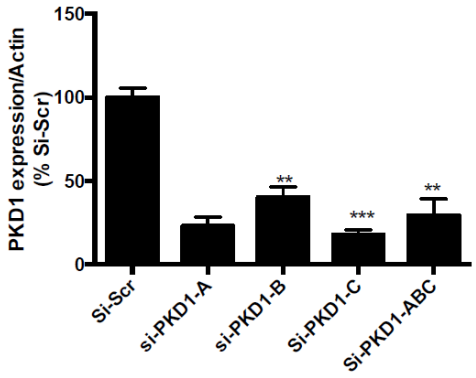
Supplemental data Figure 2.



Supplemental data Figure 3: Measuring PKD1 siRNA efficiency

Dispersed mouse pancreatic β cells were transfected with scrambled or PKD1 siRNA. Measurement of PKD1 mRNA expression by quantitative RT-PCR in dispersed mouse islets after 48 hours, data collection by MF.

Supplemental data Figure 3

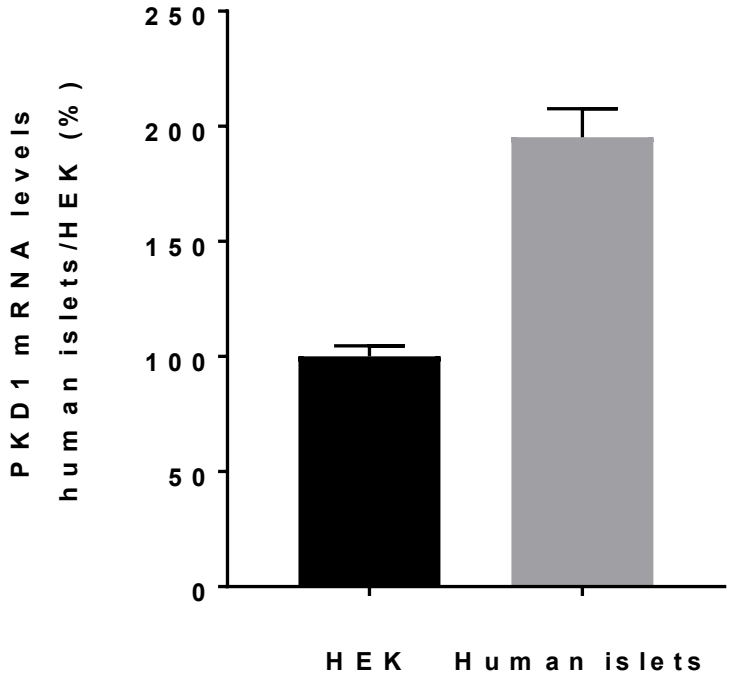


	Data Set-A		
	Mean	SEM	N
Si-Scr	100.000	5.689	6
si-PKD1-A	23.105	5.331	4
si-PKD1-B	40.130	6.316	6
Si-PKD1-C	17.822	2.900	6
Si-PKD1-ABC	29.408	9.810	6

Supplemental data Figure 4: Human islets express PKD1 mRNA

Measurement of PKD1 mRNA expression by quantitative PCR in HEK cells and in isolated human islets from healthy donor (n=3 replicates from 1 donor), data collection by SK.

Supplemental data Figure 4.



CHAPTER 4: SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS

4. SUMMARY

4.1 Summary of research findings

The stimulus-secretion coupling pathway that connects potentiation of insulin secretion to autocrine regulation of the pancreatic β cell is needs to be established. Among others, purinergic receptors present on the pancreatic β cells are responsible for the potentiation of insulin secretion (98,100,107,196,277). Insulin secretory vesicles contain high concentrations of adenine nucleotides, which are co-released with insulin during exocytosis (97). There is strong evidence that ATP and ADP serve as autocrine messengers via the purinergic receptors in pancreatic β cells (185). However, the handful of studies conducted to investigate the predominant purinergic receptor subtype present on the human pancreatic β cell and the pathways involved gave rise to considerable controversy (100,107). While one study proposed that ATP acts principally via $P2X_3$ receptors, membrane depolarisation and increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (100), a more recent study suggested a prominent role for $P2Y_1$ receptors and activation of protein kinase C (107). This thesis has provided evidence and resolved the controversy regarding the predominant purinergic receptor subtype and the downstream mechanism of actions involved. It has also demonstrated that both PLC/$InsP_3$ and the PLC/DAG pathways are involved in the autocrine action of ATP. And finally, it examined the role of PKD1 upon autocrine activation of $P2Y_1$ receptor. The following is a point form summary of the major finding of this thesis:

- The expression of $P2Y_1$ receptors in human β cells was verified by PCR and immunostaining.
- Patch-clamp recordings from dispersed islet cells demonstrated that ATP and ADP evoked inward depolarising currents in β cells. This response could not be reproduced by P2X receptor activation and was instead mimicked by $P2Y_1$ receptor agonist and inhibited by $P2Y_1$ receptor antagonists.
- The $[Ca^{2+}]_i$ responses of human β cells to extracellular ATP consist of a distinct peak followed by a sustained plateau. In the Ca^{2+} imaging experiments, the peak was insensitive to removal of extracellular Ca^{2+}, but sensitive to endoplasmic reticulum Ca^{2+} store depletion by thapsigargin or intracellular infusion of the $InsP_3$ receptor inhibitor heparin. In contrast, the plateau response required the presence of Ca^{2+} in the extracellular medium and thus reflected Ca^{2+} influx through the plasma membrane.

- In response to voltage clamp depolarisations, both ATP and ADP strongly potentiated exocytosis. The $P2Y_1$ antagonists hyperpolarised glucose-stimulated β cells and lowered $[Ca^{2+}]_i$ in the absence of exogenously added ATP.
- In human β cells overexpressing $P2X_2$ receptors, stimulation of exocytosis by Ca^{2+} infusion into the β cell via the patch pipette induced transient inward currents, reflecting autocrine action of ATP on $P2X_2$. This showed that human β cells release ATP which act in an autocrine manner, in response to elevated intracellular Ca^{2+}.
- A series of depolarisations in a subpopulation of cells produced an increase of cytosolic Ca^{2+} and this increase suddenly showed a marked acceleration coinciding with a pronounced increase of exocytosis which was prevented by $P2Y_1$ inhibition.
- The $P2Y_1$ receptor antagonist reduced insulin secretion by 35% in human islets exposed to a stimulatory glucose concentration
- Activation of PKD1 in mouse islets depolarized with KCl at low glucose was confirmed. This response was inhibited by the $P2Y_1$ antagonist.
- The ability of $P2Y_1$ agonist to potentiate glucose stimulated insulin secretion in isolated control mouse islets was confirmed but this ability to potentiate glucose stimulated insulin secretion was lost in βPKD1KO islets.
- The ability of siPKD1 to decrease exocytosis in the presence of $P2Y_1$ agonist to the first depolarisation is lost to successive depolarisations in both the control and thapsigargin pretreated cells. This showed that PKD1 is important for the very initial step of the first phase of insulin secretion.
- Islets from obese donors were more sensitive to the inhibitory effects of $P2Y_1$ antagonist and PKD1 inhibitor as opposed to the lean donors meaning autocrine $P2Y_1$ signalling, possibly acting via PKD1, makes a greater contribution to insulin secretion from islets of obese donors.

4.2 Discussion

4.2a General discussion

ANIMAL AND CELLULAR MODELS USED: Mice remain the most studied animal model in pancreas research because the development, body plan, physiology, behavior, and diseases have much in common with humans, based on the fact that 99% of the human genes have a mouse ortholog (285). The findings of the research in mice are often extrapolated to humans. However, beside the apparent difference in size and macroscopic organization of the pancreas in the 2 species, there are a number of other differences such as the distribution, composition, and architecture of the endocrine islets of Langerhans (286). Chapter 2 of this thesis was completely conducted in human islets, and therefore, it doesn't require any such extrapolation of results. It provides strong evidence regarding a particular purinergic receptor subtype and delves into the possible mechanism through which it works in humans. Nevertheless, it must be remembered that the studies of the purinergic signalling were performed in dispersed pancreatic human β cells which has a different milieu compared to the physiological milieu in the intact pancreatic islet where additional aspects (for e.g. spatial arrangement of cells, gap junctions, paracrine actions etc.) come into play and present a considerably more complex situation (287). Moreover, isolated islets or dispersed pancreatic human β cells in culture are denervated, devoid of vascular supply and can quickly lose function in vitro due to hypoxia, typified by diminished glucose-stimulated insulin secretion (288).

Chapter 3, on the other hand, used insulin secreting cell line, cells, mouse islets and human islets to establish the activation of the downstream protein kinase upon autocrine activation of the particular purinergic P2 receptor. The studies were initially attempted in the insulinoma cell model to establish proof of concept because they can be grown in large numbers very quickly. However, inherent issues with this model interfered with some experiments. A major and serious issue associated with cell lines was the change of cell characteristics over a period of continuous growth (289) and therefore, reliable and reproducible results were difficult to produce and had to be timed within a narrow window of only a few weeks. In addition, the ability of many of these cell cultures to grow without limits is related to their tumour origin, possible abnormal chromosomal content, other genetic mutations, abnormal protein expression and modified metabolism (289). Another

major disadvantage of these cells is the requirement of mercaptoethanol (toxic, irritating and irreversibly denatures the proteins) for the propagation of the cells in their culture media which causes loss of many important functional characteristics (290). In line with this, the results of the Western blotting performed in this thesis in INS 832/13 cell lines also changed with increasing passage number (Figure 21). Also, no single cell line entirely recapitulates the properties of human β cells. As a result, a switch to isolated mouse and human islets were imperative for the experiments.

The MIP-CreERT and βPKD1KO mouse islets were a generous gift from the Poitout laboratory, University of Montreal. Two considerations needed to be made about the islets. The first involved a recent study which reported a mechanism whereby inclusion of a human growth hormone (*hGH*) minigene as a component of the transgene construct impaired β cell function in β cell specific transgenic mice. Since the *hGH* minigene is the second cistron in the transgene-encoded mRNA, it was believed for a long time that it was not expressed. But this study provided compelling data otherwise (291). As suspected, expression of hGH has a profound influence on the interpretation of certain types of experiments, especially those pertaining to the control of pancreatic β cell mass and the regulation of insulin secretion (291). The study published by Bergeron et al. using MIP-CreERT and βPKD1KO mouse islets also observed a similar phenomenon in their transgenic mouse models. Under a chow diet, both MIP-CreERT and βPKD1KO mice became glucose intolerant with age compared to the WT mice showing that the phenotype observed was due to the MIP-CreERT transgene rather than β cell specific deletion of PKD1 (278). The second consideration was the sexes of the mice from which the islets were isolated and shipped as there are well documented differences in susceptibility to T2DM between males and females.

Estrogen, the major female sex hormone, is suggested to protect against development of the metabolic syndrome, and the prevalence of obesity, insulin resistance, and T2DM increases in post-menopausal women (292). Female mice are also protected against high-fat diet induced metabolic syndrome (293). However, Gonzalez et al. published different outcomes that insulin hypersecretion in islets from diet-induced hyperinsulinemic obese female mice is associated with several functional adaptations in individual β cells (294). Epidemiological studies have shown that males have a higher risk of insulin resistance, type 2 diabetes, and metabolic syndrome than

females, whereas females tend to have more severe disease (295–297). Insulin secretion experiments conducted in this thesis in MIP-Cre-ERT and βPKD1KO also generated different results with male and female islets (Figure 22). The secretory dysfunction observed in female mice was much less than that observed in male mice. Therefore, data obtained from the experiments conducted using islets from both female and male mice needed to be interpreted with care as they represent only half the population. It would be prudent to use both male and female mice for experiments to get a full understanding of how the diseases affect the entire population.

METHODOLOGICAL CONSIDERATIONS: Western blotting experiments conducted in this thesis used antibodies in INS 832/13 cell lines and in mouse islets. Antibodies are among the most commonly used tools in the biological sciences to identify proteins. But it is now clear that they are also among the most common causes of problems, too. In 2009, a study assessed the antibodies that are used to study GPCRs which are targeted by drugs to treat various disorders. In the analysis of 49 commercially available antibodies that targeted 19 signalling receptors, most bound to more than one protein, meaning that they could not be trusted to distinguish between the receptors (245). Another news feature published in Nature in 2015 pointed out the most obvious issues with bad antibodies, namely, cross-reactivity (binds to other proteins in addition to target proteins), variability (difference in performance from one batch to another) and wrong application (changing experimental condition affecting protein's binding ability) (298). The antibody used to identify total PKD1 in INS 832/13 cell lines were reproducible and consistent. However, the same antibody didn't pick up the total PKD1 in mouse islets later maybe because of batch to batch variability. Moreover, the antibody soon became unavailable and at the time of writing this thesis, according to the Cell Signalling Technology website, "in the United States and Canada this product is still currently unavailable". And hence, Beta-actin was used as a loading control for Western blotting experiments to normalize the levels of protein detected by confirming that protein loading is the same across the gel. Also, since the total PKD1 remained unchanged in the INS 832/13 cell lines, it was assumed that the total PKD1 remains same in the mouse islets too. Another antibody related issue faced by this thesis was the use of antibody to detect the human autophosphorylation site serine 910 (corresponding to PKD serine 916 of mouse). The antibody detected phospho-PKD serine 910 in human embryonic kidney (HEK) cells 293 but not in human islets (Figure 23) despite there being a two-fold expression of PKD1 mRNA in human islets compared to HEK cells. A

pertinent observation related to this is the antibody used was a rabbit polyclonal antibody which was later on discontinued by Santa Cruz Biotechnology.

An important consideration needed to be made while performing static glucose stimulated insulin secretion experiments involving PKD1. The free fatty acid (FFA) receptors, GPR40 and PPARβ/γ, are known to affect PKD1 and its regulation of insulin secretion (211,253). Therefore, to detect autocrine action of $P2Y_1$ receptor in activating and regulating insulin secretion through PKD1, the Krebs-Ringer Bicarbonate buffer solution made had FFA free bovine serum albumin (BSA) in place of regular BSA. This ensured that the effects observed were not a result of activation of the free fatty acid receptors but rather due to autocrine activation of the $P2Y_1$ receptor.

PURINERGIC SIGNALLING IN THE PHYSIOLOGICAL MILIEU: The endocrine cells store ATP in insulin containing large dense-core vesicles and differentially release it along with hormones to initiate purinergic chemical transmission (119). The vesicular nucleotide transporter is also a key component for vesicular ATP release and purinergic signalling in the endocrine cells of the pancreas (101). The endocrine cells in the islets, however, are not the only source of purine nucleotides such as ATP and ADP. From studies of other systems it is known that ATP is released from presynaptic terminals containing other transmitters such as noradrenaline (299). In the pancreas, ATP and acetylcholine have been known to show synergistic effects on insulin release (300). In mouse islets, transient and self-regenerating release of ATP from β cells serves as a complement to gap junctions for synchronising cytosolic Ca^{2+} oscillations between β cells to generate pulsatile insulin secretion (115,301). In addition to these two sources mentioned, a new source of ATP has been proposed. Pannexin-1 (Panx-1) are transmembrane channels that are able to release ATP (302). Experiments in β cell preparations revealed the third pathway for ATP release that depends on the $P2X_7$ receptor and Panx1 channels (98). Therefore, it is safe to say that additional aspects and sources of ATP come into play when considering the more complex situation in the intact pancreatic islet in the physiological milieu. It should be noted that most studies performed in both Chapter 2 and 3 were in cultured isolated β cells where the complexities of intact islets couldn't be reproduced. Intact islets form a functional syncytium integrates and propagates information encoded by secretagogues, producing a "gain-of-function" in hormone release through the generation of coordinated cell to cell activity which as mentioned right before is lost in cultured dispersed β cells.

Purinergic signalling in vivo is further controlled by E-NTPDases and P1 purinergic receptors. E-NTPDases regulate extracellular ATP levels by degrading ATP and related nucleotides. Their activity affects the relative proportion of ATP and its metabolites, which in turn impacts the level of purinergic receptor stimulation exerted by extracellular ATP (204). NTPDases are also known to participate in the formation of the metabolite adenosine. In addition to P2 receptors, pancreatic β cells are also equipped with purinergic P1 receptors that are preferentially activated by adenosine, a product of ATP degradation (303,304). Adenosine inhibits glucose-induced insulin secretion via activation of A1 adenosine receptor coupled to inhibition of the adenylase cyclase activity. Therefore, the resulting effect of extracellular ATP on the extent and duration of insulin secretion is determined by two major factors in vivo: purinergic receptor desensitization and the kinetics of ATP conversion into other nucleotides in the extracellular compartment (204).

Figure 21: Phosphorylation of PKD1 and positive control diacylglycerol (DAG) at serine 916 affected by passage number of INS 832/13 cells.

Immunoblots of protein extracts from INS 832/13 cells at treated for 20 min at 1 mmol glucose with P2Y1 agonists, MRS2365 with concentrations as indicated and DAG (100µmol/l) at passage number (a) 54 and (b) 56 , data collection by SK (c and d) Quantification of phospho-PKD1 (serine 916) normalized to total PKD1.

Figure 21.

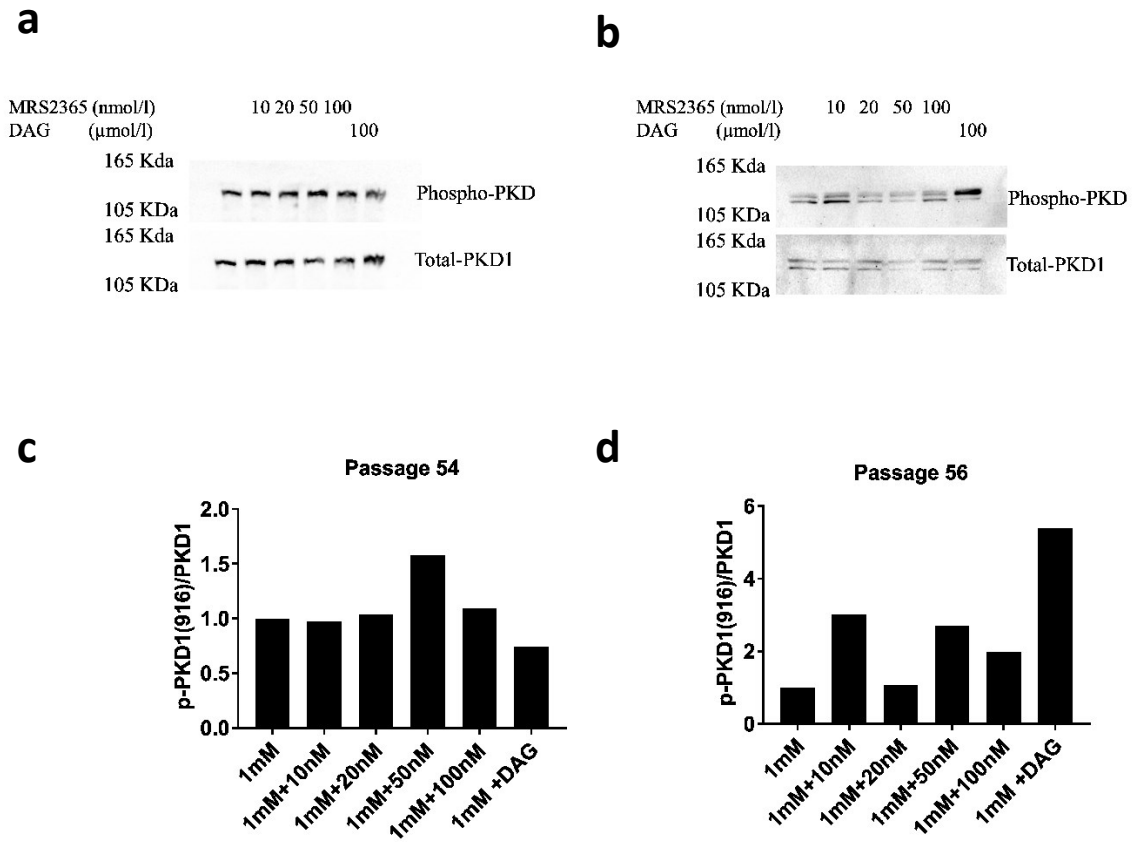

a
MRS2365 (nmol/l) 10 20 50 100
DAG (μmol/l) 100
165 Kda
105 KDa
Phospho-PKD
165 Kda
105 KDa
Total-PKD1
b
MRS2365 (nmol/l) 10 20 50 100
DAG (μmol/l) 100
165 Kda
105 KDa
Phospho-PKD
165 Kda
105 KDa
Total-PKD1
c
Passage 54
p-PKD1(916)/PKD1
2.0
1.5
1.0
0.5
0.0
1mM
1mM+10nM
1mM+20nM
1mM+50nM
1mM+100nM
1mM +DAG
d
Passage 56
p-PKD1(916)/PKD1
6
4
2
0
1mM
1mM+10nM
1mM+20nM
1mM+50nM
1mM+100nM
1mM +DAG

Figure 22: Insulin secretory dysfunction between βPKD1KO male and female mouse

(a) Insulin secretion was measured from female βPKD1KO and MI-Cre-ERT mouse islets at 1 and 10 mmol/l glucose in the presence of MRS2365 (100nmol/l), data collection by SK. (b) Insulin secretion was measured from male βPKD1KO and MI-Cre-ERT mouse islets at 1 and 10 mmol/l glucose in the presence of MRS2365 (100nmol/l), data collection by SK. Data are mean ± SEM for 3-5 independent experiments, $p < 0.05$ compared to 10 mmol/l glucose.

Figure 22:

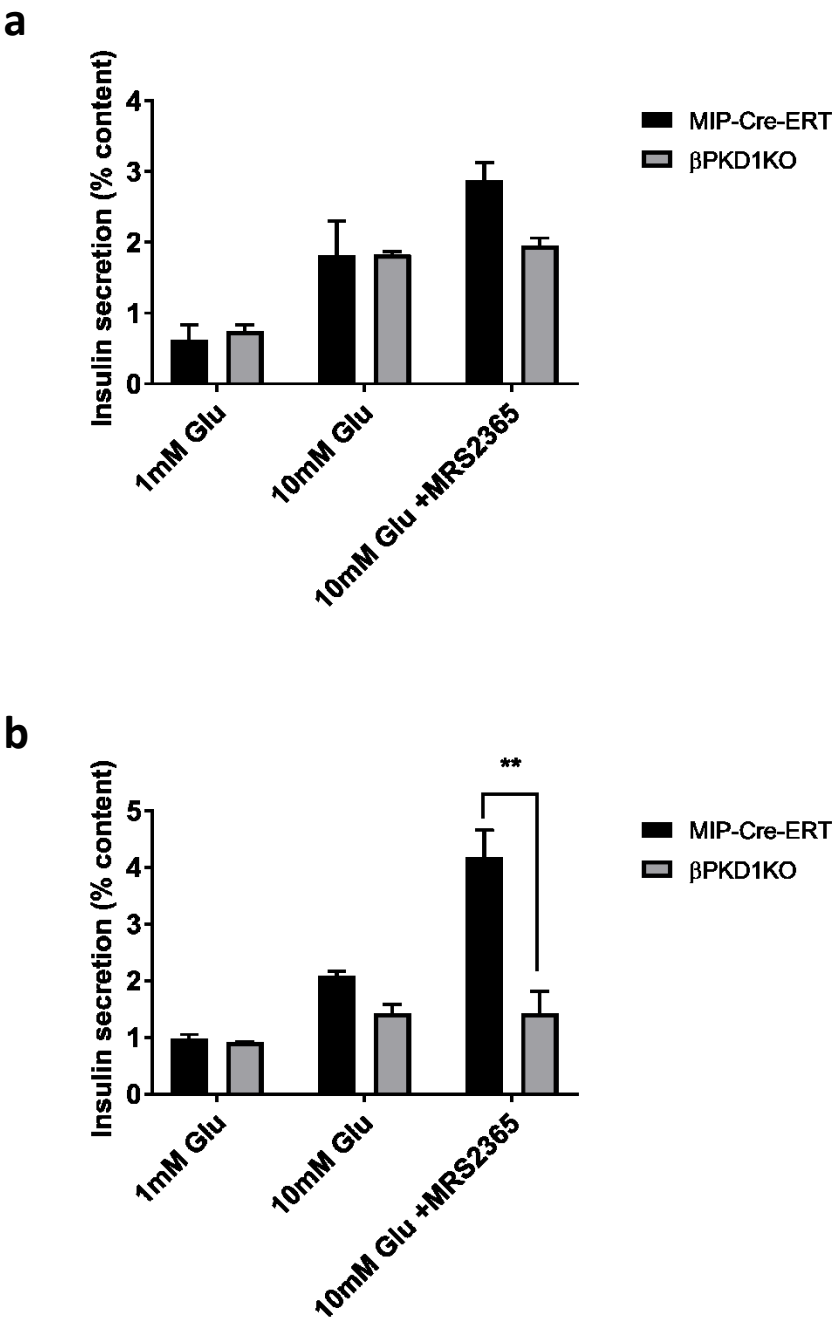
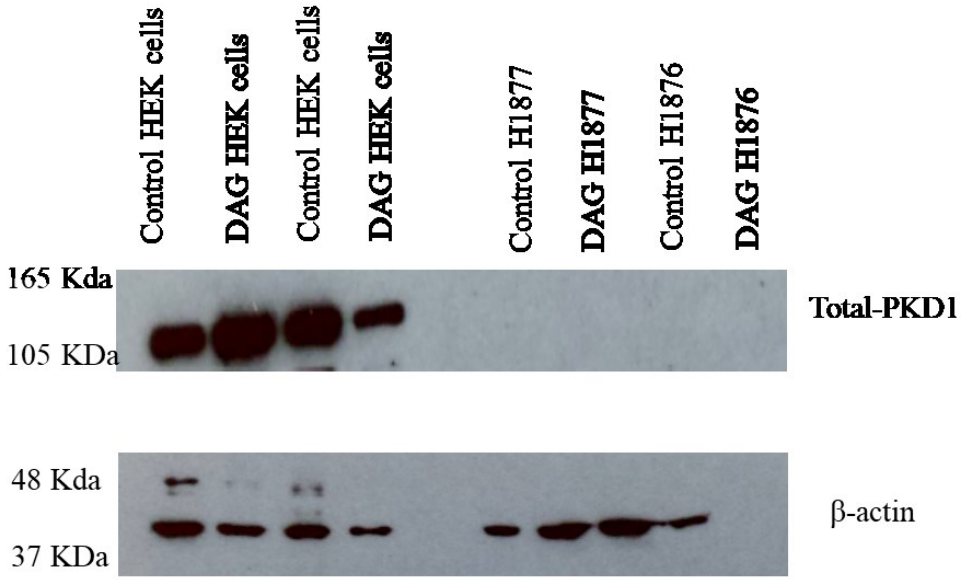

a
Insulin secretion (% content)
0
1
2
3
4
1mM Glu
10mM Glu
10mM Glu +MRS2365
MIP-Cre-ERT
βPKD1KO
b
**
Insulin secretion (% content)
0
1
2
3
4
5
1mM Glu
10mM Glu
10mM Glu +MRS2365
MIP-Cre-ERT
βPKD1KO

Figure 23: Detection of PKD1 in HEK 293 cell line and human islets

Immunoblots of protein extracts from HEK 293 cells and human islets treated for 20 min ± DAG (100µmol/l), data collection by SK. Data are from two donors.

Figure 23.


Control HEK cells
DAG HEK cells
Control HEK cells
DAG HEK cells
Control H1877
DAG H1877
Control H1876
DAG H1876
165 Kda
105 KDa
Total-PKD1
48 Kda
37 KDa
β-actin

4.2b. Discussion on Chapter 2

Despite the few technical limitations of Chapter 2 discussed above, this thesis provides compelling evidence towards the autocrine signalling action of the receptor $P2Y_1$ in human pancreatic β cells. Recent literatures had arrived at different conclusions regarding receptor involvement favouring either $P2X_3$ or $P2Y_1$ receptors in humans. In line with the discussion in Chapter 2 regarding the effects of the modulators on the $P2X_3$ receptor only very modest or no effects were observed for the $P2X_3$ receptor by Wuttke et al. and of course, by this thesis (99,277). Wuttke et al. went on to further publish on autocrine signalling action of the receptor $P2Y_1$ recruiting DAG and activating PKCs downstream (196). As observed by this thesis that the $P2X_3$ agonist, α,β-meATP, did not increase the electrical activity or the $[Ca^{2+}]_i$ levels and the selective $P2X_3$ blocker, TNP-ATP, did not affect the $[Ca^{2+}]_i$ (277). Hence, this thesis is not in agreement with the results of the study suggesting $P2X_3$ receptor playing the dominant role in human pancreatic β cells. One possible explanation includes but is not limited to the fact that the modulators, pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid (iso-PPADS) and oxidised ATP (oATP), employed by the study to inhibit $P2X_3$ were used at concentrations that also strongly inhibited the $P2Y_1$ receptor (147,305).

The first report of $P2X_7$ receptor in human pancreatic β cells was by Glas et al. Their study showed a correlation of $P2X_7$ receptor activation, interleukin-1 receptor antagonist (IL-1Ra) secretion and regulation of β cell mass and function (254). Later on, another study explored the association of hypofunctioning $P2X_7$ polymorphs with glucose homeostasis in mice and humans and found that the hypofunctioning $P2X_7$ was associated with increased insulin sensitivity and secretion in humans (191). And the latest study published this year by Ureste-Riveri et al. suggested that the 1068 G>A polymorphism of the *P2RX7* gene is associated with an increased β cell function and IL-1Ra release in T2DM patients (192). However, $P2X_7$ has a very low affinity for ATP (half maximal effective concentration [ED50] 0.78 mmol/l) in humans as reported by Chessell et al. (260). They also reported that human $P2X_7$ is insensitive to ADP and AMP (260). Therefore, based on the very high concentration of ATP required for $P2X_7$ receptor activation and the insensitiveness to ADP and AMP, the likelihood of its role in human pancreatic β cells becomes questionable. However, it may be interesting to investigate the different variants further before reaching that conclusion.

Few studies had contradictory findings regarding the role of $P2Y_1$ receptors. In 2005, León et al. performed experiments in $P2Y_1^{-/-}$ mice and observed a 15% increase in glycemia, a 40% increase in insulinemia in non-fasted animals and a tendency for glucose intolerance (106). Another group that worked with MIN6 cells showed that selective stimulation of their $P2Y_1$ and $P2Y_6$ receptors induced the insulin secretion that was blocked by selective antagonists of these receptors (195). On the other hand, all the other studies provided staggering evidence that ATP stimulates insulin secretion. In 1999, Fisher et al. tested 2-thioether-5'-Ophosphoro-thioate adenosine derivatives as potential insulin secretagogues using isolated and perfused rat pancreas. Their studies showed that ATP analogues stimulated an almost 500% increase in glucose-induced insulin secretion (306). Similar effects were observed on human pancreatic islets, although at different glucose concentrations (108). In 2006, Farret and colleagues evaluated $P2Y_1$ receptor agonists based on a C2-substitution of the adenosine 5'-O-(1-boranotriphosphate) scaffold in the isolated perfused pancreas of male rats. This work also demonstrated increased insulin release through $P2Y_1$ receptor stimulation (307). In addition to all the work mentioned above, this thesis found no evidence for a negative role of ATP in insulin secretion from human islets. And the data from this thesis is consistent with Wuttke et al. who also observed the potentiation of insulin secretion from human islets from $P2Y_1$ receptors (99,196,277).

The experiments conducted in this thesis had ATP increase $[Ca^{2+}]_i$ in a biphasic manner, with an initial peak reflecting Ca^{2+} release from stores and a plateau reflecting Ca^{2+} influx. This release of Ca^{2+} was via the heparin-sensitive InsP3 receptors of the endoplasmic reticulum. Another class of receptor, Ryanodine receptor (RyR), can trigger release of $Ca^{2+}$from the endoplasmic reticulum (308). Whether pancreatic β cells display classical CICR triggered by RyRs is hotly debated. The pancreatic β cells strongly express the three InsP3 receptor isoforms (I-III), although to different levels depending on the species (309–313). Hence, it is not surprising that this thesis identified InsP3 as the intracellular Ca^{2+} channel underlying ATP-induced Ca^{2+} release (277). The presence of RyRs in pancreatic β cells is controversial. Although several studies evaluating its expression reported its presence (314), it has been acknowledged that RyR expression is much lower in β cells than in control tissues (315). Using two microarray platforms and RT-PCR, Gilon et al. could not find significant expression of the three RyR isoforms in FACS purified normal mouse β cells but found high expression in control tissues (276,316). Also, the findings of this thesis is different from those of Jacques-Silva et al. who concluded that Ca^{2+} stores

contribute little to the ATP-evoked Ca^{2+} signal in human β cells (100). Chapter 2 already suggested that this difference in observation may be explained by experimental differences.

An important question of this thesis was whether purinergic signalling i.e. the Ca^{2+} induced Ca^{2+} release pathway is altered in diabetes. A major strength of this study was its access to T2DM donor which enabled it to attempt to answer the question. It includes intriguing limited data that intracellular Ca^{2+} mobilisation from thapsigargin-sensitive stores in response to ATP stimulation tended to be reduced in islets from a T2DM donor (277). This possibly indicates a link between deficient $P2Y_1$ signalling and impaired insulin secretion. Though it may be prudent to reproduce the findings in more T2DM donors before drawing firm conclusions. However, there is evidence that ER stress is involved in the pathogenesis of T2DM in humans (273). Thivolet et al. demonstrated that one of the isoforms of InsP3 receptor expression was altered in β cells from T2DM patients (317). They suggested that altered InsP3 receptor amounts may reflect important changes in Ca^{2+} homeostasis and release of Ca^{2+} from the ER (317). There is also evidence that exposure of human islets to high glucose concentrations led to reduced levels of sarco-endoplasmic reticulum Ca^{2+} ATPase 2b (SERCA 2b) pump, the main ER Ca^{2+} pump in β cells (274,275,318). All these evidence points towards an important role of Ca^{2+} release to the glucose-induced Ca^{2+} signal in T2DM patients.

4.2c Discussion on Chapter 3

The considerations and limitations of Chapter 3 have been addressed in detail in the general discussion. There is not much PKD1 data available in the context of the pancreatic β cells and to the best of my knowledge, no study has been done in human islets. Therefore, this thesis clearly advances our understanding of PKD1 and its possible role in obesity and diabetes.

PKD1 plays a positive role in insulin secretion from the pancreatic β cells. This thesis has shown that in C57Bl/6 mice, it is required for the potentiation of glucose stimulated insulin secretion in response to purinergic signalling. The potentiation of glucose stimulated insulin secretion by purinergic signalling is intact in the MIP-Cre-ERT islets but not in the βPKD1KO islets. Bergeron et al. suggested that there was there was no difference in the chow diet fed βPKD1KO and the MIP-Cre-ERT under stimulated conditions (i.e. PKD1 is dispensable for normal glucose homeostasis in chow diet fed mice) (208). Although this thesis observes an apparent difference between the groups in response to glucose stimulation of isolated islets, it was not statistically significant. This raises the question of why I observe a potentiation in the MIP-Cre-ERT group in the presence of a purinergic agonist. One reason may be that Bergeron et al. observed a significant reduction in insulin secretion in the chow diet fed βPKD1KO versus wild type islets but not in the chow diet fed MIP-Cre-ERT versus wild type islets in the presence of the free fatty acid, oleate (208). This goes to show that despite the groups responding similarly to stimulation of glucose, there are differences in response to potentiating factors such as oleate and $P2Y_1$ agonist.

In Chapter 2 it was demonstrated that ATP acts as a positive autocrine signal in human β cells by activating $P2Y_1$ receptors and increasing $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx and evoking Ca^{2+} release via $InsP_3$-receptors in the ER (277). Therefore, the effect on depolarization-induced exocytosis while excluding an effect on intracellular Ca^{2+} was investigated by pretreating the cells with thapsigargin (depletes $InsP_3$-sensitive intracellular Ca^{2+} stores). Though not significantly, PKD1 knockdown prevented the $P2Y_1$ induced enhancement of the exocytotic response to the first depolarization in thapsigargin treated cells. However, the exact same response was observed in cells without thapsigargin suggesting that Ca^{2+} release via $InsP_3$-receptors possibly does not contribute to the activation of PKD1 downstream. Therefore, the $P2Y_1$ signalling possibly exerts a direct effect on the exocytotic machinery through the PLC/DAG pathway but not the PLC/$InsP_3$

pathway. It was previously shown that PKD1 plays a role in priming neurotensin vesicles and regulating peripheral steps of exocytosis (279,280). It is well established that insulin granule trafficking is a key step in the secretion of glucose stimulated insulin from pancreatic β cells. Hence to study the effect of $P2Y_1$ agonist on insulin granule trafficking, electron micrographs were generated. A strong trend in decreased number of docked insulin granules and increased number of empty vesicles was observed in cells exposed to $P2Y_1$ agonist compared to the glucose group alone suggesting a role for granule docking and exocytosis (Figure 24).

Ferdaoussi et al. have shown that PKD1 promotes second-phase insulin secretion and that this promotion was due to filamentous actin (F-actin) depolymerisation (211). In β cells, filamentous F-actin forms a cortical web below the plasma membrane, functioning as a barrier between granules and the plasma membrane. Disruption of the cortical F-actin barrier enhances insulin secretion secondary to increased insulin granule access to the plasma membrane (60,320).To this effect, this thesis also attempted to observe changes in F-actin depolymerisation in response to $P2Y_1$ inhibition under stimulated conditions. Apparent inhibition of F-actin depolymerisation was observed for the cells treated with $P2Y_1$ antagonist but there was no significant F-actin depolymerisation observed in the $P2Y_1$ agonist group or the DAG group. (Figure 25). Perifusion experiments were also conducted to detect at which phase PKD1 plays a role. Unfortunately, expired PKD1 inhibitor rendered completely opposite results to what was expected (data not shown). Therefore, this thesis was unsuccessful to arrive at any conclusion regarding its role in F-actin depolymerisation.

Chapter 3 had the intriguing finding that islets from obese donors had greater inhibition of insulin secretion, in response to P2Y1 or PKD1 antagonism, compared to lean donors. Ferdaoussi et al. has provided convincing data that the free fatty acid receptor GPR40 potentiates insulin secretion via PKD1(211). It is well recognized that plasma free fatty acid levels are elevated in most obese individuals (321). It may be important to summarize that on the one hand, the free fatty acids are efficient stimulants of insulin release from pancreatic β cells (322), whereas, on the other hand, elevated free fatty acid concentrations in obesity via elevated oxidative stress and low-grade inflammation result in impaired insulin secretion and may lead to the disease progression of fully developed T2DM (323). Bergeron et al. in their high fat diet fed βPKD1KO mouse model observed increased hyperglycemia, hyperinsulinemia and glucose intolerance under the high-fat

diet condition (208). Interestingly, the authors also observed that high fat diet fed βPKD1KO mice show an insulin secretion deficiency in response to glucose during hyperglycemic clamps after 12 weeks. This is supported ex vivo by reduced insulin secretion in response to glucose in high fat diet fed βPKD1KO islets (208). Therefore, it becomes difficult to account for the hyperglycemia and hyperinsulinemia in this model despite the impaired glucose stimulated insulin secretion. In contrast, the high fat diet fed GPR40KO mice develop hyperglycemia more rapidly than wild type mice and it has been proposed that this hyperglycemia results from reduced insulin secretion (324). This thesis observes that obese donors are more sensitive to both the $P2Y_1$ and PKD1 and this possibly is due to elevated free fatty acids taken up through free fatty acids receptors which may lead to defective insulin secretion in obesity and presentation of overt T2DM.

Figure 24: Effect of $P2Y_1$ agonist on docked insulin granules

Representative electron micrographs of mouse β cells at (a) 1 mmol/l glucose or (b) 1 mmol/l glucose and MRS2365 (100nmol/l), data collection by SK. (c) Quantification of number of docked insulin granules and (d) number of empty vesicles plotted against distance from the plasma membrane.

Figure 24.

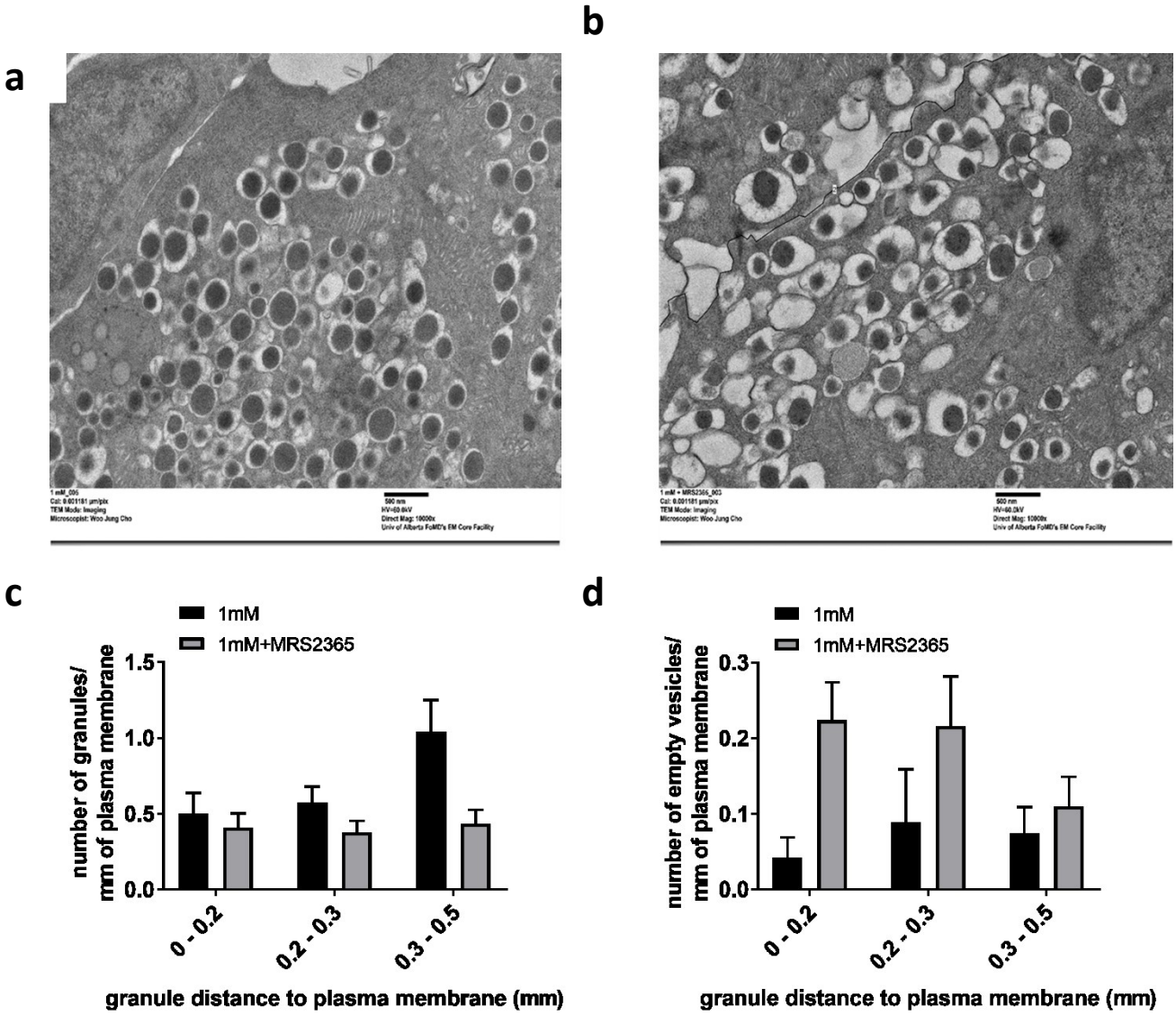
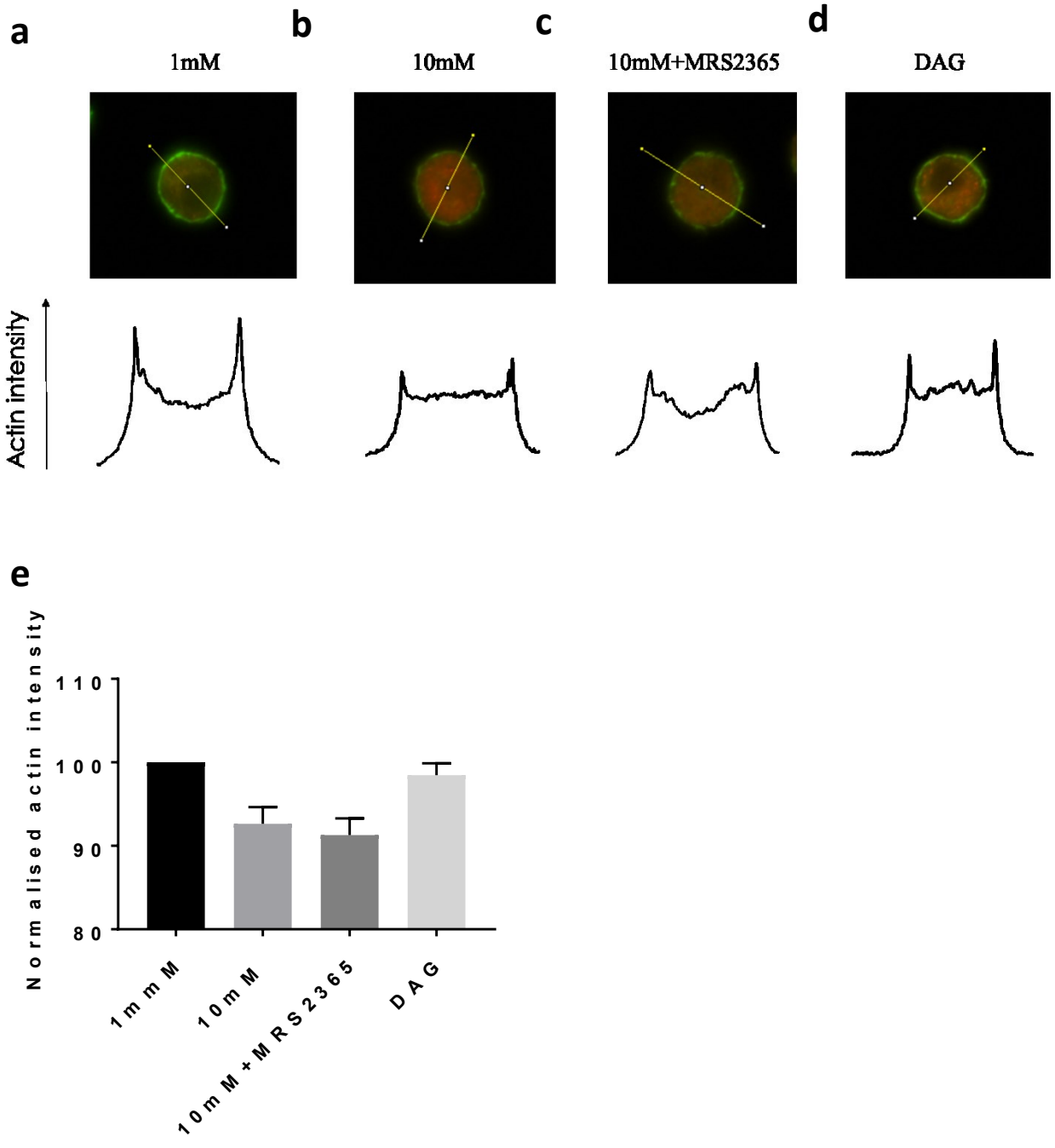

a
b
c
d
1mM
1mM+MRS2365
number of granules/ mm of plasma membrane
1.5
1.0
0.5
0.0
0 - 0.2
0.2 - 0.3
0.3 - 0.5
granule distance to plasma membrane (mm)
number of empty vesicles/ mm of plasma membrane
0.3
0.2
0.1
0.0
0 - 0.2
0.2 - 0.3
0.3 - 0.5
granule distance to plasma membrane (mm)

Figure 25: Effect of $P2Y_1$ agonist and DAG on actin depolymerisation

Representative images (after 20-minute treatment) of dispersed mouse β cells with (a) 1 mmol/l glucose, (b) 10 mmol/l glucose, (c) 10 mmol/l glucose + MRS2365 (100nmol/l) and (d) DAG (100µmol/l) stained for insulin (red) or filamentous actin (F-actin; green), data collection by SK. Example line scan analysis (middle). (e) Normalised average peak F-actin intensities as arbitrary units are shown.

Figure 25.


a
b
c
d
1mM
10mM
10mM+MRS2365
DAG
Actin intensity
e
Normalised actin intensity
110
100
90
80
1mM
10mM
10mM+MRS2365
DAG

4.3 Relevance to Diabetes

It is well established that impaired insulin secretion from pancreatic β cells is a fundamental pathogenic factor in virtually all forms of diabetes. The present thesis significantly advances our understanding of stimulus-secretion coupling in these cells. The data of this thesis suggest that signalling via $P2Y_1$ receptors represents a novel indirect form of Ca^{2+}-induced Ca^{2+} release in pancreatic β cells. It also activates PKD1 downstream to potentiate glucose stimulated insulin secretion and this pathway possibly becomes more important in obese individuals.

The passage from obesity to diabetes is made by a progressive defect in insulin secretion coupled with a progressive rise in insulin resistance (325). It is estimated that about 90% of T2DM is attributable to excess weight (326). Obesity causes sustained elevation in plasma FFA levels, both in the basal state and following glucose load which present a major factor for insulin resistance (327,328). Hyperglycemia and compensatory hyperinsulinemia associated with insulin resistance and glucose intolerance lead to a pancreatic β cell secretory failure and apoptosis (329). The observations that 1) PKD1 becomes necessary for the compensatory increase in glucose stimulated insulin secretion in response to high-fat feeding in mice (208), 2) it is activated downstream of the free fatty acid receptor GPR40 and potentiates glucose stimulated insulin secretion (211) combined with the observation of thesis that 3) PKD1 may be dispensable in lean donors but not in obese donors point towards an important role of PKD1 in regulating insulin secretion in obesity and diabetes.

The G-protein coupled receptor, $P2Y_1$ has been studied here and G-protein coupled receptors are established important drug targets. Till date, there have been no clinical trials testing the potential of $P2Y_1$ receptor modulators as a new therapeutic class to treat T2DM. Currently, the majority of drugs used to treat T2DM are K_{ATP} channel blockers, such as sulfonylureas, which are known to cause hypoglycemia as a side effect as their activity is independent of the glucose level (330). Nevertheless, novel P2Y receptor modulators are candidates for future trials based on in vitro and in vivo studies provided by this thesis and several other groups. Fischer et al. already synthesized a new $P2Y_1$ receptor agonist based on the structure of 2-MeS-ADP (331). The activation of $P2Y_1$ receptor by this ADP analogue stimulates insulin secretion in in vitro and in vivo models with a high receptor affinity, with an EC_{50} value at the nanomolar level of 38 nmol/l

(331). Additionally, this $P2Y_1$ receptor agonist is stable in gastric juice, which is a molecular advantage for the oral route of administration (331). When this ADP analogue was tested in mice and rats, it diminished blood glucose to almost normal levels. This effect was marked in relation to glibenclamide, which caused hypoglycemia (331).

The effect of purinergic signalling genes on glucose homeostasis and T2DM risk in humans was studied by Todd et al. (191).They found that a single nucleotide polymorphism in the minor allele (C) of *P2RY1* was associated with the glycemic trait, 2-hour elevated glucose level with a p value of 0.0015 (191). This finding makes the role of $P2Y_1$ even more intriguing in the context of glucose homeostasis and therefore, diabetes.

4.4 Future Directions

In an attempt to understand the role of $P2Y_1$ and PKD1 on glucose homeostasis, particularly insulin secretion, several questions have arisen from this thesis that still remain unanswered:

- The intriguing observation that intracellular Ca^{2+} mobilisation via $InsP_3$-receptors in the ER in response to ATP tended to be reduced in islets from one donor with T2DM indicated a link between deficient $P2Y_1$ signalling and impaired insulin secretion. But this finding definitely needs to be reproduced in more donors.
- E-NTPDases are crucial for the duration and magnitude of purinergic signalling. Therefore, application of the functional CD39 (E-NTPDase; expressed in human β cells) inhibitor ARL67156 (50 μM) at basal or stimulated condition and measuring insulin secretion will provide further insight into the $P2Y_1$ signalling.
- SERCA 2b, the main ER Ca^{2+} pump, had decreased expression levels with exposure to high glucose concentrations in human islets. It may be interesting to investigate if $P2Y_1$ signalling affected the expression of SERCA 2b.
- Since the single nucleotide polymorphism of *P2RY1* significantly associated with the 2 hour glucose tolerant test. Hence, it definitely merits further investigation in in vivo mouse model. Glucose tolerant tests and insulin tolerant test in vivo will shed light upon its role in glucose homeostasis.
- It may also be interesting to obtain effect estimates and p values for all single nucleotide polymorphisms provided for public download from available databases and examine each for association with T2DM risk and insulin secretion/β cell or islet function in lean and obese individuals.
- This thesis and others made the observation that PKD1 affects peripheral steps of exocytosis. Total internal reflection fluorescence (TIRF) microscopy and Transmission electron microscopy (TEM) may help delineate its impact on granule trafficking and exocytosis.
- It is widely accepted that PKD1 causes F-actin depolymerisation and enhances the second phase of glucose stimulated insulin secretion in pancreatic β cells. Therefore, perifusion

and immunostaining experiments will help understand whether it has any effect on the second phase of insulin secretion or not

- Bergeron et al. arrived at the conclusion that PKD1 was dispensable at basal condition. It has been shown that the PKD isoforms exhibit great redundancy. Therefore, it may be intriguing to explore whether the isoforms, PKD2 and PKD3, play any role in basal condition or not.
- This thesis had anthropometric data collected from 16 donors where two of the donors didn't respond to $P2Y_1$ inhibition and 3 donors didn't respond to PKD1 inhibition. This opens up an important question whether this donor-donor variability in response is also applicable to different ethnicities or different geographical location etc. It may be important to analyse data from much larger cohorts to arrive at definite conclusions.
- PKD1 can be activated downstream by both GPR40 and $P2Y_1$ and therefore, it will be interesting to study the cross talk between the receptors to see if they both coordinate and contribute to potentiation of glucose stimulated insulin secretion.

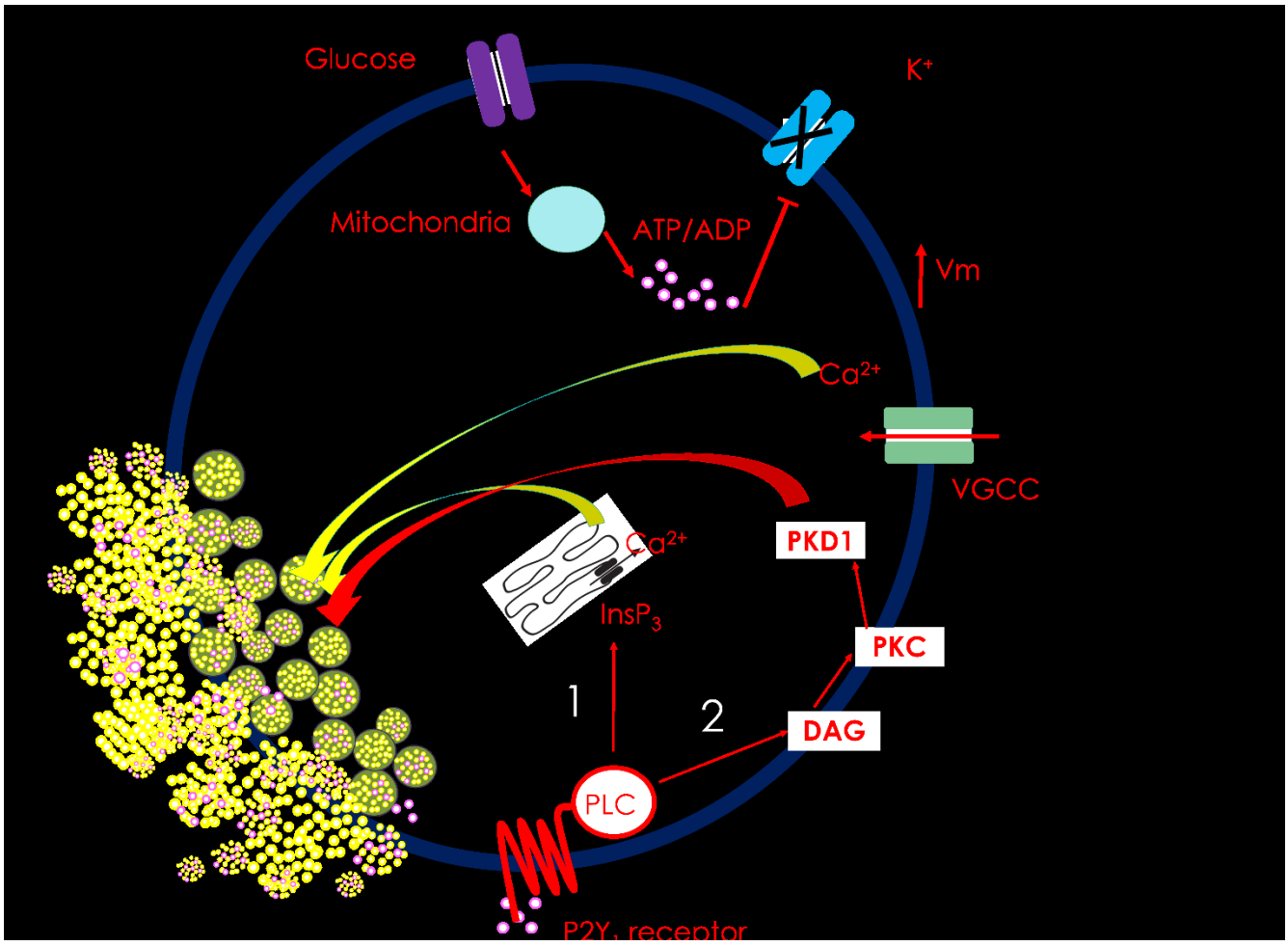
4.5 Conclusion

Glucose stimulated insulin secretion is central to maintaining glucose homeostasis. This thesis has shown that ATP acts as a positive autocrine signal in human β cells by activating $P2Y_1$ receptors, stimulating electrical activity and coupling Ca^{2+} influx to Ca^{2+} release from ER stores. It has also provided evidence that activation of PKD1 downstream contributes to the autocrine regulation of potentiation of insulin secretion (Figure 26) and that this pathway may be important in obesity and diabetes. The $P2Y_1$ receptor is not only important for the potentiation of insulin secretion but also for glucose homeostasis as indicated by the single nucleotide polymorphism study. The $P2Y_1$ receptor is currently being exploited in drug design for the treatment of diabetes, although integrated understanding is needed. A $P2Y_1$ analog is already available but the drug needs to go through preclinical trials. PKD1 on the other hand has been reported to be involved in the transcription of insulin receptor gene, glucose transport in cells, granule formation and cleavage and of course, potentiation of insulin secretion. All in all, this thesis points to a significant modulatory role of $P2Y_1$ signalling in pancreatic β cell pathophysiology which may be exploited therapeutically to treat diabetes.

Figure 26: Proposed mechanism of $P2Y_1$ signalling in pancreatic β cell.

ATP secreted upon stimulation with glucose act as an autocrine signal to activate $P2Y_1$ receptors and on one hand, 1) lead to increased $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx and evoking Ca^{2+} release via $InsP_3$ receptor in the ER and on the other, 2) production of DAG. The elevated plasma membrane DAG concentration activates novel PKCs which activate PKD1 downstream to potentiate the insulin secretory response.

Figure 26.


Glucose
K+
Mitochondria
ATP/ADP
Vm
Ca2+
VGCC
Ca2+
InsP3
PKD1
PKC
1
2
DAG
PLC
P2Y1 receptor

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