The effects of cannabinoids on insulin secretion

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Abbreviations

$[Ca^{2+}]_c$	Cytoplasmic calcium ion concentration
$[Ca^{2+}]_i$	Intracellular calcium ion concentration
2-AG	2-arachidonylglycerol
95% CI	95% confidence intervals
AA	Arachidonic acid
ACEA	Arachidonoyl-2-chloroethylamide
ADP	Adenosine diphosphate
AEA	Anandamide
Akt	Also known as Protein Kinase B
Arg	Arginine
ATP	Adenosine 5'-triphosphate
ATPase	ATP hydrolase
BMI	Body mass index
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
CaNAT	Ca^{2+}-dependent *N*-acyltransferase
CAP	Cbl-adapter protein
CB_1 receptor	Cannabinoid receptor 1
CB_2 receptor	Cannabinoid receptor 2
CB receptor	Cannabinoid receptor
Cbl	Casitas B-lineage lymphoma
CNS	Central nervous system
CoA	Coenzyme A
COX	Cyclooxygenase
CPH	Carboxypeptidase H
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
ddH_2O	Double distilled H_2O
DMSO	Dimethyl sulfoxide
DTZ	Diphenylthiocarbazone
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Endocannabinoid membrane transporter
Epac	Also known as cAMP-dependent guanine nucleotide exchange factor
ER	Endoplasmic reticulum
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein

F-actin	Filamentous actin
FFA	Free fatty acid
G protein	Guanine nucleotide-binding protein
G3P	Glycerol-3-phosphate
GCPR	G protein coupled receptor
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GSIS	Glucose-stimulated insulin secretion
GTP	Guanosine 5'-triphosphate
GTPase	GTP hydrolase
H^+	Hydrogen ion/proton
Hb	Haemoglobin
HbA_{1c}	Glycated haemoglobin
HDL	High density lipoprotein
HEPES	(4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid)
IAB	Insulin assay buffer
IC_{50}	Concentration at which a drug causes a 50% inhibition of the parameter being measured
IFG	Impaired fasting glucose
IFNγ	Interferon γ
IGT	Impaired glucose tolerance
iNAT	Ca^{2+}-independent *N*-acyltransferase
IP_3	Inositol-1,4,5-trisphosphate
$iPLA_2\beta$	Ca^{2+}-independent phospholipase A_2 type β
IRS	Insulin receptor substrate
JNK	c-Jun NH_2-terminal kinase
K^+	Potassium ion
K_{ATP} channel	ATP-sensitive K^+ channel
K_m	Concentration of substrate at which 50% of maximal enzyme activity occurs
KO	Knock-out – contains two non-functional alleles of a gene
LOX	Lipoxygenase
LPI	Lysophosphatidylinositol
Lyso-PLD	Sphingomyelin phospholipase D
MAG	Monoacylglycerol
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
metAEA	Methanandamide

MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mV	Millivolts
NA	*N*-arachidonyl
Na^+	Sodium ion
NAAA	*N*-acylethanolamine-hydrolysing acid amidase
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADA	*N*-arachidonoyl dopamine
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAE	*N*-acylethanolamine
NAGly	*N*-arachidonoyl glycine
NAPE	*N*-acyl-phosphatidylethanolamine
NAPE-PLD	*N*-acyl-phosphatidylethanolamine hydrolysing phospholipase D
NO	Nitric oxide
NOD mice	Non-obese diabetic mice, model of autoimmune-mediated (type 1) diabetes
OEA	*N*-oleoylethanolamide
PA	Phosphatidic acid
PC	Phosphatidylcholine
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
PE	Phosphatidylethanolamine
PEA	*N*-palmitoylethanolamide
PEG	Polyethylene glycerol
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PTPN22	Protein tyrosine phosphatase type 22
RhoA	Ras homologue gene family, member A
Rho-ROCK	RhoA associated kinase
RIA	Radioimmunoassay
ROS	Reactive oxygen species

SD	Standard deviation
SEA	*N*-stearoylethanolamide
siRNA	Small interfering ribonucleic acid
SNARE	Soluble [*N*-ethylmaleimide sensitive fusion factor] attachment receptor
Sst-14	Somatostatin-14
Sst-28	Somatostatin-28
SSTR	Somatostatin receptor
TCA cycle	Tricarboxylic acid cycle, also referred to as the Krebs cycle
THC	Δ^9-tetrahydrocannabidiol
TNFα	Tumour necrosis factor α
TREK-2	TWIK-related K^+ channel 2
TRPV1	Transient receptor potential vallinoid type 1
TRPV2	Transient receptor potential vallinoid type 2
t-SNARE	Membrane-associated SNARE
TZD	Thiazolidinedione
VGNC	Voltage-gated Na^+ channel
V_{max}	Concentration of substrate at which 100% of maximal enzyme activity occurs
v-SNARE	Vesicle-associated SNARE
WT	Wild-type
ZDF	Zucker Diabetic Fatty rat
ZF	Zucker Fatty rat
ZLC	Zucker Lean Control rat

Abstract

Type 2 diabetes mellitus is a chronic condition caused by a deficiency in the secretion of insulin from the islets of Langerhans and/or impaired insulin signalling, resulting in hyperglycaemia. The role of the endocannabinoid system is well-recognised in the CNS and immune system, but its role in glucose homeostasis is poorly understood. The aim of this study was to define the roles of cannabinoids in insulin secretion, to provide insights into their therapeutic potential (or limitation) in the treatment of type 2 diabetes.

Isolated islets were used, from Wistar rats, in static incubation studies measuring changes in insulin secretion rates.

The endocannabinoid anandamide (AEA) was found to inhibit insulin secretion in a glucose- and concentration-dependent manner, with an IC_{50} of 1.6μM (95% CI: 227nM to 4.0μM; n= 10). Upon further analysis of the concentration-response data islet sensitivity to AEA appeared to vary, with islets either appearing to be sensative (IC_{50} 220nM; 95% CI: 21.9nM to 2.2μM; n= 5) or less sensative (IC_{50} 12.3μM; 95% CI: 6.8μM to 19.4μM; n= 5) to AEA. Pre-incubation of islets with a fatty acid amide hydrolase inhibitor did not affect islet responsiveness to AEA. AEA-mediated inhibition of insulin secretion was not consistently affected by cannabinoid receptor 1 (CB_1) or CB_2 antagonism. Surprisingly, the CB_1 receptor antagonist AM251 was found to inhibit insulin secretion in a glucose- and concentration-dependent (IC_{50} 1.6μM; 95% CI: 507nM to 3.3μM; n= 6) manner.

Results from this study suggest that differences in CB-receptor signalling pathways, rather than endocannabinoid metabolism, could be responsible for the variations in the potency of AEA between islet preparations. Characterisation of cannabinoid signalling in islets was hindered as the CB receptor antagonists used in this study also affected insulin secretion. This study highlights the dynamics of endocannabinoid signalling in islets, which may be linked to their physiological function.

Publications

Abstracts

Anderson, R., Randall, M.R., Chan, S.L.F. (2008) The effects of anandamide and N-acylethanolamine hydrolysis on insulin secretion in rat isolated islets of Langerhans. *Abstract presented at the British Pharmacological Society's winter meeting http://www.pA2online.org/abstracts/Vol6Issue4abst121P.pdf*

1

General introduction

This chapter will begin with a brief description of insulin, regarding its effects on peripheral tissue function, insulin signalling and its role in diabetes mellitus. This will then be followed by a review of three key areas of interest which form the basis of this thesis. The first area will focus on islets of Langerhans, and their physiological role in glucose homeostasis. A discussion on how glucose stimulates insulin secretion and how insulin secretion is physiologically regulated will be presented. The review will continue by describing the aetiology and treatments for type 2 diabetes mellitus, with a focus on β-cell dysfunction and regulation of glycaemia. The final section will be an overview of the endogenous cannabinoid system describing the enzymes which produce and degrade the endogenous ligands (endocannabinoids), and their associated receptors. The introduction will then be concluded with a description of the project and its aims.

1.1 Effects of insulin on peripheral tissues

Insulin is a hormone formed from two peptides that is produced in the β-cells of the islets of Langerhans (Section 1.2), and it is an important hormone in glucose homeostasis, as it lowers plasma glucose. As shown in Table 1.1, insulin signalling alters the activity of the liver, skeletal muscle and adipose tissue in order to lower plasma glucose levels. These effects are mediated through the insulin receptor, which upon binding insulin autophosphorylates, with the subsequent phosphorylation of a number of signalling proteins called insulin receptor substrate (IRS) molecules (Rhodes & White, 2002). In total, 5 IRS (IRS-1, -2, -4, -5 and -6) molecules have been identified in human tissues (Fritsche *et al.*, 2008). Of the five isoforms, IRS-1 and IRS-2 have received the most interest with regard to glucose homeostasis, as interference with their expression leads to peripheral insulin resistance and IRS-2 blockade in β-cells leads to type 2 diabetes (see review by Rhodes & White (2002)). Activation of IRS1/2 is known to activate phosphatidylinositol 3-kinase (PI3K), PI3K then activates mammalian target

Table 1.1 The effects of insulin and glucagon on hepatic, white adipose tissue and skeletal muscle metabolism

Hormone	Hormone effect		
	Liver	Skeletal Muscle	Adipocyte
Insulin	↓ Gluconeogenesis[1]	↑ Glucose uptake	↑ Glucose uptake
	↓ β-oxidation	↓ β-oxidation	↓ Lipolysis
	↓ Glycogenolysis	↑ Fatty acid uptake	↑ Lipogenesis
	↑ Glycogenesis	↑ Glycolysis	
	↑ Glycolysis	↓ Glycogenolysis	
	↑ Lipogenesis	↑ Glycogenesis	
Glucagon	↑ Gluconeogenesis		↑ Lipolysis
	↑ Glycogenolysis		
	↓ Glycogenesis		
	↓ Glycolysis		
	↑ β-oxidation		
	↓ Lipogenesis		

[1] Reduction in gluconeogensis is achieved via the activation of hepatic (reduced transcription of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) and hypothalamic (via the vagal nerve) insulin receptors (Fritsche *et al.*, 2008; Lam *et al.*, 2009). Data has been adapted from data in Fritsche *et al.* (2008) , Lam *et al.* (2009), Bouzakri *et al.* (2006), Palanivel *et al.* (2006), Leney & Tavare (2009), Giorgino *et al.* (2005), Ali & Drucker (2009) and Klain (1977).

of rapamycin (mTOR), protein kinase C (PKC) and Akt (also known as PKB; Fig 1.1). Insulin receptor activation also leads to the activation of mitogen-activated protein kinase (MAPK) and CAP/Cbl cascades (see reviews by Giorgino *et al.* (2005), Krook & Zierath (2009), Leclercq *et al.* (2007), and Fritsche *et al.* (2008)). In peripheral tissues, promotion of cell survival, proliferation and differentiation in peripheral tissues is mediated by MAPK and Akt signalling and increased protein synthesis is mediated by mTOR and MAPK signalling (see reviews by Giorgino *et al.* (2005), Krook & Zierath (2009), Leclercq *et al.* (2007), and Fritsche *et al.* (2008)). Alterations in glucose transporter 4 (GLUT4) translocation and glucose uptake are controlled by PKC, Akt and CAP/Cbl signalling, while alterations in metabolism are mainly controlled by Akt but may also involve PKC signalling (see reviews by Giorgino *et al.* (2005), Krook & Zierath (2009), Leclercq *et al.* (2007), and Fritsche *et al.* (2008)). The two most important effects of insulin signalling in peripheral tissues (with regard to glucose clearance) are the reductions in hepatic glucose production and increased glucose uptake (by GLUT4 translocation to the plasma membrane from storage vesicles) in skeletal muscle and adipose tissue (Bouzakri *et al.*, 2006; Fritsche *et al.*,

2008; Lam *et al.*, 2009; Leney & Tavare, 2009). In addition to glucose clearance, insulin signalling also causes hepatic cell, skeletal muscle, and adipocyte metabolism to switch from a catabolic state to an anabolic state (Table 1.1).

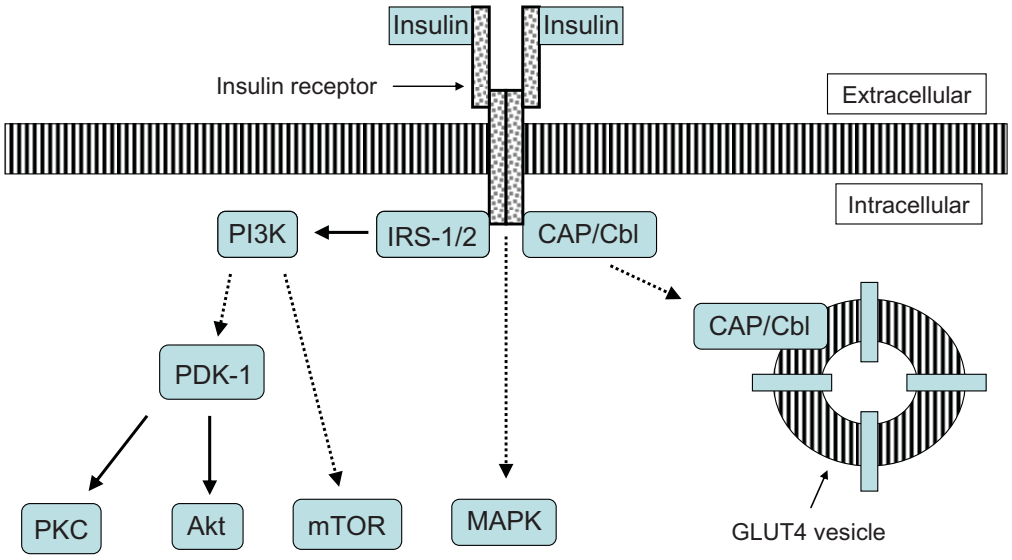


Figure 1.1 A schematic diagram of insulin receptor signalling. On binding insulin, the insulin receptor is then able to undergo autophosphorylation. This then allows various proteins to bind to and be phosphorylated by the receptor, which activates insulin receptor substrate 1 & 2 (IRS1/2), mitogen-activated protein kinase (MAPK) and CAP/Cbl signalling pathways. Block arrows represent direct activation, and broken arrows represent several steps. PI3K (phosphatidylinositol 3-kinase), PKC (protein kinase C), Akt (also known as PKB), mTOR (mammalian target of rapamycin). Figure is based on information in the reviews by Giorgino *et al.* (2005), Krook & Zierath (2009), Leclercq *et al.* (2007), and Fritsche *et al.* (2008).

Together with the counter regulatory hormones, glucagon (Section 1.2.3), cortisol, growth hormone and adrenaline, insulin is involved in the complex regulation of glycaemia, ensuring that there is a sufficient (but not an excessive) supply of glucose during periods of fasting, feeding and exercise. Therefore, insulin plays a key role in glucose homeostasis as it is the only hormone to lower blood glucose. Diabetes mellitus is a chronic endocrine disorder caused by a deficiency in amount of insulin secretion and/or a deficiency in insulin signalling resulting in hyperglycaemia. It has been estimated that 171 million people worldwide had diabetes in 2000 (Wild *et al.*, 2004). The two most prevalent types of diabetes mellitus are type 1 and type 2 diabetes. In 2005, it was estimated that ~10% and ~90% of the UK population who had diabetes mellitus had type1 and type 2 diabetes, respectively (Gonzalez *et al.*, 2009). Type 1 diabetes is caused by an absolute deficiency in insulin secretion, which

is thought to be the result of autoimmune-mediated destruction of the insulin secreting β-cells (see the review by Santamaria (2010)). Type 2 diabetes is caused by islet dysfunction and/or insulin resistance in peripheral tissues (Ferrannini, 1998; Kahn, 2003; Spellman, 2007). Islet dysfunction in type 2 diabetes was typically thought to be the result of insufficient insulin release from β cells, but evidence over recent years now suggests that excessive secretion of glucagon from α-cells also contributes to persistent hyperglycaemia (Spellman, 2007; Burcelin *et al.*, 2008). Briefly glucagon secretion during hypoglycaemia appears to be attenuated in type 2 diabetes, while glucose induced inhibition of glucagon is suppressed leading to increased hyperglycaemia by upregulating hepatic gluconeogensis (Burcelin *et al.*, 2008; Quesada *et al.*, 2008). A discussion of diabetes mellitus, and its management continues in Section 1.3.

1.2 The islets of Langerhans and insulin secretion

The islets of Langerhans are clusters of endocrine cells interspersed throughout the exocrine pancreas, and are formed from α-, β-, δ- and PP-cells which secrete glucagon, insulin, somatostatin-14 (sst-14) and pancreatic polypeptide (PP), respectively. Insulin and glucagon have opposing effects on plasma glucose levels, with insulin promoting glucose clearance while glucagon stimulates increases in plasma glucose levels (Table 1.1). Somatostatin-14 helps to regulate the secretion of glucagon and insulin (Hauge-Evans *et al.*, 2009; Mazziotti *et al.*, 2009). The effects of insulin and the roles of glucagon and sst-14 are discussed in more detail elsewhere (Sections 1.1 and 1.2.3). The effects of PP on islet function are not understood. As discussed in the review by Kojima *et al.* (2007), PP has been found to negatively regulate exocrine pancreatic activity, gastric emptying and appetite while increasing energy expenditure. There is also some evidence to suggest that there is a fifth endocrine cell within islets, the ghrelin-secreting ε-cells, but ε-cells may be only be important in foetal islet development (Heller *et al.*, 2005; Andralojc *et al.*, 2009). However, it should be noted that ghrelin has also been reported to be expressed in α-

cells in rat and human islets (Date *et al.*, 2002; Heller *et al.*, 2005), but the expression of ghrelin in human α-cells has been disputed by Andralojc *et al.* (2009).

Islet cell composition and the organisation of islets are also known to differ between different species. For instance, the study by Brissova *et al.* (2005) found that in murine islets, β-cells accounted for approximately 80% of the islet cell population, whereas in humans, this number ranged from 28-75%. Primate islets (including human) have non-β cells (α- and δ-cells) interspersed throughout the islets, whereas rodent islets are highly organised with β-cells forming the core of the islets surrounded by non-β cells (Brissova *et al.*, 2005). The composition of islets is known to vary between different regions of the pancreas, and islet function is influenced by their size (Tasaka *et al.*, 1989; Elayat *et al.*, 1995; Aizawa *et al.*, 2001). Aizawa *et al.* (2001) found that islet size influenced islet responsiveness to glucose, with larger islets typically releasing more insulin at lower glucose concentrations than smaller islets. Weaver & Sorenson (1989) have also reported that blood flow from islets is dependent on islet size, with blood from smaller islets first draining into acinar tissues before entering the wider circulation (i.e. to the rest of the body), whereas blood from larger islets drains directly into the wider circulation as well as into acinar tissues (Weaver & Sorenson, 1989). As to why islets vary is currently unclear and this aspect of islet organisation remains poorly understood.

Islets are also highly vascularised and so islets contain numerous endothelial cells, which promote β-cell survival, increase insulin content and improve insulin secretory responses through deposition of extracellular matrix proteins and growth factors (Johansson *et al.*, 2006). In turn, β-cells release various angiogenic growth factors, such as vascular endothelial growth factor A, which promote and maintain islet vascularisation (see review by Eberhard *et al.* (2010)). The islet capillaries are surrounded by a double basal membrane (Figure 1.2), which primarily consists of collagen IV and laminin extracellular matrix proteins (Virtanen *et al.*, 2008; Eberhard *et al.*, 2010). Islets are also encapsulated by a peri-insular cap (Figure 1.2). The peri-

insular cap consists of glial (Schwann) cells, whose projections run along the islet perimeter and sometimes into the islet themselves, and a basment membrane (Donev, 1984; Wang & Rosenberg, 1999). The physiological function of the glial cells is currently unknown. The coverage of the peri-insular cap differs between species. Canine islets are fully enclosed by the peri-insular cap, human and rodent islets have intermediate coverage, while porcine islets have little or no coverage. The physiological relevance of this difference is unclear but may affect islet preparation yields (Section 2.4.1). In addition islets are also highly innervated, which alters the secretion of insulin, glucagon, sst-14 and PP in response to different stimuli and is described further in Section 1.2.4.

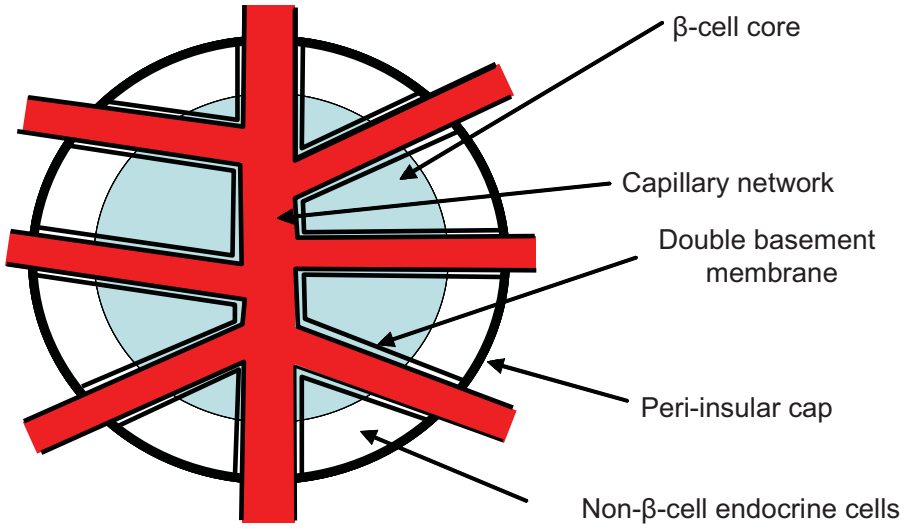


Figure 1.2 A cross-sectional representation of a rodent islet (not to scale).

1.2.1 Insulin

Human insulin is produced from a single 110 amino acid precursor polypeptide preproinsulin, which consists of a signalling peptide (residues 1-24), the B-chain (residues 25-54), C chain (residues 55-89) and the A-chain (residues 90-110; NCBI (2010)). Preproinsulin is encoded by a single gene in humans (*INS*) and glucose is known to be involved in the regulation of preproinsulin mRNA transcription, stability and translation (see reviews by Melloul *et al.* (2002), Poitout *et al.* (2006) and Fred & Welsh (2009)). As described in the review by Fred & Welsh (2009), short-term

maintenance (>3 hours) of islet insulin levels in the presence of glucose is dependent on translation of preproinsulin mRNA, while longer-term maintenance of islet insulin levels is achieved through transcription of *INS* and stabilisation of preproinsulin mRNA.

Once preproinsulin has been synthesised and inserted into the endoplasmic reticulum (ER), the signal peptide section of preproinsulin is proteolytically cleaved to yield proinsulin (Weiss, 2009; NCBI, 2010). Within the ER, proinsulin folds and forms two disulphide bridges between A- and B-chains (A7-B7 and A20-B19, as well as a third disulphide bridge within the A chain (A6-A11)). Once this has occurred, proinsulin is then translocated to the Golgi apparatus (Weiss, 2009). Once inside the Golgi apparatus, proinsulin is then sequestered into granules containing Zn^{2+}, prohormone convertase 1 (PC1), PC2 and carboxypeptidase H (Goodge & Hutton, 2000). For proinsulin to be converted to insulin, the granules need to become acidified which, is achieved by the dual actions of Cl^- (via the Cl^- channel protein, chloride channel protein 3) and H^+ (via V-type H^+-ATPase, this integral membrane protein transports H^+ ions across the vesicle membrane by converting ATP to ADP) influx (Barg *et al.*, 2001). It is hypothesised that Cl^- influx allows acidification to occur by counteracting the increasing positive membrane potential caused by H^+ influx. Once the granule has been acidified, PC1, PC2 and carboxypeptidase H then cleave the C-chain from the proinsulin polypeptide (Figure 1.3) forming insulin and C-peptide (Goodge & Hutton, 2000). Upon removal of the C-chain, insulin crystallises with Zn^{2+} (6 insulin molecules: 2 Zn^{2+}), which is thought to stop fibrillation of insulin until exocytosis (fusion of the insulin granule to the β-cell plasma membrane, see Section 1.2.2.2) occurs when the insulin crystals disassociate (Noormagi *et al.*, 2010).

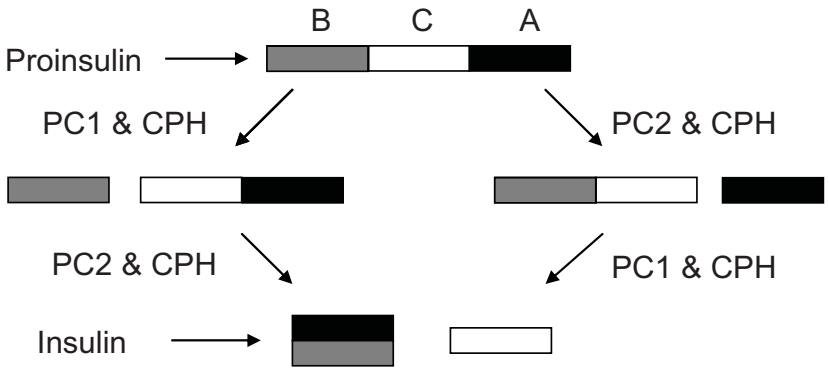


Figure 1.3 A schematic representation of the conversion of proinsulin and C-peptide by the proconvertases (PC), PC1 and PC2. The conversion of proinsulin to insulin requires endopeptidase cleavage of residues 32-33 (which reside between the B/C chain) by PC1 and residues 65-66 (which reside between the C/A chain) by PC2 (Goodge & Hutton, 2000). At the end of each stage, carboxypeptidase H (CPH) removes the residues exposed by PC1 and PC2 activity (Goodge & Hutton, 2000). The preferred reaction is shown on the left hand side although conversion of insulin can occur by PC2 initiating the first step (Goodge & Hutton, 2000). For simplicity di-sulphide bonds between A- and B-chains were not included.

1.2.2 Glucose-stimulated insulin secretion (GSIS)

Insulin is released from β-cells in response to changes in plasma glucose levels but can also be induced by free fatty acids and certain amino acids (Henquin *et al.*, 2006). How these metabolites stimulate insulin secretion is still not completely understood despite several decades of research. The following section will focus on glucose-stimulated insulin release with amino acid and free fatty acid stimulated insulin secretion briefly described in Section 1.2.2.4.

In man and the rat, constitutive (basal) insulin secretion approximately occurs at glucose concentrations up to 4mM glucose (Pick *et al.*, 1998; Henquin *et al.*, 2006). At glucose concentrations higher than 4mM glucose, insulin release is then regulated by glucose and is called glucose-stimulated insulin secretion (GSIS). Maximal rates of GSIS occur at glucose concentrations ≥ 16mM with ~50% of the maximal GSIS rates occurring at 8mM (Pick *et al.*, 1998; Henquin *et al.*, 2006). In mice, basal insulin secretion occurs at up to 8mM glucose and ~50% maximal and maximal GSIS occurs at 12 and 20mM glucose, respectively (Vieira *et al.*, 2007). GSIS is biphasic, with the first phase of insulin secretion being a transient process lasting up to 10 minutes, while the second phase of insulin secretion persists until glucose levels return to non-

stimulatory levels (Zawalich *et al.*, 2000; Shigeto *et al.*, 2006). The kinetics of insulin secretion are species-dependent, with humans and mice displaying a large primary phase and a smaller secondary phase, whereas in rats the opposite is true (Figure 1.4). The regulation of the biphasic response is dependent on ion channel activities, metabolic activities and insulin granule processing but there is still an incomplete understanding of how these processes interact. Therefore, each component will be discussed separately.

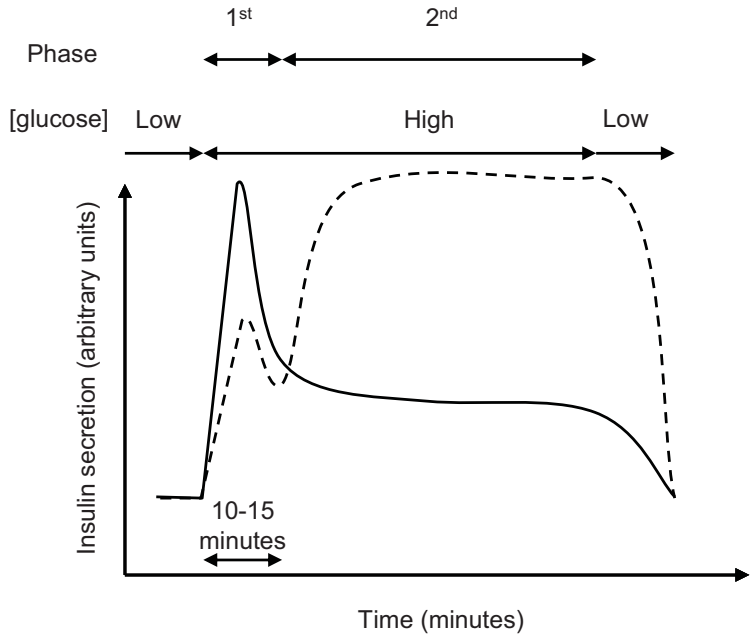


Figure 1.4 Diagrammatic representation of species-specific differences in biphasic glucose-stimulated insulin secretion responses from perifused isolated islets. The solid line is representative of insulin secretion from human and mouse isolated islets; whereas the broken line represents biphasic insulin release from rat isolated islets. Low and high glucose concentrations refer to non-stimulatory and stimulatory levels of insulin secretion. The first phase of insulin secretion is transient (lasting 10-15 minutes) whereas, the second phase persists until glucose returns to non-stimulatory levels. Figure is based on data from Henquin *et al.* (2006), Aizawa *et al.* (2001) and Hauge-Evans *et al.* (2009).

1.2.2.1 ATP-sensitive K^+ (K_{ATP}) channel-dependent insulin secretion

The widely-accepted model of GSIS induction is the K_{ATP} channel-dependent signalling pathway (also known as the triggering pathway; Figure 1.5). During the resting state, K_{ATP} channel activity is primarily responsible for maintaining the β-cell membrane potential at -70mV (Rorsman, 1997). Upon increases of plasma glucose above the threshold for GSIS, glucose enters the β-cells via the glucose transporter (GLUT) 2 which has a K_m of ~17mM glucose and a V_{max} at glucose concentrations ≥

32mM glucose, thus allowing physiological concentrations of glucose to rapidly enter the β-cell without uptake becoming saturated (Johnson *et al.*, 1990). Upon entering the β-cell, glucose is then phosphorylated by glucokinase which stops glucose from diffusing back out of the cell and is the first step in glycolysis (Ashcroft & Randle, 1970; Giroix *et al.*, 1984). With regard to GSIS, glucokinase is the most important glucose phosphorylating enzyme expressed in β-cells as it has a low affinity of 16mM glucose and is not subject to glucose-6-phosphate feedback, thus allowing high rates of glycolysis to occur (Ashcroft & Randle, 1970; Giroix *et al.*, 1984). Glucose is rapidly metabolised (by conversion to pyruvate in glycolysis and subsequent oxidation of pyruvate in the Krebs cycle) to produce ATP causing a shift in the ATP:ADP ratio (Longo *et al.*, 1991). This leads to the closure of the K_{ATP} channels and the intracellular accumulation of K^+ causes the β-cell to depolarise (Rorsman, 1997). Once the membrane potential reaches -40mV, in rat and human β-cells, this leads to the activation of L-type voltage-gated Ca^{2+} channels which allow the influx of extracellular Ca^{2+}, and peak L-type Ca^{2+} channel activity occurs at 0mV (rat) and -10mV (human) (Tamarina *et al.*, 2003; Suga *et al.*, 2004). This in turn leads to insulin granules fusing with the plasma membrane (exocytosis), allowing the insulin crystals to dissolve leading to the subsequent entry of insulin into the bloodstream. How the insulin granules are processed and reach the plasma membrane is discussed in more detail below in Section 1.2.2.2. Repolarisation of the β-cell is known to be K^+ channel activity dependent with voltage-gated, small conductance Ca^{2+}-activated and/or large conductance Ca^{2+}-activated K^+ channels all being suggested (Roe *et al.*, 1996; Tamarina *et al.*, 2003; Suga *et al.*, 2004).

The rapid change in β-cell membrane potential from a resting state to a depolarised state and subsequent repolarisation is referred to as an action potential. When islets are exposed to raised levels of glucose, the β-cells will continually cycle between depolarised and repolarised states until glucose levels fall to non-stimulatory levels (see review by Rorsman (1997)). As mentioned above, depolarisation of the β-cell is associated with increased concentrations of cytoplasmic Ca^{2+} ($[Ca^{2+}]_c$), whereas repolarisation of the β-cell corresponds with reductions in $[Ca^{2+}]_c$ when removal of

cytoplasmic Ca^{2+} exceeds Ca^{2+} influx (Hughes *et al.*, 2006). The majority of the Ca^{2+} is used to replenish ER stores (by sarcoendoplasmic reticulum Ca^{2+} ATPase), while the remaining Ca^{2+} is exported (by Na^{+}/Ca^{2+} exchanger and plasma membrane Ca^{2+} ATPase activity) out of the β-cells (Hughes *et al.*, 2006). To increase insulin secretion, the time between action potentials decreases which means that there is less time for $[Ca^{2+}]_c$ to return to basal levels, thus allowing $[Ca^{2+}]_c$ to rise, allowing for greater rates of insulin secretion to occur (Hughes *et al.*, 2006). As with action potentials in membrane potential, $[Ca^{2+}]_c$ eventually reaches a plateau corresponding with increasing amounts of glucose, whereas the amount of insulin secreted increases further, which has led to the notion of K_{ATP} channel-independent signalling (also known as the amplifying pathway); this is discussed further in Section 1.2.2.3. As GSIS is Ca^{2+}-dependent, this results in insulin secretion being pulsatile; this is physiologically important as irregular patterns in pulsatile insulin release coincide with insulin resistance (Zarkovic *et al.*, 1999).

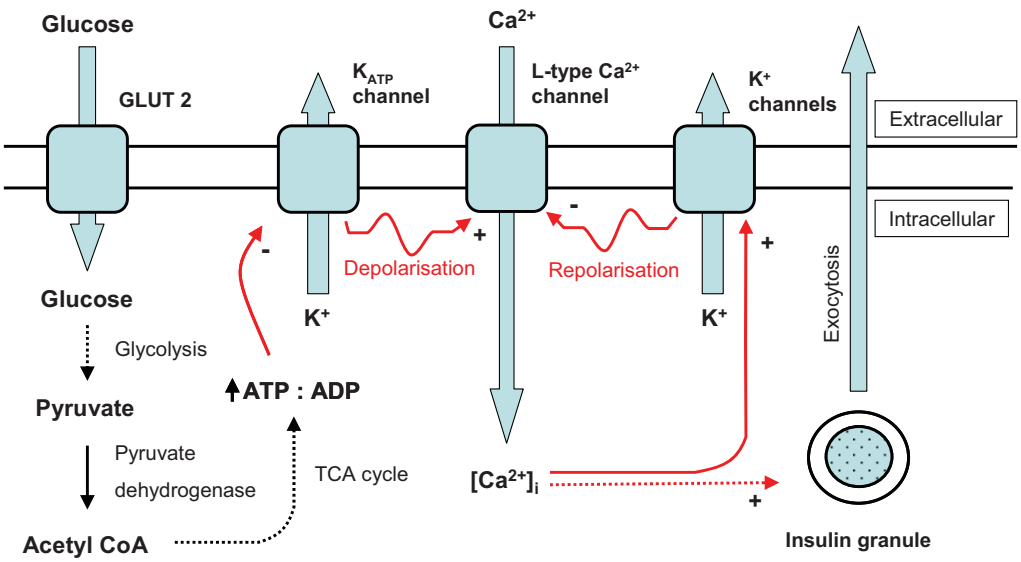


Figure 1.5 K_{ATP} channel-dependent signalling. Briefly, glucose enters the β-cells through the low affinity glucose transporter, GLUT2. It is subsequently metabolised leading to an increase in the ATP:ADP ratio this leads to the closure of the K_{ATP} channels. Closure of the K_{ATP} channels causes the β-cell to depolarise and the subsequent influx of Ca^{2+}, thus triggering insulin granule exocytosis by increasing the cytoplasmic calcium concentration ($[Ca^{2+}]_c$). Ca^{2+} either directly or through β-cell depolarisation activate K^{+} channels which repolarise the β-cells and inhibits further insulin secretion. Block arrows represent the direction the flow of substrates through integral membrane proteins (GLUT 2 transporter and the ion channels). Arrows with dotted lines represent complex processes. Arrows with + and – signs represent positive and negative influences respectively.

While the K_{ATP} channel-dependent signalling model accounts for a large proportion of insulin granule exocytosis in the first phase of insulin secretion, it has been found that this model does not account for the second phase of insulin secretion (Jing *et al.*, 2005; Shigeto *et al.*, 2006). R- and P/Q-type voltage-gated Ca^{2+} channels in mouse and humans β-cells (respectively) have been implicated to play a larger role than L-type Ca^{2+} channels in the second phase of insulin secretion (Jing *et al.*, 2005; Braun *et al.*, 2008). In addition, voltage-gated Na^{+} channels (VGNC) have been also been suggested to play an important role in GSIS responses, as pharmacological blockade of these channels in MIN6 (β-cell line) was observed to result in a loss of the first phase and much of the second phase of insulin release at 10mM glucose (Shigeto *et al.*, 2006). In a recent study, the genetic knockout of a regulatory subunit of a VGNC (in C57Bl6/J mice) resulted in loss of glucose- and KCl-stimulated but not Ca^{2+} induced insulin secretion in isolated islets, and caused glucose intolerance *in vivo* (Ernst *et al.*, 2009).

1.2.2.2 Insulin granule processing

The mobilisation of insulin granules to the plasma membrane, their subsequent processing and exocytosis is also a key factor in the phasic control of insulin secretion. The majority (~90%) of insulin granules reside in the cytoplasm, in what is referred to as the reserve pool, while the remaining granules (~10%) are located in close proximity to the plasma membrane and are usually called the docked pool (Straub & Sharp, 2002). The docked pool is thought to consist of granules in various states of readiness for exocytosis (Straub & Sharp, 2002). The process proposed by Straub & Sharp (2002) is that insulin granules dock below the plasma membrane, they then undergo a priming phase to form the readily-releasable pool which consists of mature insulin granules. Additionally, a subset of the readily-releasable pool associates with the exocytotic machinery to form the immediately-releasable pool (Straub & Sharp, 2002). The first phase of insulin in response to depolarising agents (e.g. KCl at concentrations ≥ 30mM) and/or K_{ATP} channel-dependent signalling is linked to the exocytosis of the immediately-releasable granules which make up ~0.5-

2% of the total islet granule population (Straub & Sharp, 2002; Wang & Thurmond, 2009). The study by Ohara-Imaizumi *et al.* (2007) suggests that not all insulin granules involved in the first phase originate from the docked pool, but there is an involvement of the reserve pool. Similarly, the maintenance of the second phase of insulin secretion is dependent on insulin granule mobilisation from the reserve pool (Straub & Sharp, 2002; Ohara-Imaizumi *et al.*, 2007). The processing of granules is dependent on β-cell cytoskeleton re-arrangement which is, in turn, dependent on various classes of small GTPases (guanosine triphosphate hydrolases, which are also involved in granule processing and eventual exocytosis), which have been reviewed by Wang & Thurmond (2009). In summary, movement of granules from the reserve pool involves transport of granules to the plasma membrane docking area along microtubules; where filamentous-actin (F-actin) remodelling is required for the replenishment of the docked pool and for granule exocytosis. Finally, the process of exocytosis is dependent on soluble [*N*-ethylmaleimide sensitive fusion factor] attachment receptor (SNARE) proteins, of which several vesicle SNARE (v-SNARE), t-SNARE (membrane-associated) and accessory factors (t-SNARE associated) are known to be expressed by islets (refer to the review by Wang & Thurmond (2009)). The composition of the t-SNARE complexes appears to be phase-dependent, with t-SNARE complexes believed to be pre-assembled for the first phase of insulin secretion, whilst in the second phase of insulin secretion, t-SNARE complexes must first undergo calcium-dependent assembly before granule exocytosis can occur (Ohara-Imaizumi *et al.*, 2007; Takahashi *et al.*).

1.2.2.3 K_{ATP} channel-independent signalling

As discussed in the review by Wang & Thurmond (2009), in addition to increasing the ATP:ADP ratio (which triggers K_{ATP} channel-dependent signalling), glucose metabolism leads to the generation of other metabolic signalling which amplifies the insulin secretory response. With respect to changes in $[Ca^{2+}]_c$ (cytoplasmic concentration of Ca^{2+}) in response to higher levels of GSIS, this is achieved by increased duration of action potentials (Section 1.2.2.1) rather than significant

alterations in the amplitude of action potentials (Wang & Thurmond, 2009). As demonstrated in the study by Heart *et al.* (2006), mean changes in $[Ca^{2+}]_c$ correlate poorly with GSIS beyond 8mM in mouse islets, whereas increases in mitochondrial membrane potential strongly correlate with increases in glucose and insulin secretion. This indicates that mitochondrial activity plays an integral role in GSIS aside from ATP generation. In addition, various second messenger molecules are also produced during GSIS which are known to potentiate insulin secretion (Wolf *et al.*, 1991; Tamarina *et al.*, 2005; Kim *et al.*, 2008a).

Due to high rates of glycolysis in GSIS, β-cells require high rates of NADH (reduced form of nicotinamide adenine dinucleotide) conversion to NAD^+ (oxidised form) to maintain metabolic rates. As lactate dehydrogenase activity in β-cells is low, NAD^+ regeneration is dependent on glycerol-3-phosphate (G3P) and malate/aspartate shuttles which link the oxidation of cytoplasmic NADH to the production of ATP in the mitochondria (Tan *et al.*, 2002; McKenna *et al.*, 2006; Jitrapakdee *et al.*, 2010). Briefly, in the malate-aspartate cycle, NADH is oxidised to NAD^+ by the conversion of cytoplasmic aspartate to malate. Malate is then transported into the mitochondria where it is converted back to aspartate (this is transported back into the cytoplasm) and is also used to reduce mitochondrial NAD^+ to NADH. In the glycerol-3-phosphate NAD^+ shuttling system, cytoplasmic NADH is used to reduce dihydroxyacetone phosphate to glycerol-3-phosphate, which regenerates NAD^+. Oxidation of G3P (to dihydroxyacetone phosphate) is used to oxidise Coenzyme Q by mitochondrial glycerol-3-phosphate, using flavin adenine dinucleotide as an intermediate in the redox reaction. Both shuttling systems are described in greater detail in the review by McKenna *et al.* (2006).

Another important aspect of the β-cell is the expression of pyruvate carboxylase. Pyruvate carboxylase converts pyruvate to oxaloacetate which increases the input of carbon atoms into the tricarboxylic acid (TCA, also referred to as the Krebs cycle) cycle (Jensen *et al.*, 2008; Nolan & Prentki, 2008; Jitrapakdee *et al.*). Pyruvate

carboxylase allows cataplerosis (removal of substrates from the TCA cycle) to occur, so that TCA substrates can be used for the potentiation of insulin secretion. Pharmacological inhibition of pyruvate carboxylase causes a large inhibition GSIS in rat islets, thus demonstrating its importance (Farfari *et al.*, 2000). One particular use of TCA substrates is in the generation of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) as intracellular levels of NADPH increase in proportion to glucose (Sener *et al.*, 1984; Rocheleau *et al.*, 2004). Three shuttling systems have been proposed as being important (see reviews by Jensen *et al.* (2008), Jitrapakdee *et al.* (2010), and Nolan & Prentki (2008)). In all three shuttling systems, intermediates from the Krebs cycle are imported into the cytoplasm by specific carrier models. In the pyruvate-malate shuttle, NADPH is produced by the conversion of malate to pyruvate by the cytosolic malic enzyme (see reviews by Jensen *et al.* (2008) and Jitrapakdee *et al.* (2010)). In the pyruvate-citrate shuttle, citrate is converted to oxaloacetate and acetyl Coenzyme A (acetyl CoA) by adenosine triphosphate citrate lyase. The oxaloacetate is converted to malate and then pyruvate producing NADPH, while the acetyl CoA is converted to malonyl-coA by acetyl CoA carboxylase, which is then used in the production of long chain acyl-Coenzyme As (see reviews by Jensen *et al.* (2008) and Jitrapakdee *et al.* (2010)). In the pyruvate-isocitrate cycle, isocitrate is imported into the cytoplasm from the mitochondrial matrix by the 2-oxoglutarate carrier, it is then converted to α-ketoglutarate (by NADP-dependent isocitrate dehydrogenase) which generates NADPH (Ronnebaum *et al.*, 2006; Odegaard *et al.*, 2010). However, use of siRNA (small interfering ribonucleic acid) has only been successful in demonstrating the importance of the pyruvate-isocitrate cycle in GSIS in islets (Ronnebaum *et al.*, 2006; Odegaard *et al.*, 2010) but not the other two shuttling systems, which may be caused by compensatory alterations in other enzyme activities (Jensen *et al.*, 2006; Pongratz *et al.*, 2007). GTP, produced by the GTP-producing form of succinyl-CoA synthase and potentially the phosphoenolpyruvate cycle, may also be another molecule produced by mitochondria that leads to the potentiation of GSIS, as reduction of intracellular β-cell GTP levels negatively affects GSIS (Kibbey *et al.*, 2007; Stark *et al.*,

2009). It remains unclear how GTP potentiates insulin secretion (Metz *et al.*, 1993), however it is likely that GTP is at least partially utilised by the small GTPases involved in granule processing (see Section 1.2.2.2).

β-cells are also capable of generating arachidonic acid (AA), inositol-1,4,5-trisphosphate (IP3), diacylglycerol (DAG) and cyclic adenosine monophosphate (cAMP) that contribute to the amplification of insulin release in response to increased levels of glucose (Wolf *et al.*, 1991; Tamarina *et al.*, 2005; Kim *et al.*, 2008a). AA potentiates insulin release by increasing $[Ca^{2+}]_c$ (through activation of arachidonic-acid regulated Ca^{2+} channels on the plasma membrane and ER ryanodine receptors) as well as down-regulating voltage-gated K+ channel activity (Woolcott *et al.*, 2006; Jacobson *et al.*, 2007; Yeung-Yam-Wah *et al.*, 2010). In primary β-cells, AA production has been linked to the activities of phopholipase (PL) A_2 (three isoforms PLA_2 that are thought to be involved in different stages of GSIS) and a Ca^{2+}-dependent diacylglycerol lipase (Konrad *et al.*, 1994; Ma *et al.*, 1998; Juhl *et al.*, 2003; Bao *et al.*, 2008). IP_3 and DAG are generated by PLC (either by glucose, or following activation of GqPCRs) from phosphatidylinositol-4,5-bisphosphate (Wolf *et al.*, 1988). IP_3 then binds to IP_3 receptors which leads to the increased release of Ca^{2+} from intracellular Ca^{2+} stores which increases $[Ca^{2+}]_c$ (Blondel *et al.*, 1993). The increase in $[Ca^{2+}]_c$ and DAG levels in the β-cells then activates several different isoforms of protein kinase C (PKC) that are thought to play different roles in β-cell function (Ishikawa *et al.*, 2005; Shimono *et al.*, 2005). Functions of PKC signalling in β-cells include insulin granule processing, insulin granule exocytosis, β-cell proliferation and the regulation of several genes, including those encoding GLUT2 and both subunits of the K_{ATP} channel (Buteau *et al.*, 2001; Hashimoto *et al.*, 2005; Ishikawa *et al.*, 2005; Warwar *et al.*, 2006; Uchida *et al.*, 2007). Finally, cAMP is generated from ATP by adenylyl cyclase (either during GSIS or folloing activation of $G_{\alpha s}$PCRs) and leads to the activation of PKA and Epac 2 (also known as cAMP-dependent guanine nucleotide exchange factor II), which are both known to potentiate insulin secretion (Kashima *et al.*, 2001; Hatakeyama *et al.*, 2006; Kim *et al.*, 2008a). Islets also express

Epac1, an isoform of Epac2, but Epac1 has received less interest with regards to GSIS (Kang *et al.*, 2006). PKA-mediated potentiation of insulin secretion is thought to involve multiple mechanisms such as activation of $_{i}PLA_2\beta$ (a Ca^{2+}-independent isoform of PLA_2) and Ca^{2+} release from intracellular stores through the activation of IP_3 receptor activity (Dyachok & Gylfe, 2004; Bao *et al.*, 2008). PKA is also known to phosphorylate several ion channels leading to increased closure of K_{ATP} channels, increased voltage-gated L-type Ca^{2+} activity and decreased voltage gated K^+ ion channel activity (Holz *et al.*, 1993; Kanno *et al.*, 1998; Kim *et al.*, 2005). Epac2 signalling leads to the potentiation of GSIS by increasing the number of insulin granules that translocate to the plasma membrane but Epac2 signalling does not appear to induce exocytosis (Eliasson *et al.*, 2003; Shibasaki *et al.*, 2007). Research in transfected non-β- and β-cell lines suggest that Epac1 activation, by cAMP, may also potentiate insulin secretion by increasing K_{ATP} channel ATP sensitivity and activation of PLC (Evellin *et al.*, 2002; Suzuki *et al.*, 2006; Kang *et al.*, 2008). It should be noted that the positive effects of the incretin hormones (see Sections 1.2.5 & 1.3.3) on β-cell function, mass and survival, are mediated though the activation of adenylyl cyclase and the subsequent activaties of PKA and Epac2 (Eliasson *et al.*, 2003; Kwon *et al.*, 2004; Kawasaki *et al.*, 2005).

1.2.2.4 Stimulation of insulin secretion by amino acids and free fatty acids

In addition to glucose, insulin secretion can also be stimulated by certain amino acids (Sener & Malaisse, 2002; Zhou *et al.*, 2010). Of the amino acids, only leucine has been demonstrated to stimulate insulin secretion from islets by itself in the absence of glucose (Sener & Malaisse, 2002; Li *et al.*, 2003), while glutamine and alanine can only stimulate insulin secretion in the presence of other compounds, such as leucine (Sener & Malaisse, 2002; Zhou *et al.*, 2010). With respect to glutamine, leucine and α-ketoisocaproate were found to allow glutamine to stimulate insulin secretion, which are thought to lead to the conversion of glutamate to α-ketoglutarate by two separate mechanisms (Zhou *et al.*, 2010). Potentiation of leucine-stimulated insulin secretion by glutamate is known to occur as leucine positively stimulates glutamate

dehydrogenase activity, which converts glutamate to α-ketoglutarate (Li *et al.*, 2003). With regard to free fatty acids (FFA), it is known that FFA such as palmitate and oleate potentiate glucose-stimulated insulin secretion but cannot stimulate insulin secretion alone (Latour *et al.*, 2007). It has since been observed that islets express high levels of GPR40 (also known as free fatty acid receptor 1). Studies that have measured insulin secretion from islets isolated from WT (wild type) and GPR40 KO (knock out) mice suggest that oleate- and palmitate-mediated potentiation of GSIS occurs through this receptor alone (Latour *et al.*, 2007; Tan *et al.*, 2008).

1.2.3 Autocrine and paracrine regulation of islet secretory activities

It is known that β-cells express insulin receptors as well as IRS-1 and IRS-2 suggesting that insulin signalling occurs within β-cells and may affect its own secretion (Burks & White, 2001). As discussed in the review by Leibiger *et al.* (2008), the earliest experiments with insulin suggest that it negatively regulates its own secretion. However, this view point has become more controversial as various studies have reported that insulin either has no effect or potentiates its own secretion (Leibiger *et al.*, 2008). A study by Hee-Park *et al.* (2007) suggests that some of this confusion may been due to the effects of insulin being dependent on when insulin was applied to islets in relation to the addition to glucose. Hee-Park *et al.* (2007) reported that when islets were first exposed to insulin, glucose-stimulated increases in $[Ca^{2+}]_i$ (which coincides with the release of insulin) were inhibited. Yet if insulin were added after glucose, insulin transiently increased $[Ca^{2+}]_i$ suggesting a positive regulation of insulin release. Insulin signalling is known to be essential in β-cells as loss of insulin receptor signalling results in reductions in β-cell mass, decreased insulin content, attenuated GSIS and mitochondrial dysfunction (Otani *et al.*, 2004; Cantley *et al.*, 2007; Okada *et al.*, 2007; Liu *et al.*, 2009b).

In addition to autocrine signalling, insulin release is also affected by paracrine signalling from α- and δ-cells (Zambre *et al.*, 1999; Sorensen *et al.*, 2006). However,

there is controversy surrounding the role of paracrine signalling within rodent islets as the most widely supported model of blood flow within islets is the inner-to-outer model (Figure 1.6A), in which blood flows from the core of the islet to the periphery and finally into wider circulation (Nyman *et al.*, 2008). As the core of rodent islets is populated by β-cells, this suggests that molecules involved in paracrine signalling can only flow from β-cells to non-β-cells. A counter argument in rodent islets have been that paracrine signalling in islets occurs at cell-to-cell interfaces and the signalling molecules difuse through the interstitial spaces (Hauge-Evans *et al.*, 2009). The study by Nyman *et al.* (2008) using *in vivo* imaging techniques, suggests that there are three patterns of blood flow through murine islets (Figure 1.6). Nyman *et al.* (2008) suggests that in ~ 40% of islets, non-β cell to β-cell paracrine signalling can occur through by the release of signalling molecules into the islet capillaries.

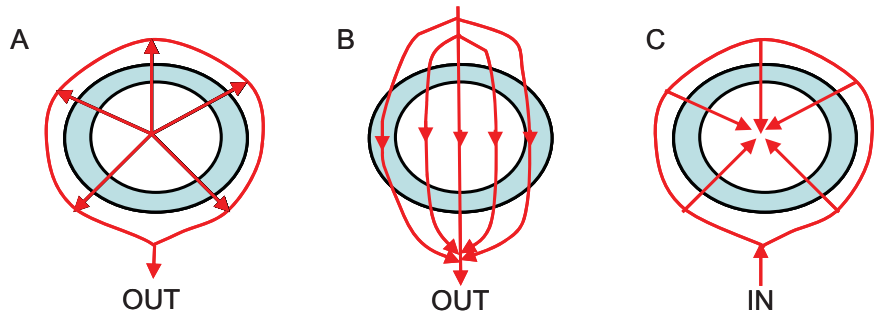


Figure 1.6 Diagrammatic representations of three different islet blood flow patterns reported to occur *in vivo* in mouse pancreas by Nyman *et al.* (2008). In each of the three figures, islets are represented as a ring with the inside representing the β-cell core and the shaded areas representing the periphery where α-, δ- and PP-cells are found. Arrows represent the direction of blood flow. A) Inner-to-outer flow. Blood enters the core of the islet and flows outwards to the periphery. This blood flow pattern supports β- to non β-cell paracrine interactions and was found in 60% of islets. B) This blood flow pattern occurred in 35% of islets tested where blood enters through one side of the islet and flows through the islet. This blood flow pattern supports non β- to β- to non β-cell paracrine interactions. C)s Outer-to-inner flow. Capallaries pass through the islet periphery and proceed to the core where blood empties into a venule/s. This was observed in 5% of islets and supports non β- to β-cell paracrine interactions.

The release of glucagon is influenced by glucose, with glucagon secretion highest in the absence of glucose and decreases to a minimal (basal) secretory rate at the glucose concentrations which first induce GSIS. During hypoglycaemia (when plasma glucose levels ≤ 3.3mM), cognitive function is impaired as the brain is dependent on glucose and prolonged periods of hypoglycaemia can result in loss of consciousness,

neuronal cell death, and ultimately death (Warren & Frier, 2005; Suh *et al.*, 2007). Therefore, glucagon is an important counter to insulin, as it prevents plasma glucose levels from falling too low by increasing plasma glucose levels through hepatic glucose production (Gelling *et al.*, 2009).

Paracrine interactions from β- and δ-cells have been observed to decrease the rate of glucagon release in whole islets (Vieira *et al.*, 2007). Despite this, glucose is believed to be the main contributing factor in the control of α-cell secretory activity (MacDonald *et al.*, 2007; Vieira *et al.*, 2007). Glucagon is known to directly potentiate insulin release by activating β-cell glucagon receptors, which are $G_{\alpha s}$-protein-coupled, leading to the activation of adenylyl cyclase (Gelling *et al.*, 2009). Loss of glucagon receptor signalling in islets (through receptor knock-out) does not affect basal or intermediate rates of insulin release in relation to wild-type islets (Sorensen *et al.*, 2006). However, insulin secretion at 16.7mM and 22.2mM glucose from islets with defective glucagon receptor signalling, was attenuated in comparison to wild-type mice (Sorensen *et al.*, 2006). Therefore, glucagon released from α-cells acting via glucagon-receptor signalling in β-cells is required for achieving higher rates of glucose-stimulated insulin secretion.

The release of sst-14 from δ-cells in islets is positively correlated with the external glucose concentration (Vieira *et al.*, 2007). Animal and clinical data suggest that the physiological role of somatostatin signalling in islets is to reduce the amount of glucagon and insulin released from α- and β-cells, respectively, rather than to alter the glucose responsiveness of either cell type (Hauge-Evans *et al.*, 2009; Mazziotti *et al.*, 2009). The four main islet cell types in rodent and human islets are known to express all five somatostatin receptors (SSTR-1, -2, -3 -4 and -5, which are also referred to as $sst_{1\text{-}5}$) but each islet cell type may not express each somatostatin receptor subtype to the same level (Ludvigsen *et al.*, 2004; Ludvigsen *et al.*, 2005; Taniyama *et al.*, 2005). In addition, SSTR expression patterns may also be sensitive to pathological state as it has been observed in non-obese diabetic (NOD) mice that SSTR receptor expression

patterns for α-, β-, δ- and PP-cell types differed between non-diabetic and diabetic mice (Ludvigsen *et al.*, 2005). While each SSTR can be expressed by each islet cell type, the consensus from numerous studies suggests that SSTR2 is responsible for the inhibition of glucagon release (Table 1.2). Both SSTR2 and SSTR5 have been suggested to be the primary sst-14 receptor responsible for the inhibition of insulin secretion, but the majority of these studies indicate that SSTR5 is key receptor responsible for sst-14 mediated inhibition of GSIS.

1.2.4 Neuronal regulation of islet secretory activities

Islets are known to be highly innervated by sympathetic, parasympathetic, and sensory neurons (Ahren, 2000). Islets are also innervated with cholecystokinin (CCK) releasing neurons, and when activated potentiate insulin secretion (Ahren, 2000). The effects of sympathetic and parasympathetic signalling on islet secretory activities and their physiological effects have been extensively reviewed by Ahren (2000) and Ahren *et al.* (2006). In summary, parasympathetic neurotransmission increases rates of insulin, glucagon, sst-14 and PP secretion, while sympathetic signalling upregulates glucagon and PP signalling but downregulates insulin and sst-14 secretion. Parasympathetic signalling has been found to potentiate insulin secretion at the onset of feeding but before the initial rise in blood glucose occurs (cephalic phase). Parasympathetic signalling also occurs during hypoglycaemia and stimulates both glucagon and PP secretion. The parasympathetic neurotransmitter receptors expressed by islets include the G_qPCR muscarinic receptor type 3 (m3) and $G_{\alpha s}$PCR pituitary adenylyl cyclase activating polypeptide 1 receptor type 1 and vasoactive intestinal polypeptide receptor 2 (Ahren, 2000). Sympathetic signalling occurs during exercise and increases plasma glucose levels by stimulating hepatic glucose output. It was observed, through loss of sympathetic nerve terminals, that sympathetic signalling also affects glucose-stimulated insulin secretion, insulin gene expression and β-cell mass. The sympathetic neurotransmitter receptors expressed by islets are the α_2 ($G_{\alpha i}$PCR, inhibits insulin secretion) and β_2 ($G_{\alpha s}$PCR, potentiates glucagon secretion) adrenoceptors and the $G_{\alpha i}$PCR (inhibits insulin secretion) neuropeptide Y

Table 1.2 The role of somatostatin receptor (SSTR) signalling on insulin and glucagon secretion

Study	Model used	Approach used	Effects of SSTR on hormone secretion	
			Glucagon	Insulin
Strowski *et al.* (2003)	Islets from WT and SSTR5-KO mixed breed mice	sst-28 and SSTR-specific agonists at 20mM glucose		SSTR5 ↓ SSTR2 ↓ (minor)
Wang *et al.* (2004)	Islets from WT and double SSTR1- and SSTR5-KO mice	sst-14 and sst-28 at various glucose concentrations		SSTR1 & SSTR5 ↓
Strowski *et al.* (2000)	Islets from WT and SSTR2-KO C57BL/129 mice	sst-14 and sst-28 at various glucose concentrations	SSTR2 ↓ SSTR5 -	SSTR5 ↓ SSTR2 -
Cejvan *et al.* (2003)	Wistar rat isolated islets	sst-14 and a SSTR2-specific antagonist at 3.3/5.5mM glucose + 20mM Arg	SSTR2 ↓	SSTR2 -
Rossowski & Coy (1994)	*In vivo* CD rats	sst-14 and SSTR receptor-specific antagonists	SSTR2 ↓	SSTR5 ↓
Yao *et al.* (2005)	Mixed breed hamster islets and HIT-T15 β-cells	SSTR5-selective agonists and sst-14 with a SSTR2-specific antagonist at 20mM glucose		SSTR1, 3,4 and 5 - SSTR2 ↓
Atiya *et al.* (1997)	Isolated perfused human pancreas	anti-sst-14 antibody with SSTR receptor-specific agonists at 16.7mM glucose		SSTR2 ↓ SSTR5 -
Zambre *et al.* (1999)	Human islets	24-hour incubation at 6.1mM glucose with SSTR-specific agonists		SSTR2 - SSTR5 ↓
Singh *et al.* (2007)	Human islets	sst-14 or SSTR-specific agonists Insulin secretion at 20mM glucose, glucagon secretion stimulated by 20mM Arg	SSTR2 ↓ SSTR1 ↓ SSTR5 ↓ (minor)	SSTR2 ↓ SSTR5 ↓ SSTR1 ↓

Minor refers to relative level of inhibition caused by activation of the specified receptors on insulin secretion. - no effect.

receptor Y1 (Moltz & McDonald, 1985; Lacey *et al.*, 1991; Ahren, 2000; Ahren *et al.*, 2006).

With regard to sensory neurons, there are two types associated with islets, substance P and calcitonin gene-related peptide (CGRP) containing neurons (Ahren, 2000). The effects of CGRP and substance P are controversial, as factors such as species differences, concentrations used and model used (isolated islets *vs.* perifused pancreas) appear to influence the effects of these neurotransmitters on islet secretory activities (Chiba *et al.*, 1985; Hermansen & Ahren, 1990; Kogire *et al.*, 1991; Rasmussen *et al.*, 1998; Ahren, 2000). However, evidence over recent years is mounting that sensory neuron activities in islets may contribute to β-cell dysfunction. TRPV1-associated neurons have linked sensory neuron dysfunction to autoimmune-mediated destruction of β cells in a spontaneously type 1 diabetic mouse model (Razavi *et al.*, 2006). In addition, ablation of sensory neurons in the Zucker Diabetic Fatty rat model of type 2 diabetes was also found to improve glucose tolerance by increasing insulin secretion both *in vivo* and *in vitro* (Gram *et al.*, 2007).

1.2.5 Gastrointestinal tract-mediated regulation of islet secretory activities

The study by McIntyre *et al.* (1965) was one of the first studies to identify that orally ingested glucose produced greater insulin secretory responses than that produced by an identical amount of glucose directly infused into the blood stream. This phenomenon is known as the incretin effect and, as described in the review by Peters (2010), this was later found to be due to the actions of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1).

Glucose-dependent insulinotropic polypeptide (GIP) is primarily secreted by K-cells, endocrine cells present in the duodenum and distal jejunum (Damholt *et al.*, 1999). A truncated form of GIP, $GIP_{(1-30)}$, is also actively secreted by α-cells in mouse, python and human islets (Fujita *et al.*, 2010). Both forms of GIP, at physiological levels,

potentiate GSIS (but not basal rates of insulin secretion), and glucagon secretion too (De Marinis *et al.*, 2010; Fujita *et al.*, 2010). In addition to affecting islet secretory function, use of exogenous GIP has been found to increase β-cell mass and reduce rates of β-cell apoptosis under physiological conditions, but its protective effects are diminished in diabetic states (Winzell & Ahren, 2007; Maida *et al.*, 2009).

GLP-1 is produced by L-cells and, like GIP, potentiates GSIS (but not basal insulin secretion rates) but inhibits the release of glucagon from α-cells (De Marinis *et al.*, 2010; Fujita *et al.*, 2010). As such, manipulation of GLP-1 signalling has been exploited in the treatment of type 2 diabetes (discussed below). With regard to information generated from animal models of diabetes, GLP-1 receptor signalling in islets has been found to not only improve glycaemic control as it also promotes β-cell proliferation as well as causing a reduction in the rates of β-cell apoptosis (Maida *et al.*, 2009; Lupi *et al.*, 2010).

Ghrelin is an appetite-stimulating peptide that is primarily produced by A-like cells (an endocrine cell) in the stomach (Yada *et al.*, 2008). It exists in two forms, an acylated form (referred to as ghrelin) which can activate the ghrelin receptor, and a non-acylated (also referred to a des-acyl-/desacyl-ghrelin) form which cannot act via the ghrelin receptor (Granata *et al.*, 2007). The *in vivo* and *in vitro* effects of ghrelin on glucose homeostasis and β-cell function have been reviewed by Yada *et al.* (2008), which has primarily focused on ghrelin receptor antagonism and genetic knock-out studies. Ghrelin receptor signalling in islets promotes a hyperglycaemic state by potentiating glucagon secretion and inhibiting insulin release. Therefore, use of ghrelin receptor antagonists have received interest with regard to improving glucose tolerance (Salehi *et al.*, 2004; Yada *et al.*, 2008). It is also of note that there appears to be at least one other ghrelin receptor expressed in β-cell lines and islets, which can bind both forms of ghrelin (Camina, 2006; Granata *et al.*, 2007). Granata *et al.* (2007) suggest that this receptor induces β-cell proliferation and reduces apoptotic signalling. As discussed in Section 1.2, the ghrelin is also expressed in islets by ε-cells (appear

to be important in feotal islet development) and may also be secreted by α-cells. With regard to α-cells, ghrelin may be used in autocrine and paracrine signalling as its known to potentiate glucagon secretion and inhibit insulin secretion (Salehi *et al.*, 2004; Dezaki *et al.*, 2006).

1.3 Diabetes mellitus

As described above (Section 1.1), diabetes mellitus is a chronic endocrine disorder, with type 1 and type 2 diabetes being the two most prevalent forms of the disease and is clinically defined by several criteria (Table 1.3). Type 1 diabetes is known to have a prevalent genetic element with ~50 chromosomal locations linked to the development of type 1 diabetes (see the review by Santamaria (2010)). As discussed in the reviews by Todd (2010) and Santamaria (2010), highest risk of type 1 diabetes is conferred by specific alleles of class I and II MHC (major histocompatibility complex) proteins that are expressed by antigen-presenting cells. During the onset of type 1 diabetes, these cells present β-cell specific autoantigens (such as preproinsulin) to naïve T-cells, which generates autoantigen recognising $CD8^+$ cytotoxic T-cells which then destroy the β-cells. However, the onset of type 1 diabetes requires an environmental trigger; Schulte *et al.* (2010) suggest that the infection of β-cells by enteroviruses is an environmental trigger, which leads to the phagocytosis of β-cells by dendritic cells (an antigen presenting cell). Unlike type 1 diabetes, environmental factors instead of a genetic disposition are thought to be the major factors in the onset of type 2 diabetes and have been discussed in more detail below in Section 1.3.1.

Hyperglycaemia is associated with a wide array of secondary complications ranging from short-term to long-term health issues. Short term symptoms of hyperglycaemia include abnormal thirst, abnormal hunger, increased urination frequency, blurred vision and/or increased susceptibility to infection (American Diabetes Association, 2010). If the hyperglycaemia is left untreated, this can lead to diabetic ketoacidosis (type 1 diabetes) or hyperosmolar hyperglycaemic state (type 2 diabetes; Umpierrez

et al., 1996; American Diabetes Association, 2010). Long-term complications of chronic hyperglycaemia are blindness (retinopathy), nephropathy (potentially leading to end stage kidney failure), peripheral neuropathy and amputations due to advanced infection. Additionally, patients with diabetes are at a significantly increased risk of cardiovascular disease (Schramm *et al.*, 2008). Therefore, effective management of plasma glucose levels is essential in any form of diabetes. Due to the loss of β-cells, glycaemic management in type 1 diabetes is currently dependent on insulin replacement therapy. As type 2 diabetes is caused by a progressive loss of β-cell function and/or insulin resistance (Section 1.1) there are different regimes available for its treatment discussed in more detail in Section 1.3.3.

Table 1.3 Criteria for the diagnosis of diabetes mellitus.

Criteria	Pre-diabetes / at risk		Gestational Diabetes	Diabetes
	IFG	IGT		
FBG (mM)	6.1 - 6.9	< 7	≥ 5.3	≥ 7.0
75g OGTT 2 hour plasma glucose (mM)	< 7.8	7.8 < 11.0	≥ 8.6	≥ 11.1
Random glucose (mM)	-	-	-	≥ 11.1
HbA_{1C} (millimoles/mole)	-	-	-	≥ 65

IFG- impaired fasting glucose, IGT- impaired glucose tolerance, FBG- fasting blood glucose (taken after a ≥ 8 hour fasting period), OGTT- oral glucose tolerance test, random glucose- plasma glucose levels taken from a non-fasted individual, HbA_{1C}-glycosylated haemoglobin- units expressed as mmol/mol (HbA_{1c}/total Hb). Information has been primarily taken from the World Health Organisation (2006) with gestational diabetes and HbA_{1c} data taken from American Diabetes Association (2010).

1.3.1 Type 2 diabetes risk factors

The progression of type 2 diabetes takes place over many years, so considerable effort has been made to identify factors which influence the occurrence of the disease. People with either impaired glucose tolerance (IGT, Table 1.3), impaired fasting glucose (IFG, Table 1.3), or IGT together with IFG are known to be at higher risk of developing type 2 diabetes and so have been classed as “pre-diabetic states” (Unwin *et al.*, 2002; Li *et al.*, 2008; Hippisley-Cox *et al.*, 2009; Tabak *et al.*, 2009; Wang *et al.*, 2010). Similarly, women who become diabetic during pregnancy (gestational diabetes;

Table 1.3) are another group at higher risk of developing type 2 diabetes as they also display defects in β-cell function and insulin signalling (Kousta *et al.*, 2003).

The occurrence of type 2 diabetes has been found to coincide with numerous other risk factors. Age has been described as a risk factor, as the incidence of type 2 diabetes is significantly higher in people who are aged 40 years or over but can also occur in people under the age of 10 (Ramachandran *et al.*, 2004; Gonzalez *et al.*, 2009; Amed *et al.*, 2010). The incidence of type 2 diabetes has been found to significantly increase at BMIs above 23, with highest risks observed at BMI scores ≥ 35, but it should be noted that BMI is a contributing, not a causative, factor (Ramachandran *et al.*, 2004; Nijpels *et al.*, 2008; Lehtovirta *et al.*, 2010; Lorenzo *et al.*, 2010). The incidence of type 2 diabetes is also increased in certain ethnic populations (Hippisley-Cox *et al.*, 2009), is associated with low birth weight (as discussed in the review by Pinney & Simmons (2010)) and increased in people with first order relatives who have type 2 diabetes (Lorenzo *et al.*, 2010). The latter risk factor indicates that there are specific alleles associated with the incidence of type 2 diabetes, and in 2009 single nucleotide polymorphisms in 19 genes were implicated as conferring increased risk (see reviews by Elbein (2009) and McCarthy & Zeggini (2009)).

1.3.2 Onset of type 2 diabetes

The progression from being at risk of developing type 2 diabetes to developing overt type 2 diabetes in not clearly understood but is linked to further decreases in β-cell function and/or insulin resistance. Leahy (2009) suggests that the progression from pre-diabetic to developing type 2 diabetes is linked to how resilient an individual's β-cells are, as 17 out of 19 genes (as mentioned above) implicated in susceptibility for type 2 diabetes are either involved in β-cell survival, function or development. Histological studies of pancreata obtained from non-diabetic and type 2 diabetic people all suggest that β-cell mass is decreased without similar decreases in α-cell and δ-cell numbers (Butler *et al.*, 2003; Yoon *et al.*, 2003; Iki & Pour, 2007). The loss

of β-cell mass and function has been ascribed to glucotoxicity, glucolipotoxicity and/or β-cell fatigue and are briefly described below (for more detailed mechanisms see reviews by Leahy (2009), Poitout & Robertson (2002) and Robertson (2009)). Glucotoxicity refers to chronic exposure of β-cells to supraphysiological levels of glucose, associated with a non-reversible reduced transcription of insulin mRNA, which leads to reductions in islet insulin content and GSIS (see reviews by Poitout & Robertson (2002) and Robertson (2009)). It is also recognised that glucotoxicity increases the incidence of apoptosis through oxidative stress in β-cells (Robertson, 2009; Zhang *et al.*, 2010). β-cell fatigue is based on a similar concept as glucotoxicity. This hypothesis suggests that the effects of glucotoxicity are reversible, by resting the β-cells (i.e. no longer actively secreting insulin), before a point where their insulin reserves fall too low (as discussed in the reviews by Leahy (2009) and Robertson (2009)). The concept of glucolipotoxicity is based on the observation that chronic exposure of β-cell lines and islets to high concentrations of saturated fatty acids (e.g. palmitate) does not affect β-cells survival unless cultured under elevated glucose concentrations ≥ 11mM (El-Assaad *et al.*, 2003). Additionally, under glucolipotoxic, conditions palmitate has also been found to reduce maximal levels of GSIS (Sun *et al.*, 2008; Popescu *et al.*, 2010).

1.3.3 Current treatments for type 2 diabetes

On diagnosis of type 2 diabetes, a patient is given advice on permanent changes in diet which are aimed not only to reduce the peak amount of glucose absorbed into the blood stream but as a means to regulate the intake of specific types of lipids too (Holt & Kumar, 2010). In addition to changes in diet, 30 minutes of moderate aerobic exercise 5 days a week is recommended as excercise improves peripheral insulin sensitivity. Collectively, these alterations not only aid weight loss in overweight patients but also help regulate glycaemia and improve several risk factors associated with cardiovascular disease (Sibal & Home, 2009; Holt & Kumar, 2010). If HbA_{1c} (Glycated haemoglobin) levels are > 70 millimoles/mole or patients are displaying symptoms (e.g. retinopathy), then oral drugs are prescribed, in addition to changes in

lifestyle modifications to manage hyperglycaemia. According to the study by Gonzalez *et al.* (2009), the most commonly prescribed oral drugs by people with type 2 diabetes to regulate their hyperglycaemia were metformin, sulphonylureas and thiazolidinediones (also known as glitazones). Since 2005, GLP-1 receptor agonists and dipeptidyl peptidase 4 (DPP4) inhibitors have also been introduced for the treatment of type 2 diabetes (Janosz *et al.*, 2009). It should be noted that subcutaneous injections of insulin are also used in the treatment of type 2 diabetes, either alone or in combination with oral drugs, as a final resort should other treatments fail to effectively regulate glycaemia (Gonzalez *et al.*, 2009; Sibal & Home, 2009).

Metformin is typically the first oral drug to be used for the management of hyperglycaemia and is known to reduce hepatic gluconeogenesis (and lipogenesis) via the activation of adenosine monophosphate kinase signalling (Shaw *et al.*, 2005). Additionally, metformin may also increase skeletal muscle and other peripheral tissue insulin sensitivity, reduce plasma lipid levels, inhibit DDP4 activity and increase GLP-1 production (see reviews by Setter *et al.* (2003) Tahrani *et al.* (2010)). Another benefit of metformin is a low occurrence of hypoglycaemia but a concern with metformin is that it can also induce, albeit rarely, lactic acidosis (as discussed in the review by Setter *et al.* (2003)). Sulphonylureas bind to the SUR1 subunit on β-cell K_{ATP} channels which causes channel closure leading to insulin release (see above), and are used to enhance insulin secretion from β-cells (see review by Gribble & Reimann (2003)). Sulphonylureas have significant drawbacks, including weight gain and increased occurrence of hypoglycaemia (Sibal & Home, 2009). Additionally, the study by Tzoulaki *et al.* (2009) suggests that sulphonylurea monotherapy is associated with significantly higher risks of myocardial infarction, congestive heart failure and general mortality rates in comparison to metformin monotherapy.

Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor gamma (PPARγ) agonists and are primarily associated with increasing peripheral tissue insulin sensitivity (Kahn *et al.*, 2006; Scheen *et al.*, 2009). In the ADOPT trial (Kahn *et*

al., 2006), rosiglitazone (a TZD) treatment was not only attributed to the improvement in insulin sensitivity but increased preservation of β-cell function with respect to the metformin and sulphonylurea treatment groups. These effects have been confirmed in animal models of diabetes and in human islets *in vitro*, where TZD treatment was found to preserve islet mass, islet morphology, islet insulin content, insulin secretory activity and it reduced islet triglyceride content (Shimabukuro *et al.*, 1998; Ishida *et al.*, 2004; Kawasaki *et al.*, 2005; Vandewalle *et al.*, 2008). *In vitro* studies suggest that the protective effects of rosiglitazone (a TZD) in islets are primarily due to PPARγ receptor-dependent signalling but rosiglitazone is also able to reduce some aspects of palmitate-induced oxidative stress by antagonising free fatty acid receptor 1 signalling (Meidute Abaraviciene *et al.*, 2008; Vandewalle *et al.*, 2008). There are several health concerns associated with TZDs, which include weight gain (observed in both ADOPT and PROactive trials), as well as increased risk of fractures, oedema and cardiovascular events (Scheen *et al.*, 2009; Tzoulaki *et al.*, 2009; Tahrani *et al.*, 2010).

GLP-1 was identified as a potential drug for type 2 diabetes as it is an incretin hormone which potentiates insulin secretion and also inhibits glucagon secretion (Section 1.2.5). However, *in vivo* GLP-1 is rapidly degraded by DPP4, which led to the development of GLP-1 receptor agonists and DPP4 inhibitors, with both approaches proving successful in the treatment of type 2 diabetes (see reviews by Mentlein (1999) and Peters (2010)). With regard to the efficacy of GLP-1 receptor agonists in comparison to DPP4 inhibitors, the reviews by Janosz *et al.* (2009) and Tahrani *et al.* (2010) suggest that GLP-1 agonists offer greater glycaemic control and also are associated with weight loss too. Both classes of drugs are associated with improved β-cell function and survival as well as improvements in cardiovascular risk factors through reductions in plasma triglycerides and blood pressure (Janosz *et al.*, 2009; Tahrani *et al.*, 2010). However, there are concerns that both forms of treatment may be associated with pancreatitis (see review by Butler *et al.* (2010)). The 2008 National Collaborating Centre for Chronic Conditions (NCC-CC, 2008) guidelines are the latest

(full) guidelines for the treatment of type 2 diabetes used by the UK's National Health Service but the section dealing with the clinical use of oral anti-hyperglycaemic drugs was updated in 2009 (NICE, 2009).

As discussed above, type 2 diabetes is a progressive disease with many secondary complications and it has a considerable economic cost if quality of life is to be assured. Therefore, studies are now focusing on how to prevent the onset type 2 diabetes in people who at highest risk of developing the disease. The meta-analysis by Gillies *et al.* (2007) suggests that lifestyle interventions (diet, exercise or both) reduced the incidence of type 2 diabetes by ~50%. Overall, lifestyle interventions were found to be more effective than oral diabetics such as acarbose (α-glucosidase inhibitor that reduces intestinal absorption of carbohydrates) and metformin (Gillies *et al.*, 2007; Tahrani *et al.*, 2010).

1.4 The endogenous cannabinoid system

Cannabis has been used for medical and recreational purposes for thousands of years (Pacher *et al.*, 2006), but it was not until the 1960's that the main psychoactive component of cannabis, Δ^9-tetrahydrocannabinol (THC), was discovered (Gaoni & Mechoulam, 1964). Despite this it wasn't until the early 1990's that the first cannabinoid (CB) receptor, CB_1, and an endogenous ligand, *N*-arachidonoylethanolamide (otherwise known as anandamide or AEA) were discovered (Matsuda *et al.*, 1990; Devane *et al.*, 1992). Since then, another cannabinoid receptor (CB_2) and the endocannabinoid, *sn*-2 monoacylglycerol 2-arachidonoylglycerol (2-AG), have been identified (Munro *et al.*, 1993; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). Early investigations into the effects of anandamide reported that AEA had anti-nociceptive, hyperphagic (over-eating) and vasorelaxatory effects, suggesting that the endocannabinoid system was involved in several physiological systems (Randall *et al.*, 1996; Stein *et al.*, 1996). The endocannabinoid system is now known to be expressed in a wide range of cells and tissues throughout

the body. The endocannabinoid system is involved in the physiological regulation of a number of systems and is discussed further in Section 1.4.3, with regard to receptor-specific signalling.

Several other compounds have been suggested to be putative endocannabinoids. They include noladin ether (2-arachidonyl glyceryl ether), virodhamine (*O*-arachidonoylethanolamine) and *N*-arachidonoyl dopamine (Hanus *et al.*, 2001; Huang *et al.*, 2002; Porter *et al.*, 2002). While these compounds have been found to be cannabinoid agonists, it remains unclear whether they are endogenous ligands as biosynthetic pathways have not been established. Additionally, it has been found that AEA is part of a wider class of lipid signalling molecules called *N*-acylethanolamines, which include *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA). However, OEA and PEA are devoid of CB receptor activity and are subsequently called endocannabinoid-like molecules (Matias *et al.*, 2007).

1.4.1 Endocannabinoid synthesis

Described below are the biosynthetic pathways for three endocannabinoids. A key aspect of their production is that they are made *de novo* as they can readily diffuse across membrane and so cannot be stored intracellularly.

1.4.1.1 N-acylethanolamine (NAE) synthesis

The *de novo* production of *N*-acylethanolamines involves two key stages, the formation of *N*-acyl-phosphatidylethanolamine (NAPE) and then the removal of the *N*-acylethanolamine from the NAPE (Figure 1.7). The formation of NAPE first involves the transfer of a *sn*-1 fatty acid moiety from a phosphatidylcholine (PC) glycerophospholipid to the ethanolamine head group of a phosphatidylethanolamine (PE) glycerophospholipid (Cadas *et al.*, 1996; Cadas *et al.*, 1997); Ca^{2+}-dependent or -independent *N*-acyltransferase (CaNAT and iNAT, respectively) catalyse this step (Cadas *et al.*, 1996; Cadas *et al.*, 1997; Jin *et al.*, 2007). The second stage is the liberation of the *N*-acylethanolamine from NAPE; for AEA this can be achieved by multiple pathways. One pathway is the direct hydrolysis of AEA from NAPE by a

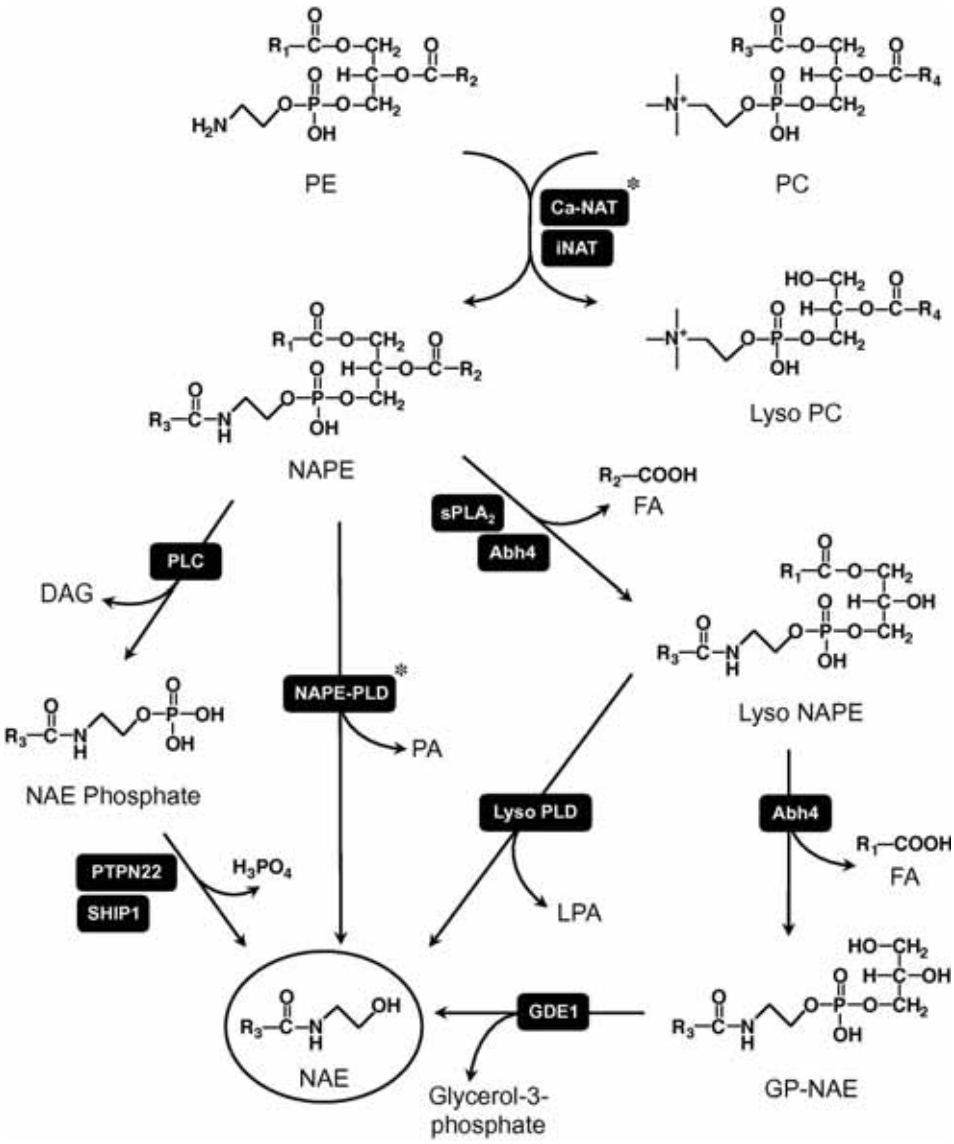


Figure 1.7 The different biosynthetic pathways by which anandamide (*N*-arachidonoylethanolamine) can be produced. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; LPA, lysophosphatidic acid; DAG, diacylglycerol; NAPE, *N*-acyl-phosphatidylethanolamine; Ca-NAT, Ca^{2+}-dependent *N*-acyltransferase; iNAT, Ca^{2+}-independent *N*-acyltransferase; NAPE-PLD, NAPE-specific phospholipase D; PLC, phospholipase C; PTPN22, protein tyrosine phosphatase, non-receptor type 22; $sPLA_2$, secretory phospholipase A_2; GP-NAE, glycerol-3-phosphate *N*-acylethanolamine; NAE, *N*-acylethanolamine; Abh4, α/β-hydrolase 4; GDE1, glycerophosphodiesterase 1; SHIP1, Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1; FA, fatty acid. The figure has been taken from Ueda (2010).

NAPE-specific phospholipase D (NAPE-PLD), whose activity is known to be Ca^{2+}-dependent (Cadas *et al.*, 1996). In a second pathway, the fatty acid at the *sn*-1 position is removed by type IB secretory phospholipase A_2 to produce lyso-NAPE, from which AEA is liberated by lyso-PLD (Sun *et al.*, 2004). A third pathway involves phospholipase C hydrolysing NAPE producing a phosphorylated form of AEA, which

is then dephosphorylated by PTPN22 (protein tyrosine phosphatase, non-receptor type 22) yielding AEA (Liu *et al.*, 2006). There are additional pathways and alternate enzymes by which AEA can be produced from NAPE which are described in the review by Ueda *et al.* (2010).

The expression of these pathways differs between tissues. For instance, the activities of CaNAT and NAPE-PLD are highest in the brain (Cadas *et al.*, 1997; Leung *et al.*, 2006). This may also reflect tissue-specific requirements in endocannabinoid signalling, as mouse NAPE-PLD knock-out studies suggest that *N*-acylethanolamine formation in the brain is primarily dependent on NAPE-PLD, whereas in testis *N*-acylethanolamines are produced by NAPE-PLD independent pathways (Leung *et al.*, 2006). The utilisation of different NAPE-PLD hydrolytic pathways may allow cells to produce specific NAEs. This was observed in the study by Leung *et al.* (2006), as saturated and monounsaturated NAE levels in brains from NAPE-PLD KO mice were significantly reduced but the levels of polyunsaturated NAEs, such as AEA, were least affected. However, this could also be explained by a compensatory mechanism in which another AEA synthetic pathway was upregulated in specific regions of the brain.

1.4.1.2 2-Arachidonyl glycerol (2-AG) synthesis

As discussed in the review by Sugiura *et al.* (2002), the existence of 2-AG, a monoacylglycerol (MAG), was long known before its identification as an endocannabinoid, with 2-AG as an intermediate in the production of triacylglycerols, glycerophospholipids, and arachidonic acid. With regard to the production of 2-AG, for the purposes of endocannabinoid signalling, it is viewed that the biosynthetic pathways are Ca^{2+}-dependent (Bisogno *et al.*, 1997; Stella *et al.*, 1997). This led to the identification of two Ca^{2+}-dependent signalling pathways which produce the diacylglycerol (DAG), *sn*-1-acyl-2-arachinodyl-glycerol (Bisogno *et al.*, 1997; Stella *et al.*, 1997). The pathway identified by Stella *et al.* (1997) was the production of DAG from phosphatidylinositol (PI) by a PI-specific phospholipase C. The second pathway indentified by Bisogno *et al.* (1997) was the production of DAG from phosphatidic acid (PA) by PA phosphatase. As with NAE biosynthesis, there may also be cell-specific

expression of the two DAG producing pathways, as Stella *et al.* (1997) used hippocampal neurons, whereas Bisogno *et al.* (1997) used the $N_{18}TG_2$ neuroblastoma cells (neuronal cell line). Both studies recognised that the conversion of DAGs to MAGs was catalysed by a Ca^{2+}-dependent DAG lipase (DAGL), of which two isoforms were identified, DAGLα and DAGLβ (Bisogno *et al.*, 2003). Both isoforms were found to be co-expressed together in tissues but the two isoforms show differential activities for DAGs. DAGLα showing equal preference for linoleic-, oleic-, arachidonic-, and stearic-acid based DAGs, whereas DAGLβ had the highest activity for linoleic-acid ≥ oleic-acid > arachidonic-acid > stearic-acid based DAGs (Bisogno *et al.*, 2003).

1.4.1.3 N-arachidonoyl dopamine (NADA) synthesis

N-arachidonoyl dopamine is the only putative endocannabinoid (of those listed above) to have its biosynthetic pathway at least partially characterised. NADA appears to be restricted to dopaminergic nerve terminals in rat brain, and two possible biosynthetic pathways have been described (Hu *et al.*, 2009). The first pathway proposed that *N*-arachidonyl (NA) tyrosine (produced from arachidonic acid [AA] and tyrosine) was converted to NA-dopamine and then to NADA, but this pathway was discredited as only one intermediate (*N*-arachidonyl tyrosine) was found to be present in rat brain. The second pathway proposed by Hu *et al.* (2009) was a fatty acid amide hydrolase (FAAH)-dependent formation of NADA from AA and dopamine, which was observed *in vivo* and in rat brain membrane assays. However, the formation of NADA by micelles containing FAAH was poor, suggesting that other proteins may be involved (Hu *et al.*, 2009).

1.4.2 Endocannabinoid degradation

Another key feature of endocannabinoid signalling is that endocannabinoids are rapidly degraded by cells. As the degradative enzymes are expressed intracellularly, endocannabinoids first need to be internalised and so endocannabinoid degradation is typically considered to be a two-stage process. Three main endocannabinoid degrading enzymes are involved (FAAH, *N*-acylethanolamine-hydrolysing acid

amidase and monoacylglycerol lipase) and these are discussed further in the following sub-sections. The main endocannabinoid degradative pathways are represented in Figure 1.8. In addition to these pathways, endocannabinoids can also be oxygenated to form compounds with different pharmacology (van der Stelt *et al.*, 2002). Briefly, cyclooxygenases (COX) and lipoxygenases (LOX) are the two main classes of enzymes that can oxygenate endocannabinoids (van der Stelt *et al.*, 2002). Both COX and LOX can also oxygenate AA to produce biologically active molecules (Han *et al.*, 2002; Lichtman *et al.*, 2002).

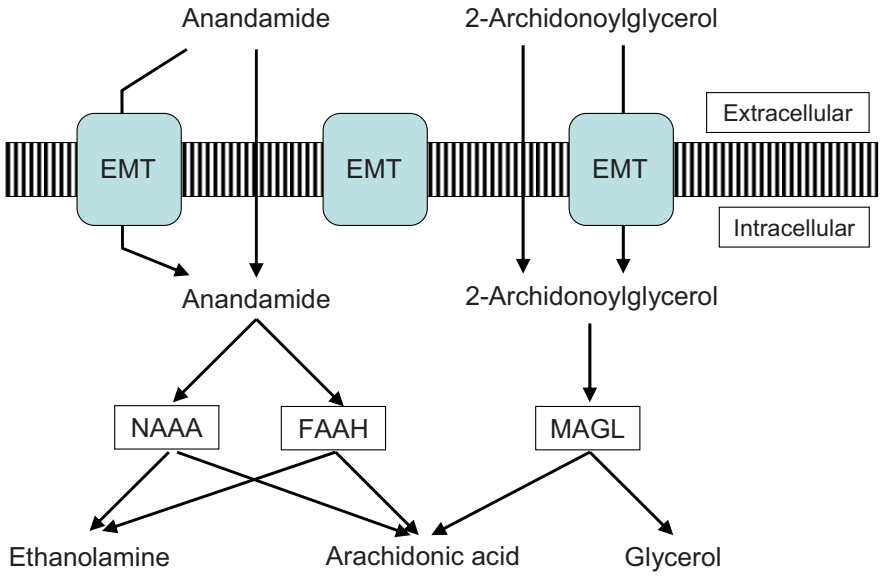


Figure 1.8 The major degradative pathways of anandamide and 2-arachidonoylglycerol. For anandamide and 2-arachidonoylglycerol to be degraded, they first need to enter the cell: this can occur either by diffusion across the plasma membrane or through endocannabinoid membrane transport (EMT) proteins (Kaczocha *et al.*, 2009). Once in the cytoplasm, the endocannabinoids can then be metabolised by FAAH (fatty acid amide hydrolyase), NAAA(*N*-acylethanolamine-hydrolysing acid amidase) or MAGL (monoacylglycerol lipase), with enzyme involvement being dependent on the endocannabinoid and cell type (Sun *et al.*, 2005; Tsuboi *et al.*, 2005; Blankman *et al.*, 2007).

The study by Ortega-Gutierrez *et al.* (2004), using neuronal cells isolated from FAAH wild type and knock-out mice, found that AEA uptake was dependent on multiple factors. They concluded that 24% of AEA uptake was FAAH-dependent, 18% CB_1 receptor-dependent, 30% EMT-dependent and 28% diffusion-dependent. However, the diffusion-dependent uptake of AEA is reliant on the metabolism of cytosolic AEA in order to prevent the intracellular and extracellular concentrations of AEA from

equilibrating. Maccarrone *et al.* (2000) suggested that CB_1 receptor-stimulated AEA uptake occurs through potentiation of nitric oxide (NO) synthase leading to increased intracellular NO levels, which they hypothesised to upregulate EMT uptake rates. With regard to EMTs, the study by Kaczocha *et al.* (2009) identified that certain fatty acid carriers, fatty acid binding proteins (FABP), contributed to AEA uptake. More specifically, it was found that $N_{18}GT_2$ cells express FABP5, and that its inhibition reduced AEA uptake by 57% over a 5-minute period. Therefore, it appears likely that AEA uptake and the uptake of other endocannabinoids are achieved through diffusion gradients and membrane transport proteins, with the maintenance of the diffusion gradient being dependent on endocannabinoid clearance. As discussed in the review by Yates & Barker (2009), endocannabinoids that diffuse into cells may be sequestered into intracellular compartments. It has been proposed that endocannabinoids which enter the plasma membrane may also be captured in endocytotic vesicles.

1.4.2.1 Fatty acid amide hydrolase (FAAH)

FAAH (also known as FAAH-1) is conserved between mouse, rat (91% homology with mouse) and human (shares ~83% homology with mouse and rat FAAH genes). FAAH is a serine hydrolase, and hydrolyses AEA to form AA and ethanolamine (see review by McKinney & Cravatt (2005)). FAAH has an optimal pH of 9 and is typically reported to have highest activities for oleamide (a primary amide) and AEA (Ueda *et al.*, 1995; Giang & Cravatt, 1997; Sun *et al.*, 2005; Wang *et al.*, 2008). *In vitro* studies also suggest that FAAH can also hydrolyse 2-AG and other MAGs, but *in vivo* the contribution of FAAH in regulation of MAG levels is minor (Lichtman *et al.*, 2002). FAAH is expressed in the wide number of tissues, with FAAH being the major AEA degradative enzyme in brain, spinal cord and liver but does not appear to be expressed in heart or skeletal muscle (Cravatt *et al.*, 2001; Sun *et al.*, 2005; Mulder & Cravatt, 2006; Wei *et al.*, 2006).

In addition to FAAH, there is a second FAAH, FAAH-2, which is 20% homologous to FAAH and is expressed in humans but not in rodents (Wei *et al.*, 2006). Wei *et al.* (2006) found that the optimal pH of FAAH-2 was between pH 8 and 9 and that it displayed the highest catalytic activity for oleamide with only minor NEA hydrolytic activity. FAAH-2 is expressed in a wide range of human tissues, in particular the heart, but was not found to be expressed in brain, small intestine or liver (Wei *et al.*, 2006). Both FAAH and FAAH-2 are globular proteins with a single membrane-spanning helix and so are integral membrane proteins (Wei *et al.*, 2006). Both FAAH and FAAH-2 are expressed intracellularly with the globular domain of FAAH facing the cytosol, while the globular domain of FAAH-2 is expressed on the luminal sides of membranes (Wei *et al.*, 2006). In the study by Morozov *et al.* (2004), > 80% of FAAH was present on small vesicles, 10% was found on the outer mitochondrial membrane and 5% was expressed on the cytosolic side of the plasma membrane.

1.4.2.2 N-acylethanolamine-hydrolysing acid amidase (NAAA)

Ueda *et al.* (1999) identified a FAAH-like enzyme activity in human megakaryoblastic cells, which hydrolysed AEA to AA and ethanolamine, but, unlike FAAH, it had an optimal pH of 5 and was not inhibited by FAAH inhibitors. The FAAH-like enzyme was subsequently named *N*-acylethanolamine-hydrolysing acid amidase (NAAA), and these characteristics have been reproduced by other studies, and it has been found that NAAA is catalytically distinct from FAAH, as well as showing the highest hydrolytic activity for PEA over other NAEs including AEA (Sun *et al.*, 2005; Tsuboi *et al.*, 2005). Both Sun *et al* (2005) and Tsuboi *et al.* (2005) examined the expression (mRNA) in mouse and rat tissues (respectively) and showed that NAAA is widely-distributed throughout the body but is expressed at low levels in liver and small intestine. Cellular analysis of NAAA has revealed that NAAA appears to be expressed within lysosomes or is associated with the lysosomal membrane (see review by Ueda *et al.* (2010))

1.4.2.3 Monoacylglycerol lipase (MAGL)

Monoacylglycerol lipase (MAGL) is the only enzyme of its class to be expressed in mammals and is a serine hydrolase that hydrolyses medium to long chain fatty acids MAGs, MAGL-mediated hydrolysis of 2-AG produces AA and glycerol (Karlsson *et al.*, 1997). MAGL is a cytosolic membrane-associated enzyme and is expressed in brain, intestinal tract, islets and liver (Blankman *et al.*, 2007; Saario & Laitinen, 2007; Duncan *et al.*, 2008; Starowicz *et al.*, 2008; Tharp *et al.*, 2008). It was found that in mouse brain, 90% of 2-AG hydrolysis was membrane-localised, with MAGL accounting for 85% of this (Blankman *et al.*, 2007). Of the remaining membrane-localised 2-AG hydrolysis, FAAH accounted for < 1% of activity (Blankman *et al.*, 2007). In porcine brain preparations, MAGL was found to have an optimal pH of 7 (Goparaju *et al.*, 1999), but has also been described elsewhere as having an optimal pH of 8 (see review by Blankman *et al* (2007)). MAGL is typically described as having a higher activity for 2-AG over other MAGs (see review by Blankman *et al.* (2007)) but the study by Dinh *et al.* (2002) found that MAGL hydrolysed 2-oleyl glycerol at a faster rate (~2 fold) than 2-AG.

1.4.4 "Classical" Cannabinoid receptors

To date, there are two confirmed ('classical') cannabinoid receptors, CB_1 and CB_2, both of which have been identified as inhibitory G protein coupled receptors ($G_{\alpha i/o}PCR$), and were originally believed to localised to the CNS and immune tissue, respectively (Bayewitch *et al.*, 1995; Howlett *et al.*, 2002; Jarrahian *et al.*, 2004). Studies over recent years have confirmed the expression (at least mRNA) of CB_1 receptors in peripheral tissues such as liver, islet, adipose tissue and gastrointestinal tract (Juan-Pico *et al.*, 2006; Starowicz *et al.*, 2008; Izzo & Sharkey, 2010). CB_1 receptors are involved in a wide range of physiological systems such as the regulation of neuronal activity in the CNS by post-synaptic inhibition, nociception, and regulation of blood vessel dilation (as discussed in the review by Pacher *et al.* (2006)). The study by Liu *et al.* (2009a) has also reported the expression of CB_2 receptor mRNAs in non-immune tissues such as brain, kidney, and testes. However, the expression of CB_2

may have gone previously undetected as its expression may be inducible; for instance, the study by Viscomi *et al.* (2009) found that CB_2 expression by neurons in the cerebellum was only induced under conditions of neurodegeneration. In physiological and pathological conditions, CB_2 receptor signalling in the immune system has an immunosuppressive effect which is thought to be protective as it prevents excessive immune responses (see review by Patel *et al.* (2006)). CB_2 receptor signalling also serves a protective role in other tissues; in pathological states as it reduces inflammation and injury in the cardiovascular system, reduces liver fibrosis and stimulates neurogenesis in the CNS (as discussed in the review by Pacher *et al.* (2006))

CB_1 and CB_2 receptor activation is known to result in inhibition of adenylyl cyclase activity, and activation of mitogen-activated protein kinase (MAPK) signalling cascades (see the review by Bosier *et al.* (2010)). Additionally, CB_1 receptors are known to inhibit the activities of voltage-gated calcium channels but activate G protein-coupled inwardly rectifying potassium channels (see review by Turu & Hunyady (2010)). It should be noted that in several studies, it has been observed that some cannabinoids (typically AEA) have been found to inhibit ion channel activities independently of the CB_1 receptor (Shimasue *et al.*, 1996; Chemin *et al.*, 2001; Maingret *et al.*, 2001; Sade *et al.*, 2006).

Both CB_1 and CB_2 receptors have been described as possessing constitutive activity; this is primarily based on observations of CB-receptor antagonists having the opposite effect to CB-receptor agonists in studies *in vivo* and *in vitro* (this concept is discussed further by Pertwee (2003)). Briefly, if receptors have two states, dormant and active, the receptor can only switch to the active state upon binding of an agonist. The receptor will remain in the active state until the agonist dissociates from the receptor. In this model, antagonists block the binding of agonists to the receptor, preventing it from switching on, whereas receptors which are constitutively active can switch between the dormant and active states in the absence of any agonist. However, only

a portion of the constitutively-active receptors will be in the active state at any one time. Upon binding of an agonist, the active state of the constitutively-active receptor will be stabilised (i.e. it will not switch back to the dormant state) until the agonist dissociates from the receptor. An antagonist that blocks the binding of the agonist but does not affect either state of the receptor is referred to as a neutral antagonist, whereas an antagonist that also stabilises the dormant state of the receptor is called an inverse agonist. There is disagreement as to whether certain CB_1 and CB_2 receptors antagonists are inverse agonists; one such argument is that the constitutive CB receptor activity may be a result of low level endocannabinoid production. As discussed in the study by Savinainen *et al.* (2003), observations of inverse agonism by the putative CB_1 receptor antagonist/inverse agonist rimonabant, at micromolar concentrations, in cerebellar membranes may have been due to blockade of adenosine A_1 receptor signalling.

In addition to acting at cannabinoid receptors, it has been recognised that there may be additional receptors at which the endocannabinoids and endogenous cannabinoids exert their effects. In brain membrane preparations from CB_1 receptor KO mice, it was observed that AEA stimulated GTP binding in a concentration-dependent manner which was not affected by CB_1 or CB_2 receptor antagonism, thus, indicating that there was at least one extra uncharacterised CB-like GPCR expressed in mouse brain (Di Marzo *et al.*, 2000). Similarly, cannabinoid studies in the vascular system have also indicated that there is another cannabinoid-like receptor expressed by the endothelium aside from the CB_1 receptor (see review by Kunos *et al.* (2002)). This was first suggested by Wagner *et al.* (1999) as AEA-mediated vessel relaxation in mesenteric arteries did not appear to be CB_1 receptor-mediated and the effects of AEA were not reproduced by 2-AG or THC (Kunos *et al.*, 2002). The following sections describes several receptors which are either associated with cannabinoid signalling or are currently classed as putative cannabinoid receptors.

1.4.5 Peroxisome proliferator-activated receptors (PPARs)

There are three isoforms of PPAR receptors, α, γ, and β/δ (which have different physiological functions, different ligand selectivity and different tissues distributions), expressed by a wide range of tissues (Braissant *et al.*, 1996). The PPAR receptors when activated are known to heterodimerise with the retinoid X receptor, which bind to peroxisome proliferator response elements and recruit co-activators to form active transcription complexes (Desvergne & Wahli, 1999; Zoete *et al.*, 2007). Kozak *et al.* (2002) was the first study to suggest a link between the endocannabinoid system and PPAR receptors by observing that the 15-LOX metabolite of 2-AG was a PPARα agonist. As discussed in O'Sullivan & Kendall (2010), there is reasonable evidence to suggest that cannabinoid signalling can either directly or indirectly (via oxygenation by COX and LOX) agonise PPARα and/or PPARγ receptor. Data from several *in vitro* studies suggest that AEA (either directly or via COX-2 metabolism) may produce a significant PPARγ-mediated signalling event (Rockwell & Kaminski, 2004; Bouaboula *et al.*, 2005; O'Sullivan *et al.*, 2009).

Briefly, PPARα is typically expressed in tissues with high levels of fatty acid oxidation (e.g. skeletal muscle) and its activation results in the increased expression of genes required for fatty acid uptake and β-oxidation (see review by Ferre (2004)). PPARα receptors are targetted by the fibrates, which are PPARα agonists that are used in the treatment of dyslipidemia because they reduce plasma triglyceride levels, raise high density lipoprotein (HDL)-cholesterol levels, and have been found to have positive effects on CVD (Keech *et al.*, 2005). As discussed in the review by Stienstra *et al.* (2007), PPARα activation is also associated with anti-inflammatory effects. The expression of PPARβ/δ in rat tissue has been found to be wide-spread (Braissant *et al.*, 1996). PPARβ/δ function is the least understood amongst the PPAR isoforms but appears to be involved in the regulation of fat mass, type 1 skeletal fibre formation, cardiomyocyte growth as well as increasing fatty acid metabolism (see review by Wagner & Wagner (2010)). Two separate isoforms of PPARγ have been identified, PPARγ1 and PPARγ2, with PPARγ2 expression being most prevalent in adipose tissue (Tontonoz *et al.*, 1994). PPARγ signalling is a key step in the adipocyte

differentiation, regulates lipid deposition and also has anti-inflammatory actions (Leesnitzer *et al.*, 2002; Ferre, 2004; Stienstra *et al.*, 2007; Scheen *et al.*, 2009). As discussed in section 1.3.3, PPARγ agonists (the TZDs) are currently used in the treatment of type 2 diabetes as peripheral insulin sensitisers but may also exert benifical effects of β-cell function and activity too.

1.4.6 Transient receptor potential vallinoid type 1 (TRPV1)

The TRPV1 channel was first characterised by Caterina *et al.* (1997) and was originally called vallinoid receptor 1. TRPV1 channels were found to open in response to noxious heat and acidic pH, allowing the influx of positive mono- and divalent ions into TRPV1-expressing cells (Caterina *et al.*, 1997). TRPV1 was found to have highest permeability for Ca^{2+} ions, and its expression is limited to sensory neurons (Caterina *et al.*, 1997). Since this study, expression of TPRV1 channels has also been found in tissues such as brain, smooth muscle and lungs (Jara-Oseguera *et al.*, 2008). Zygmunt *et al.* (1999) was the first study to establish AEA as an endovallinoid as well as an endocannabinoid, as it was observed that AEA caused vasorelaxation in arteries via TRPV1 channels expressed by vessel-associated sensory neurons. As discussed in the review by Jara-Oseguera *et al.* (2008), TRPV1 activity is associated with various inflammatory disorders and pain perception but is also associated with protective effects in the cardiovascular system and gastrointestinal tract.

1.4.7 GPR119

The expression of GPR119 mRNA has been reported, albeit at low levels, in a large number of tissues including brain, heart, spleen and stomach (Soga *et al.*, 2005; Chu *et al.*, 2008). The highest levels of GPR119 expression has been reported in islets, β-cell lines, and intestinal L-cells (Sakamoto *et al.*, 2006; Chu *et al.*, 2008; Lan *et al.*, 2009). Evidence largely suggests that GPR119 is a $G_{\alpha s}$PCR as its activation leads to increases in cAMP levels and potentiates the secretion of insulin, GLP-1 and GIP from islets and L-cells, respectively (Soga *et al.*, 2005; Overton *et al.*, 2006; Chu *et al.*, 2007; Chu *et al.*, 2008). GPR119 has received interest with regard to cannabinoid

signalling as the NAEs, including AEA, have been reported as endogenous ligands (in order of potency, OEA> PEA > SEA> AEA), as have the lysophosphatidylcholines (Soga *et al.*, 2005; Overton *et al.*, 2006). However, the study by Lan *et al.* (2009) in which GPR119 KO mice were used, found that the effects of OEA and lysophosphatidylcholine on insulin secretion were similar in islets isolated from WT and KO mice. This may suggest that OEA and lysophosphatidylcholine were potentiating insulin secretion via non-GPR119 receptors.

1.4.8 GPR18

Expression of GPR18 has primarily been found in spleen, thymus, testis and leukocytes, with *N*-arachidonoyl glycine (NAGly, produced from AEA) and *N*-palmitoyl glycine being suggested as endogenous agonists for GPR18 (Gantz *et al.*, 1997; Rimmerman *et al.*, 2008; Bradshaw *et al.*, 2009). Evidence for NAGly as an endogenous GPR18 agonist is still controversial as studies in cell lines disagree as to whether NAGly possess significant GPR18 agonistic behaviour (Kohno *et al.*, 2006; Rimmerman *et al.*, 2008). Data suggest that GPR18 is a $G_{\alpha i}$PCR, the activation of which results in the influx of Ca^{2+} influx (Ikeda *et al.*, 2005; Kohno *et al.*, 2006; Rimmerman *et al.*, 2008). The physiological role of GPR18 remains unknown but there are suggestions that GPR18 may regulate sensory neuron activity and immune cell function (Rimmerman *et al.*, 2008).

1.4.9 GPR55

The expression of the GPR55 (mRNA) has been reported in a wide number of tissues, with highest expression levels reported in adrenal glands, brain and small intestine (Sawzdargo *et al.*, 1999; Ryberg *et al.*, 2007; Lauckner *et al.*, 2008; Waldeck-Weiermair *et al.*, 2008; Whyte *et al.*, 2009). The physiological effects of GPR55 signalling, thus far, are the regulation of bone mass and hyperalgesia (increased sensitivity to painful stimuli) in response to inflammatory and neuropathic pain (Staton *et al.*, 2008; Whyte *et al.*, 2009). Activation of GPR55 in natively-expressing cells has been suggested to lead to activation of RhoA (Ras homologue gene family, member

A) and Rho-Associated Kinase (ROCK) mediated by either $G_{\alpha 12}$ or $G_{\alpha 13}$ proteins (Lauckner *et al.*, 2008; Waldeck-Weiermair *et al.*, 2008; Henstridge *et al.*, 2009; Oka *et al.*, 2009; Whyte *et al.*, 2009). In addition, activation of GPR55 has also been linked to PLC, via G_q activation (Sawaki *et al.*, 1993; Iismaa *et al.*, 2000; Duttaroy *et al.*, 2004; Waldeck-Weiermair *et al.*, 2008; Henstridge *et al.*, 2009). While there is strong consensus that lysophosphatidylinositols are endogenous ligands for GPR55, there is controversy surrounding which cannabinoids also have GPR55 activity although there are more studies that suggest that the CB_1 receptor antagonists AM251 and rimonabant are GPR55 agonists than those that do not (Waldeck-Weiermair *et al.*, 2008; Kapur *et al.*, 2009; Oka *et al.*, 2009; Yin *et al.*, 2009; Henstridge *et al.*).

1.4.10 The endocannabinoid system with respect to insulin signalling and islet function

While the stimulatory effects of cannabis on appetite were widely-known before the discovery of the endocannabinoid system, it was not until studies such as Williams & Kirkham (1999) that endocannabinoid signalling was shown to induce hyperphagia. Given the rise in the prevalence of obesity, the development of appetite suppressors has received interest as a means of weight control. Therefore, research was (and still is being) conducted to assess whether CB_1 receptor antagonists could be used as viable anti-obesity treatments (Ravinet Trillou *et al.*, 2003; Pi-Sunyer *et al.*, 2006; Nogueiras *et al.*, 2008). This research subsequently led to the discovery that the endocannabinoid system, in particular CB_1 receptor signalling in the CNS and in the periphery, plays a central role in energy homeostasis (Ravinet Trillou *et al.*, 2003; Janiak *et al.*, 2007; Nogueiras *et al.*, 2008).

In a recent review by Andre & Gonthier (2010), the physiological role of the endocannabinoid system is to promote food intake and food assimilation while decreasing fatty acid oxidation and increasing lipogenesis. In obese states, the endocannabinoid system is believed to become overactive and contributes to dyslipidaemia and insulin resistance in hepatic, adipose and skeletal muscle tissues

(Andre & Gonthier, 2010). Overactivity of the endocannabinoid system in peripheral tissues will inadvertently affect β-cells, because greater amounts of insulin release will be needed from the β-cells in order to compensate the peripheral insulin resistance. Therefore, dysfunctional endocannabinoid signalling in non-islet tissues may indirectly contribute towards islet dysfunction in obese pre-diabetic states.

Review of the literature regarding the *in vivo* effects of cannabinoids on glucose tolerance and insulin secretion in humans is limited but it has been reported that injection of a 6mg dose of THC induces glucose intolerance (determined by 100g OGTT; Hollister & Reaven, 1974). This data appears to coincide with the study by Bermudez-Silva *et al.* (2006) that found that administration of AEA and ACEA (a CB_1 receptor agonist) prior to intraperitoneal glucose loading in Wistar rats also induced glucose intolerance. In a follow up study to Bermudez-Silva *et al.* (2006), it was found that administration of 2-AG and JWH-133 (a CB_2 receptor agonist) prior to intraperitoneal glucose loading in Wistar rat promoted glucose tolerance (Bermudez-Silva *et al.*, 2007). Therefore, this would suggest that THC in the study by Hollister & Reaven (1974) was acting as a CB_1 receptor agonist, assuming that the roles of the cannabinoid receptors in glucose homeostasis are conserved between rats and humans. Hollister & Reaven (1974) also measured changes in plasma insulin levels and found there was not a significant alteration in β-cell secretory activity. While it is uncertain whether THC induced glucose intolerance by peripheral insulin resistance, Hollister & Reaven (1974) did find a potentiation of plasma growth hormone levels, especially in the most glucose intolerant subjects, that corresponded with the time periods where plasma glucose were significantly higher than vehicle control data. This may suggest that excessive activation of CB_1 receptors in obese pre-diabetic states may contribute towards glucose intolerance by increasing hepatic glucose production and/or promoting peripheral insulin resistance.

Despite the observation by Hollister & Reaven (1974) that THC administration did not affect GSIS there are now several studies that have found that CB receptor activation,

in isolated islets and primary β-cells, affects insulin secretion. However, there is controversy regarding the effects of CB_1 and CB_2 receptor signalling on insulin secretion from islets and primary β-cells, which, does not appear to specie-dependent. Briefly, the studies by Juan-Pico *et al.* (2006) and Nakata & Yada (2008) suggest that CB_1 receptors are negatively coupled to insulin secretion, whereas, the studies by Bermudez-Silva *et al.* (2008), Li *et al.* (2010) and Vilches-Flores *et al.* (2010) have found that CB_1 receptor agonism potentiates insulin secretion. With regard to CB_2 receptor signalling, Juan-Pico *et al.* (2006) and Bermudez-Silva *et al.* (2008) both reported CB_2 receptor activation led to an inhibtion of insulin secretion whereas, data presented in the study by Li *et al.* (2010) suggests that the CB_2 receptor activation potentiates insulin secretion.

With regard to endocannabinoid dysfunction in islets, the study by Matias *et al.* (2006) reported that AEA and 2-AG were produced in RIN-m5F cells (a glucose unresponsive, insulin-secreting β-cell line). It was found that 2-AG biosynthesis by RIN-m5F cells cultured at 25mM glucose for 24 hours (compared to cells cultured at 13mM glucose) was upregulated in response to 33mM glucose. A subsequent study by the same group also found that PEA and OEA were produced by RIN-m5F cells, and that regulation of their production was also affected by hyperglycaemia (Matias *et al.*, 2007). Several studies have now been conducted regarding the expression of endocannabinoid synthesising and metabolising enzymes in islets and islet cell lines, which are detailed in Table 1.4.

Matias *et al.* (2006) also reported that CB_1 receptor-signalling in RIN-m5F cells kept at 25mM glucose for 24 hours (compared to cells cultured at 13mM glucose) potentiated insulin secretion indicating that CB_1 receptor signalling may have also become dysregulated as a result of hyperglycaemia. Therefore, the results from Matias *et al.* (2006) may indicate that dysregulation of the endocannabinoid system in islets contributes to β-cell dysfunction, suggesting that correction of endocannabinoid dysfunction in islets may become a future treatment of type 2 diabetes. As described

Table 1.4 Expression of endocannabinoid synthesising and degrading enzymes in islets and β-cell lines

Study	Techniques used	Resolution	Cell-line/tissue used	NAPE-PLD		FAAH		DAGLα and DAGLβ		MAGL	
				Detected	Cell specific distribution	Detected	Cell specific distribution	Detected	Cell specific distribution	Detected	Cell specific distribution
Matias *et al.* (2006)	RT-PCR	N/A	RIN-m5F β-cell line	Y		Y		Y		Y	
Starowicz *et al.* (2008)	RT-PCR IHC	β non-β	C57BL/6J mouse islet	Y	Non-β ++ β +	Y	β	α Y β N	α	Y	β
Tharp *et al.* (2008)	IHC	α, β, δ	ZF rat islet	ND		Y	α ++ β +	ND		Y	δ
			Human islet	ND		Y	α ++ β +	ND		?	
Bermudez-Silva *et al.* (2008)	Western blotting	Whole islet	Human islet	N		Y	B	α Y β Y		Y	

For RT-PCR (reverse transcriptase polymerase chain reaction) and western blotting whole islet sample were used. For western blotting and immunohistochemistry (IHC) enzyme specific antibodies were used and for immunohistochemistry cell specific distributions were confirmed by hormone-specific antibodies. Y expression detected, N expression not detected, ND not determined, ? Authors reported unreliable staining, ++ refers to high levels of expression detected in the cell type, + refers to low levels of expression detected in the cell type.

above (Section 1.4.5), cannabinoids are also known to be capable of PPARγ receptor-mediated signalling thus, the apparent dysfunction in β-cell endocannabinoid production may instead be a positive adaptation of the endocannabinoid system to increase β-cell survival in glucotoxic conditions. Again, this may suggest that manipulation of the endocannabinoid system might offer benefits in the treatment of type 2 diabetes.

1.5 Aims and Objectives

The role of the endocannabinoid system is well-recognised in the CNS and immune system but its role in glucose homeostasis is poorly understood. The principal aim of this study was to investigate the role of cannabinoid receptors and endogenous cannabinoids in the acute control and modulation of insulin secretion. Once the acute effects of cannabinoid signalling was established, the chronic effects of PPARγ receptor and cannabinoid signalling on islet function and insulin secretion were to be characterised under conditions similar to those found in type 2 diabetes.

To achieve these aims, rat isolated islets and β-cell lines were used as model systems. The initial stages of study focused on the use of the endocannabinoids and CB-receptor (CB_1- and CB_2-receptor specific) antagonists to pharmacologically characterise the acute effects of cannabinoid signalling on insulin secretion. A FAAH inhibitor was used to ascertain the effects of local metabolism either alone or with additionally applied endocannabinoids.

This study aimed to define the roles of cannabinoids in insulin secretion and provide insights into their therapeutic potential in the treatment or limitation of impaired β cell function in Type 2 diabetes.

2

Materials and Methods

2.1 Animals

Wistar and Zucker rats were purchased from Charles River UK (Kent, UK) and kept in-house for at least 4 days prior to experimentation. Animals were housed at 20-22ºC and kept in a standard 12-hour light cycle (on 7am: off 7pm). Water and standard rat chow (Taklad Global ~70% carbohydrate; Harlan, WI, USA) were available *ad libitum*. Animals were either killed according to Schedule 1 killing practices by asphyxiation (using CO_2) or stunning followed by cervical dislocation or decapitation.

2.2 Materials

Bovine serum albumin (BSA; Fraction V) and collagenase P (from *Clostridium histolytiam*, non-sterile) were purchased from Roche Diagnostics (Mannhein, Germany). Sac-Cel (Donkey anti-guinea pig secondary Ab coupled to cellulose) was purchased from Immunodiagnostic Services (Bolton, UK). Goat anti-guinea pig anti-serum was purchased from Equitech-Bio Inc. (Texas, USA). AEA (*N*-(2-Hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), R-(+)-methanandamide ((R)-*N*-(2-Hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), O-2050 ((6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran), ACEA (*N*-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), JWH-133 ((6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran), AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone), GW9662 (2-Chloro-5-nitro-*N*-phenylbenzamide), and BIM 23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2) were purchased from Tocris (Bristol, UK). Guinea-pig anti-bovine insulin anti-serum, RPMI-1640 media, URB597 (cyclohexylcarbamic acid 3´-carbamoyl-biphenyl-3-yl ester), somatostatin-14 and LPI (L-α-lysophosphatidylinositol) sodium salt (from *Glycine max)* were purchased from Sigma-Aldrich (Dorset, UK). [^{3}H]-AEA was

purchased from American Radiolabeled Chemicals (MO, USA). Any other chemicals used were of reagent grade.

AEA, methanandamide, and ACEA solutions were purchased as 5mg/ml solutions pre-dissolved in ethanol. O-2050, GW9662, JWH 133, and URB597 were first dissolved in 100% ethanol to produce 1mg/ml stock solutions. AM251 and AM630 1mg/ml stock solutions were prepared by dissolving the drug in DMSO. LPI was dissolved in ddH_2O to produce a 10mM solution. A 1mM somatostatin-14 stock was prepared by dissolving the peptide in 10% (v/v) acetic acid/ 1% (w/v) BSA. BIM 23056 was first dissolved in 10% (v/v) DMSO solution to produce a 1mg/ml solution. All drug stock solutions were kept at -20ºC. The stock solutions that were either made up in DMSO or water (i.e. frozen when stored at -20ºC) were aliquoted in order to order to minimise degradation from repeated freeze/thaw cycles. Where appropriate, drug stocks were diluted to the required concentration by serial dilution, with no greater than a 10-fold dilution per step. If the concentration of the stock solution was < 1mM, then solutions were diluted in ddH_2O. In the case of AEA, methanandamide and ACEA, where serial dilution steps used stock solutions at concentrations ≥ 1mM, then 50% (v/v) ethanol solution was used instead of ddH_2O to avoid emulsion formation.

2.3 Buffers and cell culture media

2.3.1 Gey & Gey buffer

$NaCl_2$ 111mM, $NaHCO_3$ 25.2mM, KCl 5mM, $MgCl_2$ 1mM, $MgSO_4$ 0.3mM, Na_2HPO_4 0.4mM, KH_2PO_4 0.3mM and $CaCl_2$ 1mM. Gassed for 10 minutes with 95% O_2/ 5% CO_2 at the start of the experiment to oxygenate the buffer and adjust the pH of the buffer to 7.4. Gey & Gey buffer was periodically re-gassed to ensure the pH of the buffer was maintained at 7.4.

2.3.2 Insulin Assay Buffer (IAB)

Na_2HPO_4 15.9mM, KH_2PO_4 9.9mM, NaCl 154mM, EDTA 10mM and BSA 5% (w/v). Buffer pH is 6.5.

2.3.2 RPMI-1640 media

RPMI-1640 media were supplemented with 5% (v/v) foetal calf serum, 2mM glutamine, 10mM HEPES, 1mM sodium pyruvate, 50μM 2-mercaptoethanol and either 5mM or 11.1mM glucose.

2.4 Insulin secretion studies

Insulin secretion can be studied by various methods which have been reviewed in Table 2.1. The use of isolated exocrine-free islets, as in this study, is advantageous as the effects of treatments on insulin secretion directly reflect alterations in islet cell activities. A limitation of this approach is the potential damage to cell surface proteins during the isolation procedure which could affect islet function. This can be circumvented by culturing the islets to allow protein expression recovery. The isolated islets will not solely consist of the islet endocrine cells but the islets may also contain endothelial cells as islets are highly vascularised (Weaver & Sorenson, 1989; Olsson & Carlsson, 2006).

2.4.1 Islet preparations

As the islets are interspersed throughout the pancreas, they first need to be isolated from the surrounding exocrine tissue before any isolated islet experiments can be performed. A two-step method employing collagenase digestion of pancreata is a commonly used for the isolation of islets. Collagenase enzymes are either applied in solution to chopped tissue or injected via the pancreatic bile duct (van Suylichem *et al.*, 1992). The collagenase enzymes specifically hydrolyse the collagen fibres which connect the endocrine and exocrine tissues together. However, care must be taken with collagenase enzymes as given enough time the enzymes will degrade the

intercellular proteins which form physical connections between the islet cells, causing the islets to disperse. Both methodologies require an isolation step to obtain exocrine-free islets. This is achieved either by manually picking the islets or through the use of density gradient purification techniques (van Suylichem *et al.*, 1992; Wang & Rosenberg, 1999).

While collagenase-based islet isolation techniques have been used for several decades, it is still not fully understood why preparations are more efficient in some species compared to others. The efficacy of islet isolation by collagenase is in part mediated by the peri-insular cap (Section 1.2). For instance, rat islets are mostly covered by a peri-insular cap and high yields are obtained from collagenase-based isolation techniques (van Deijnen *et al.*, 1992), whereas porcine islets have poor coverage and islet yields by collagenase digestion are poor (van Deijnen *et al.*, 1992). However, canine islets are fully covered by the peri-insular cap but are associated with intermediate yields indicating that other aspects of islet structure also contribute to efficacy of collagenase based islet isolation techniques (van Deijnen *et al.*, 1992).

It must be noted that there are several complications in isolating large quantities of islets by collagenase digestion especially as an estimated 50% of islets are lost in the most efficient of islet preparations (van Suylichem *et al.*, 1992). Commercial collagenases contain contaminating protease activity from other proteases, such as trypsin, which may affect islet activity by hydrolysing extracellular regions of cell surface proteins. In addition, proteases and other digestive enzymes are also released from the exocrine tissues which may further add to cell damage and reduction in islet yields. In this study, it was also found that the age of the rat was also a confounding factor as the older rats (with body weights > 300g) generally had more fibrous pancreata than younger rats (with body weights ≤ 300g). Fibrosis of the pancreas is known to occur as a result of aging (Reaven *et al.*, 1979) and can also be caused by hyperglycaemia (see review by Czako *et al.* (2009)). This had a negative

Table 2.1 Different cell model systems used to study insulin secretion.

System	Tissues which may affect insulin secretion					Insulin secretion	
	Neuronal[1]	Peripheral[2]	Exocrine	Endothelial[3]	Paracrine	Measurement[4]	Kinetics
In vivo	Y	Y	Y	Y	Y	Direct	Y
Perfused pancreata	Y[5]	N	Y	Y	Y	Direct	Y
Isolated islets	N	N	N	Y	Y	Direct/indirect	Y[6]
Primary β-cells	N	N	N	N	N	Direct[7]/Indirect	Y[8]
β-cell line[9]	N	N	N	N	N	Direct[7]/Indirect	Y[8]
Pseudo islets[9,10]	N	N	N	N	N	Direct[7]/Indirect	Y[6]

[1] Neuronal refers to parasympathetic, sympathetic and sensory as well as other nerve types which do not fall into these categories but also associated with islets
[2] Peripheral tissues include the liver and adipose tissues
[3] Endothelial refers to the endothelial cells which are part of the capillaries which permeate the islets
[4] Measurement refers to the manner in which insulin secretion is typically measured in studies which employ the specified system. Direct refers to quantification of insulin (e.g. radioimmunoassay) and indirectly refers to a qualitative measure of insulin secretion (e.g. changes in intracellular Ca^{2+})
[5] This is based on study by Park *et al.* (1999) which found that severed neurons could be activated by electrical field stimulation.
[6] Based on the use of islets in a perifusion system.
[7] This is based on dispersed β-cells being used in reverse haemolytic plaque assays (Malaisse *et al.*, 1998).
[8] Based on a technique described in Ashcroft *et al.* (1987) where dispersed β-cells were kept inbetween two layers of gel, which were porous enough to allow solutes (including insulin) to pass through but not the β-cells, in a perifusion system.
[9] Based on the assumption that a β-cell line is used alone and not co-cultured with non-β cell lines
[10] This is based on the studies such as by Brenner & Mest (2004) which formed islet-like structures from islet cell lines by suspension culture of the cells.

effect as pancreata from older/larger animals, at times led to inefficient handling of the tissue (distension, chooping; see later) and subsequently uneven digest.

2.4.1.1 General Procedure

The method used for the isolation of islets described below is based upon the protocol described in Howell & Taylor (1968). The protocol uses Gey & Gey buffer (Section 2.3.1) which is a bicarbonate-buffered physiological saline solution and has been supplemented with 4mM glucose for use in this protocol.

Either one or two pancreata were used per preparation. Once the pancreas had been isolated, further cleaning was required to remove contaminating tissue from the pancreatic tissue, including spleen, adipose tissue, lymph nodes and large blood vessels. The remaining tissue was then thoroughly distended using a needle and syringe to inject Gey and Gey buffer into the tissue. This step is important as it physically separates the tissues and allows oxygenation of the tissue. The tissue was then roughly chopped into ~1mm^3 pieces and transferred to a conical tube, where it was centrifuged (Eppendorf Centrifuge 5810R) at 3000rpm for 3 seconds in 12ml of buffer. The supernatant was poured off and the pellet was transferred to a 25ml plastic conical flask (Nalgene, NY, USA), to which collagenase P (~2.4mg collagenase per pancreas) and 1-2 equivalent volumes of buffer to tissue were added. The mixture was then shaken vigorously (~200 shakes/minute) at 37°C for ~5-6 minutes using a Griffin flask shaker (Griffin and George Ltd., UK). The conical flask was then hand-shaken until the digest was smooth and homologous in appearance. Samples of the digest were taken periodically during hand-shaking and viewed under a dissecting binocular SZ4045 microscope (Olympus, Essex, UK). The digestion procedure was terminated when samples of the digest contained a majority of exocrine-free islets. The digest was then spun for a further 3 seconds at 3000rpm, the supernatant was then poured off and the pellet resuspended in 10ml of buffer. Exocrine-free islets were then manually isolated from the digest and transferred to a conical tube containing buffer, using a drawn-out Pasteur pipette under a dissecting binocular microscope. Typical preparations yielded between 75-180 islets per

pancreas. Islets isolated from Wistar rats typically had a uniform morphology as the islets were white in colour and were spheroid shape. For this reason, islets were picked in a black coated-Petri dish as islets could be easily distinguished from exocrine tissues which are diffuse in appearance and grey in colour except for acinar bodies. Acinar bodies have a similar morphology to islets but are smaller in size, are brighter in appearance and have a slight blue colouration.

2.4.2 Islet static incubation studies

The buffer used for these studies (Gey & Gey buffer; Section 2.3.1) was supplemented with 4mM glucose and 1% (w/v) BSA. Initially, LP3 tubes were used for the islet incubation experiments but 96-well plates were used for the majority of the islets studies as they were more convenient. The following protocol describes the use 96-well plates. For conditions where higher glucose concentrations were required glucose solution was used to adjust stocks of 4mM glucose Gey & Gey buffer. The adjustment of the buffer glucose concentration was performed prior to addition of the buffer to wells.

Two microlitres of drug stock solution (at 100x the final concentration) were first added to the wells followed by the addition of 200µl buffer (at the required glucose concentration). Batches of 3 islets were carefully added to each well using a drawn-out Pasteur pipette; extra care was taken to avoid the addition of buffer to the well. The 96-well plates were then incubated at 37ºC in a 95% O_2 /5% CO_2 atmosphere using a Sanyo CO_2 incubator, with an incubation period of 60 minutes. An additional 5 minutes were added to the incubation period to allow buffer to warm to 37ºC. Where LP3 tubes were used instead of 96-well plates 5µL of 100x drug stock and 500µl of buffer was added to each tube. After the addition of the islets the tubes were briefly gassed with 95% O_2 / 5% CO_2, capped and then incubated at 37ºC for 60 minutes in a water bath. At the end of the incubation period, the 96 well-plate was agitated to allow mixing and transferred to a fridge/freezer for 5 minutes to inhibit further insulin release. Samples of the incubation media were then taken from each well and the

insulin content was determined by radioimmunoassay (RIA; Section 2.6.1) or samples were stored at -20ºC for later use. Insulin secretion rates were determined as ng of insulin secreted/islet/hour.

2.4.2.1 Basic experimental setup

In all experiments, islets were incubated at 4mM and 20mM glucose in order to assess for islet viability. These two concentrations represent basal and maximal levels of glucose-stimulated insulin secretion, see Figure 2.1. The islets were deemed viable (i.e. the islets are functioning normally) if there was a ≥ 2-fold difference in insulin secretion rates between 4mM and 20mM glucose. All experiments, unless stated otherwise, were compiled from a *n* of ≥ 5 experiments (performed with separate islet isolations). Compilation and statistical testing of experimental data were carried out as described in Section 2.4.5.

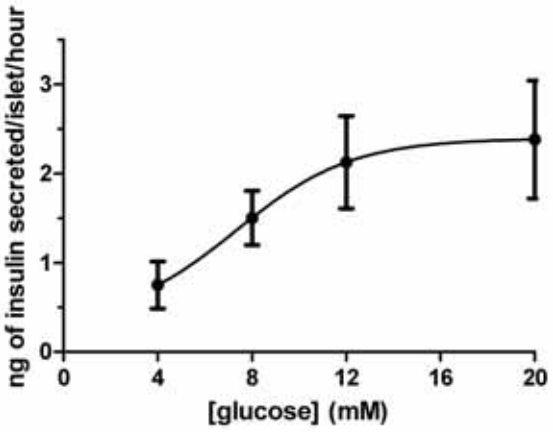


Figure 2.1 The effects of glucose on insulin secretion from Wistar rat isolated islets of Langerhans. Islets were incubated at the indicated glucose concentrations for 1 hour at 37ºC. Results shown are the mean insulin secretion rate ± 1 SD from a single experiment. n = 4-6 replicates per condition.

2.4.3 INS-1 823/13 β-cell secretion studies

INS-1 823/13 cells were used to assess whether cannabinoid signalling was directly affecting β-cell activity. INS-1 823/13 cells were seeded in 48-well plates at a density of $5x10^4$ cells per well in 0.5ml of RPMI-1640 media containing 11.1mM glucose (Section 2.3.3) and were kept under a 95% O_2/5% CO_2 atmosphere at 37 ºC until cells reached 70% confluency. 18 hours before experimentation, the RPMI-1640 media

was changed to RPMI media containing 5mM glucose. The glucose concentration of the media was reduced prior to experimentation to improve the glucose responsiveness of the INS-1 823/13 cells.

On the day of the experiment, the RPMI-1640 media was aspirated off, 1ml of pre-warmed Gey & Gey buffer (Section 2.3.1) was added to each well and then aspirated off. This washing step was repeated twice more and then 0.5ml of pre-warmed Gey and Gey buffer, supplemented with 3mM glucose and 1% (w/v) BSA, was then added to each well and the plate was incubated at 95% O_2/5% CO_2 atmosphere at 37 ºC for 30-60 minutes. Once this time period had elapsed, the media was then aspirated and 0.5ml of pre-warmed 1% (w/v) BSA supplemented Gey and Gey buffer (with the required amount glucose) was added to each well. 5µl of drug (at 100x the required concentration) or appropriate vehicle solution was added to each well as required. The plate was then gently agitated and then incubated at 95% O_2/5% CO_2 atmosphere at 37 ºC for 1 or 2 hours. Once the time period had elapsed, the plate was agitated and then transferred to a fridge/freezer for 5 minutes. Then, a 0.25ml samples were taken from each well to determine the amount of insulin secreted by RIA. Insulin secretion was determined as ng of insulin secreted/islet/hour.

Glucose (3mM) was used to determine basal insulin secretion rates for INS-1 823/13 cells instead of 4mM glucose which was used for islets. Compilation, normalisation and statistical testing of experimental data were carried out as described in Section 2.4.5.

2.4.4 Islet perifusion studies

The buffer used for the islet perifusion studies was Gey & Gey buffer supplemented with 4mM glucose and 1% (w/v) BSA (fraction V) which was kept under a 95% O_2/5% CO_2 atmosphere. The perifusion system was housed in a Stuart Scientific S160D incubator (Staffordshire, UK) and the temperature was kept at 39ºC for the duration of the perifusions to maintain the islets at 37 ºC. Batches of 65-75 islets were loaded into glass wool plugged chambers. The chambers were perifused with buffer at a rate of

1ml of buffer per minute using a Minipuls 3 peristaltic pump (Gilson, USA). The glucose concentration was adjusted by addition of an appropriate volume of 1M glucose stock solution to the perifusion buffer reservoir (the volume of glucose solution added was dependent on reservoir volume). Vehicle or drug (at 100x the working concentration) was infused at a rate of 10µl/minute by a second Minipuls 3 peristaltic pump. Figure 2.2 shows a simplified diagram of how the equipment was arranged. The islets were equilibrated for 20-40 minutes (once the temperature had stabilised) before the start of each experiment, after which the media was collected in LP3 tubes in 2 minute fractions. The fractions were either used immediately for RIA or stored at -20ºC for assaying at a later time point. Insulin secretion rates were determined as pg of insulin secreted/islet/minute.

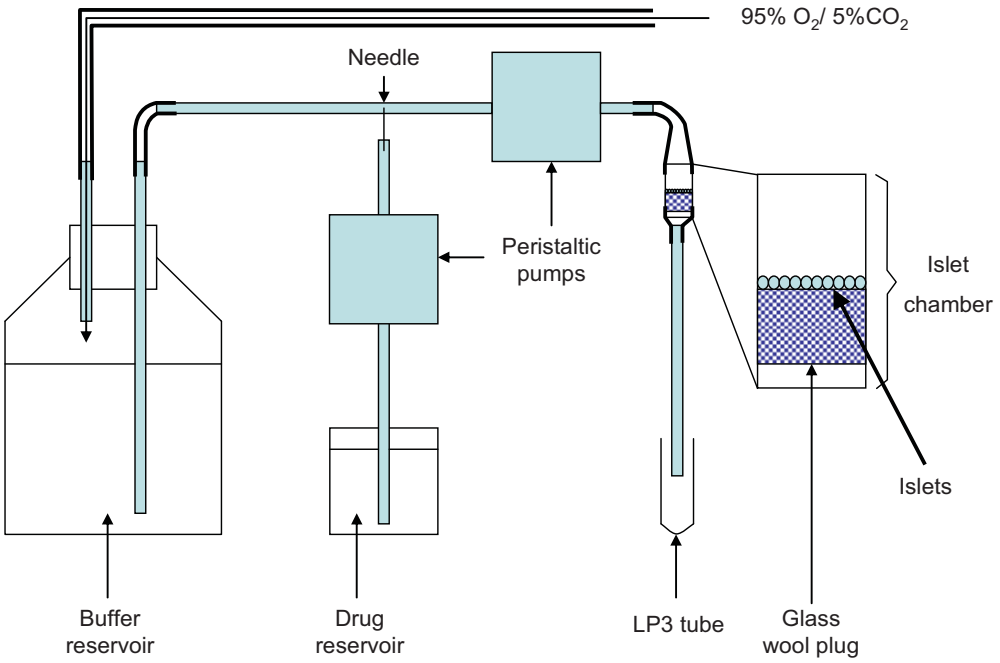


Figure 2.2 A diagrammatic representation of the perifusion equipment. Buffer is peristaltically pumped from the buffer reservoir, through the islet chamber and collected in LP3 tubes. Drug is peristaltically pumped from the drug reservoir and infused into buffer line. For simplicity, multiple islet chambers and lines (with individual drug/vehicle reservoirs), and the S106D incubator (in which the equipment was housed) were excluded from the diagram. For the duration of the experiment, the buffer reservoir was kept under a 95% O_2/5% CO_2 atmosphere. Buffer was pumped (at a rate of 1ml/minute) from the reservoir to the islets and then collected in LP3 tubes. When appropriate, the glucose concentration of the buffer was adjusted by the addition of 1M glucose solution directly to the reservoir which was then agitated to ensure thorough mixing. When required drug stock (or vehicle control) was infused at 100 times the required concentration, by peristaltic pump at a rate of 10µl/minute, upstream of the islets through the buffer line which fed to the appropriate islet chamber.

2.4.5 Data analysis

For pharmacological studies (e.g. use of endogenous compounds/receptor agonists/receptor antagonists at fixed concentrations), the mean insulin secretion rate were determined for each group (both control and test groups). Outliers were removed based on objective judgement to reduce variation (standard error of the mean ≤ 10, if possible) and was based on the following rules: each group within the experiment were considered separately, no more than half the replicates were removed with no less than three replicates left per condition; the mean insulin secretion rates within the experiment were then standardised against the 20mM glucose control group (unless stated otherwise). The mean insulin secretion rate for each condition, from each individual experiment, was then averaged again to give the overall mean insulin secretion rate. Mean insulin secretion rates were expressed as a percentage of the 20mM glucose control (which was always 100%). Variation was expressed as the mean ± 1 SD unless stated otherwise. One-way ANOVA analysis was used in co-junction with Tukey's *post-hoc* testing. For experiments where the glucose-dependent effects of a compound were being tested all conditions were included in the statistical analysis. However, for studies where the effects of treatment/s were exclusively examined at 20mM glucose, the 4mM glucose control was not included in the statistical analysis because it was only used to asses islet viability. For all experiments a P value < 0.05 was considered significant.

Firstly for concentration-response curves, different methods were used to establish IC_{50} values, and to produce the figures showing the average rate of insulin secretion at 20mM glucose at all drug concentrations. The IC_{50} value refers to the concentration of drug required to cause a 50% inhibition of the variable being examined. IC_{50} values were first calculated for each experiment with the raw insulin secretion data using Prism 5.0 software by the following method (GraphPad software Inc., CA, USA). The drug concentrations were then converted to logarithms. The data were then normalised using the built-in functions, whereby the smallest value in each data set was set to 0% and the largest to 100%. The $\log_{10}IC_{50}$ for each experiment was

calculated using the built-in non-linear regression for variable slope analysis (log[inhibitor] *vs.* normalised response). The equation for the variable slope analysis was Y=100/(1+10^(($Log_{10}IC_{50}$-X)*HillSlope))). The fitting method for the non-linear regression for variable slope analysis was the least squares ordinary fit and no constraints or weighting methods were used. Data from these experiments were expressed as the antilog of the mean $log_{10}IC_{50}$ value with the 95% confidence intervals (95% CI). The 95% confidence intervals were calculated from the antilog pIC_{50} values using the built-in PRISM column analysis feature. The figures used to display concentration-response data were compiled from 20mM glucose control normalised data in the same way as the basic pharmacological studies but were not used for calculation of IC_{50} data. Variation is expressed as mean ± 1 SD. Statistical significance ($P< 0.05$) was determined from all the data sets performed at 20mM glucose by one-way ANOVA analysis with Dunnett's *post-hoc* test using the 20mM glucose control as the control group.

Post-hoc tests were only used in conjunction with one-way and two-way ANOVA analysis when the ANOVA analysis determined that there was significant variation between the means. Where significant differences were found ($P< 0.05$), only comparisons of interest were displayed/reported in the results chapters. For example, if the insulin secretion rates were significantly different ($P< 0.05$) between the 8mM glucose control and the "20mM glucose + agonist" groups, this information was not included because the conditions were not directly comparable.

2.4.5.1 Justification for the statistical analysis tests used

The data are typically expressed as a percentage of the mean insulin secretion rate of the 20mM glucose control (unless stated otherwise). The data were normalised to reduce the amount of inter-experimental variation, as the difference between basal and maximal secretion insulin rates can vary between two to tenfold which further contributes to variation within the data sets. This variation can have a profound effect as a treatment may have a consistent effect inter-experimentally (e.g. 50% inhibition

at a specific glucose concentration compared to control) but this is not apparent in the compiled un-normalised data. By normalising the data, the variation is reduced but this does not result in a loss of data as the relative changes in insulin secretion rates are more informative than the changes in quantity of insulin released. Indeed, no external controls were included in the radioimmunoassay, meaning there was no inter-assay standardisation; it cannot be assumed that the assays estimated insulin levels to a comparable degree. The normalised data was then entered into Prism 5 software (Graphpad, CA, USA) for statistical analysis.

As data sets were standardised to a control, the use of non-parametric testing would have been statistically appropriate as normal distribution of data cannot be assumed as small experimental n numbers were used. Yet non-parametric testing has lower statistical power compared to parametric testing and use of non-parametric testing may have resulted in higher rates of type 2 statistical (false negative) errors. It was assumed that insulin secretion rates show normal distribution (as observed in most biological systems) and this was used to justify the use of parametric testing instead the use of non-parametric testing.

2.5 Endocannabinoid metabolism assay

The endocannabinoid metabolism assay is based on the methods described by Holt *et al.* (2005) and Boldrup *et al.* (2004). AEA consists of a hydrophilic ethanolamine head group covalently attached to a hydrophobic arachidonic acid (AA) molecule. When the [^{3}H]-AEA is hydrolysed, the [^{3}H]-ethanolamine resides in polar phases, whereas the liberated AA moiety and non-hydrolysed [^{3}H]-AEA will reside in non-polar phases. By measuring the level of β-radiation emitted from samples from the hydrophilic phase then the level of AEA hydrolysis can be determined by the use of internal controls included in the assay and the quantity of AEA added.

2.5.1 Pancreatic and liver tissue protocol

The buffer used for the preparation of the tissue homogenates was Gey & Gey buffer (Section 2.3.1) supplemented with 4mM glucose and 1% (w/v) BSA. Pancreatic and liver tissue (taken from the small liver lobe which was situated at the bottom of the stomach and behind the duodenum) were taken from the same male Wistar rat and were immediately placed in buffer on ice. Contaminating tissue from the pancreatic tissue including the spleen, adipose tissue, lymph nodes and large blood vessels was removed and the pancreatic tissue was placed back into buffer. The amount (wet weight) of tissue to be homogenised was weighed, after which liver tissue was transferred to a 1.5ml microfuge tube and pancreatic tissue was transferred to a 25ml universal tube. To each tube, ice cold fresh buffer was added until a pre-determined volume was reached. The tissues were then homogenised using an Ultra Turrax T8 homogeniser (Ika, Staufen, Germany) on the fourth setting for 10-30 seconds on ice until smooth and homogeneous in appearance.

The buffer used for the endocannabinoid metabolism assays was Gey & Gey buffer supplemented with 4mM glucose and 1% (w/v) BSA. Each condition consisted of three replicates of 190µl of tissue homogenate in 6ml polypropylene scintillation tubes (Sarstedt, Leicester, UK) and two additional scintillation tubes that contained 190µl of the Gey & Gey assay buffer which were used as controls (non-enzymatic AEA hydrolysis and total radiation). Where appropriate, 3µl of 1M glucose and/or 2µl of 100µM URB597 were added to conditions in order to achieve a glucose or URB597 concentration of 20mM and 1µM, respectively. The homogenates were then incubated and agitated for 15 minutes at 37ºC using a shaking water bath. Once the 15 minutes had elapsed, then 10µl of 40µM [^{3}H]-AEA solution was added to each condition to give a final AEA concentration of 2µM. The 40µM [^{3}H]-AEA solution was prepared by the addition of 2µl [^{3}H]-AEA to 200µl of non-radiolabeled 40µM AEA solution (to give ~20 000cpm/µl). The tubes were then further incubated and agitated for 30 minutes. At the end of the second incubation period, 400µl of 0.5M HCl solution with 4% (w/v) charcoal was added to each sample and one of the controls (non-enzymatic AEA hydrolysis). For the total radiation control, 400µl of 0.5M HCl solution (without

charcoal) was added. Each tube was then vortexed and left to stand. After 30 minutes, the tubes were then centrifuged at 3000rpm for 5 minutes using a Centuar 2 centrifuge (MSE, London, UK). 200µl of the supernatant was then transferred to fresh scintillation tubes. To each tube, 3ml of scintillation fluid (PerkinElmer, MA, USA) was added and the β-radiation levels for each tube were counted for 3 minutes using a Packard 1900TR liquid scintillation analyser (Perkin-Elmer, MA, USA). AEA hydrolytic activity was expressed as pmol of AEA hydrolysed per minute per mg of wet weight tissue.

2.5.2 Islet protocol

The pancreatic and liver tissue protocol was altered in the following ways for use with islets. 190µl of buffer at the required glucose concentration were added to 1.5ml microfuge or scintillation tube. To this, 3-20 islets were added to each tube. URB597 was used at a final concentration of 10µM and islets were pre-incubated for 30 minutes. This was done in order to validate, the URB597 pre-incubation protocol described in Section 3.2.3.1. All other variables were kept constant. AEA hydrolytic activity was expressed as pmol of AEA hydrolysed per minute per islet.

2.6 Radioimmunoassay

Radioimmunoassays are competition based assays that allow the quantification of ng levels of protein. The assays utilise antigen-specific (e.g. anti-insulin) antibodies, which will bind to a target ligand (referred herein as antigen) with high specificity (but not irreversibly) to form an antibody-antigen complex. In radioimmunoassays, a sample consists of a fixed volume of sample containing either a known or unknown amount of non-radiolabeled target (“cold”) antigen, to which fixed amounts of radiolabeled (“hot”) antigen and a limited amount of antibody are added in each assay sample. The samples are then left for a set amount of time to allow a stable equilibrium (in the ratio of antibody bound “hot” and “cold” antigen) to form. At the end of the equilibration period, a separation step is performed to separate antibody-

antigen complex from unbound reagents. Typically, this involves the precipitation of an antigen-antibody complex, which can be separated from the unbound “hot” and “cold” antigen by centrifugation to form a pellet. The amount of radioactivity in the pellet will be dependent on the ratio of “cold”-to-“hot” antigens. Thus, the level of radioactivity in the pellet will be inversely proportional to the concentration of “cold” antigen. Standard samples, of known amounts of “cold” antigen, are included in an assay to allow a standard (also known as a calibration) curve can be constructed, where concentration of the cold antigen (x -axis) is plotted against radioactivity (y-axis). From this standard curve, the concentration of antigen in a sample can be estimated by the amount of radioactivity in the antigen-antibody complex.

2.6.1 Insulin RIA

All solutions were made using the insulin assay buffer (IAB, Section 2.3.2). Recombinant human insulin was used as the standard and a stock was initially prepared in 10% (v/v) acetic acid/ 1% (w/v) BSA at a concentration of 1mg/ml. The standard curve consisted of insulin standards (0.125-8ng/ml) constructed by two-fold serial dilution of the 8ng/ml standard in IAB. The standard curve also includes non-specific binding and total radioactivity controls. All samples and standards were assayed in duplicate. 50µl sample or standards were incubated with 50µl of 1:6000 guinea pig anti-bovine insulin anti-serum and 50µl of ^{125}I labelled insulin (to give approximately 2500cpm/tube), at 4ºC, overnight.

Two different methods were employed to separate the primary antibody from solution. The first method used 50µl of 1:1 Sac-Cel solution(Donkey anti-guinea pig secondary Ab coupled to cellulose : IAB), which was added to each assay sample, mixed and left for 20-30 minutes at room temperature before 1ml ddH_2O was added to each tube and the tubes were then centrifuged at 4000rpm (Eppendorf Centrifuge 5810R) for 5 minutes at room temperature. The supernatant was then carefully aspirated off, making sure not to disturb the pellet. The second method used 50µl of 1:10 goat anti-guinea pig secondary antibody, the tubes were mixed and then incubated at 4ºC for

10 minutes. 500µl of 5% (w/v) PEG (molecular weight 8000) solution to the tubes which were then further incubated for a further 2-4 hours at 4ºC. The PEG solution acts as a precipitating agent for the secondary-primary antibody complex which would otherwise normally need an additional overnight incubation period (Peterson & Swerdloff, 1979). The PEG solution also contains a small quantity of Brilliant Blue (Sigma Aldrich; Dorset, UK) which is used to visually identify the pellet. The samples were then centrifuged at 3000rpm (Eppendorf Centrifuge 5810R) for 15 minutes at 4 ºC and the supernatant was then carefully aspirated off, making sure not to disturb the pellet.

The amount of γ radiation being emitted by the pellets was counted using a Packard Cobra 2 γ-counter (Perkin-Elmer, MA, USA). The insulin content within each sample was automatically determined, as ng of insulin/ml, by interpolation of the RIA standard curve by the Packard Cobra 2 γ-counter.

3

The acute effects of the endocannabinoid anandamide on insulin secretion from Wistar rat isolated pancreatic islets

3.1 Introduction

The endocannabinoid system, as reviewed in Section 1.4, is involved in a number of systems, with intensive research being conducted with respect to role of the endocannabinoid system in energy homeostasis. Chronic dosing of mice with rimonabant (SR141716A, a CB_1 receptor antagonist/inverse agonist) has been used in studies *in vivo* to examine whether CB_1 receptor antagonism would act as an appetite suppressant, therefore, promoting weight loss (Ravinet Trillou *et al.*, 2003). It was found that mice treated with rimonabant had reduced food intake and weighed less than vehicle-treated animals; however, the effects on food intake were transient, whereas weight loss persisted (Ravinet Trillou *et al.*, 2003). Rimonabant has also been used in clinical trials and has been found to cause similar persistent weight loss in people who were either overweight or obese (Despres *et al.*, 2005; Pi-Sunyer *et al.*, 2006). As it was found to be an effective weight loss therapy, rimonabant was available for clinical use in 2006 as an anti-obesity treatment, until its withdrawal from clinical use in 2009 (see below). It is now thought that the effects of chronic rimonabant treatment are mediated through the central nervous system and peripheral tissues (Ravinet Trillou *et al.*, 2003; Janiak *et al.*, 2007; Nogueiras *et al.*, 2008).

As described in Section 1.3.3, weight loss is recommended in patients with type 2 diabetes who are obese, as even modest weight loss reduces the risk of cardiovascular events. Hence, rimonabant was also trialled in overweight/obese people who have type 2 diabetes (Scheen *et al.*, 2006; Rosenstock *et al.*, 2008). It was found in these clinical trials that people not only lost weight but rimonabant treatment also significantly reduced levels of glycated haemoglobin, fasting plasma glucose levels and insulin resistance (Scheen *et al.*, 2006; Rosenstock *et al.*, 2008). The reductions in glycated haemoglobin, fasting plasma glucose levels and insulin resistance were not primarily due to weight loss, indicating that other therapeutic effects were occurring. This suggests that blockade of CB_1 receptor signalling may be

a novel treatment for type 2 diabetes. The blood glucose lowering effects of rimonabant has subsequently been reported in 12-month old Zucker obese rats (genetic model of obesity; Chapter 6) which appeared to be diabetic or have impaired fasting blood glucose with a mean blood glucose level of 10mM after 24-hour fasting (Janiak *et al.*, 2007). It was also found in the same study that rimonabant treatment prevented loss of β cell mass and increased β cell numbers per islet suggesting that rimonabant protected islet viability (Janiak *et al.*, 2007). The clinical use of rimonabant is no longer permitted in the USA and EU countries due to concerns over the development of depression and the risk of suicide (Johansson *et al.*, 2009). Therefore, a greater understanding of how the endocannabinoid systems works in the periphery may allow more targeted modulation of endocannabinoid signalling to be developed for the treatment of type 2 diabetes without significant CNS side-effects.

In further support of a possible role of the endocannabinoid system in the treatment of type 2 diabetes, it has been reported in rodent studies *in vivo* that acute application of CB_1 and CB_2 receptor specific agonists and antagonists prior to intraperitoneal glucose loading affects the subsequent clearance of excess plasma glucose (Bermudez-Siva *et al.*, 2006; Bermudez-Silva *et al.*, 2007). As described in Section 1.1, the regulation of blood glucose is the result of the interplay of the islet hormones (glucagon and insulin) and their subsequent effects on peripheral tissues. Therefore, the observed decreases in glucose clearance caused by CB_1 receptor agonism and CB_2 antagonism prior to glucose loading may be due to decreased insulin release from islets and/or decreased peripheral insulin sensitivity. Several studies have reported that various components of the endocannabinoid system are expressed within mouse, rat and human islets, suggesting that the endocannabinoid system is active in these tissues (Juan-Pico *et al.*, 2006; Bermudez-Silva *et al.*, 2007; Bermudez-Silva *et al.*, 2008; Nakata & Yada, 2008; Starowicz *et al.*, 2008; Tharp *et al.*, 2008; Bermudez-Silva *et al.*, 2009). While the expression levels and cell distributions of different components of the endocannabinoid system still remains

controversial, it appears that the *in vivo* effects of cannabinoids on glucose tolerance may have involved a direct effect of islet function.

Despite the lack of information regarding what role(s) the endocannabinoid system plays within islets, data from *in vivo* rodent and clinical studies suggest that that endocannabinoid system may play a direct role in preserving islet function in pathological disease states. To determine whether endocannabinoid signalling within islets may offer novel treatments for type 2 diabetes, it is first necessary to characterise the system in islets which have been isolated from lean non-diabetic rats. Therefore, the principal aim of this chapter was to examine whether endocannabinoid signalling acutely effects insulin secretion. In order to achieve this, anandamide (AEA) was used in freshly isolated islets of Langerhans from male Wistar rats.

3.2 Methods

3.2.1 Materials

All drugs and buffers were prepared as described in Sections 2.2.

3.2.2 Animals

All procedures were performed using tissues obtained from male Wistar rats whose weights ranged from 230-350g. Animals were housed and killed as described in Section 2.1.

3.2.3 Insulin secretion studies

Islets were isolated from male Wistar rats according to the isolation procedure described in 2.4.1.1.

3.2.3.1 Static incubations

Freshly isolated islets were used in static incubation studies, as described in 2.4.2. Once the hour incubation had finished, then samples were taken from each condition and the amount of insulin in the samples was determined by RIA as described in 2.6. Analysis of experimental data was carried out as described in 2.4.5. Where indicated, the Spearman rank (non-parametric) correlation test was used to determine whether there was a significant correlation between two parameters.

For experiments where 10μM URB597 (a fatty acid amide hydrolase specific inhibitor) was used, islets were pre-incubated for 30 minutes at 4mM glucose with either URB597 or vehicle (ethanol at a final concentration of 0.34% (v/v)). A pre-incubation period of 30 minutes was chosen as a high level of FAAH inhibition should occur within this time-frame (Ahn *et al.*, 2007) without compromising islet function, which diminishes with time. While other studies have found FAAH to be expressed within islets, the level of FAAH activity within islets has not been characterised (Bermudez-

Silva *et al.*, 2008; Starowicz *et al.*, 2008; Tharp *et al.*, 2008). It was decided that URB597 would be used at 10μM, despite the possibility of non-specific actions occurring, as it was uncertain whether URB597 at lower concentrations would successfully penetrate the islets in sufficient time to successfully inhibit FAAH activity (Ahn *et al.*, 2007; Zhang *et al.*, 2007). Following the pre-incubation period, test reagents were added and the 96-well plate was then agitated by hand to ensure thorough mixing before the plate was incubated for a further hour. AEA was used at 10μM in these URB597 experiments as the largest difference in insulin secretion rates was observed at this concentration between AEA responsive and non-responsive islets (based on compiled data, Figure 3.3).

3.2.3.1 Islet perifusion

The perifusion equipment was set up as described in 2.4.4. For the first ten minutes of the experiment, islets were exposed to 4mM glucose and after this time point the islets were exposed to 20mM glucose. Islets were then left for 20 minutes at 20mM glucose to establish glucose responsiveness. Islets were either continuously exposed to 30μM AEA or vehicle control (ethanol 0.6% (v/v) - final concentration) at 20mM glucose for 30 minutes. A concentration of 30μM AEA was chosen as it was found to cause a 50% (and reproducible) inhibition of insulin secretion in statically-incubated islets (Section 4.3.1).

Islets were considered to be glucose-responsive if the islets from the vehicle group were found to display a biphasic response of insulin release as a result of exposure to 20mM glucose, see Section 1.2.2. Additionally for experimental data to be useful, islets from the AEA treatment group had to show similar responses to glucose within the first 30 minutes as the control group (i.e. prior to exposure to AEA). This was to demonstrate islet viability and that insulin secretion rates were comparable between vehicle and test groups during the first 30 minutes- prior to the addition of AEA or vehicle. Insulin secretion for each individual time point was expressed as a percentage of the mean basal insulin secretion rate (the first 10 minutes, which was

set at 100%). The area under curve (AUC) was then calculated using the integration function in Prism 5 using the trapezium rule. The AUC was compiled into 10 minute bins and was subjected to two-way ANOVA analysis.

3.2.4 Endocannabinoid metabolism assays

Preparation of homogenised tissues and isolated islets were performed as described in Section 2.5. The experiments were performed and AEA hydrolytic rates calculated as described in Section 2.5.

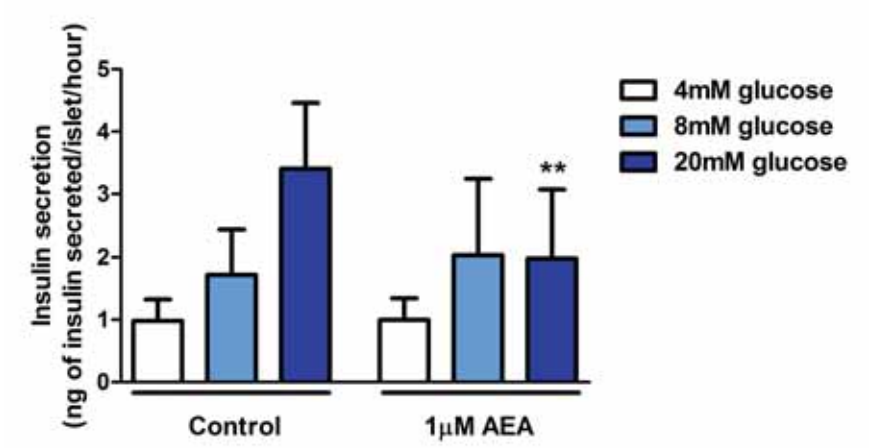
Homogenised pancreatic tissue or freshly isolated islets were incubated at either 4mM or 20mM glucose to determine whether there was glucose-dependent hydrolysis of AEA. In addition to this, pancreatic and liver tissue homogenates or islets were incubated in either the absence or presence of URB597 in order to establish FAAH activity. For homogenised pancreas studies, a pre-incubation period of 15 minutes with vehicle or 1μM URB597 was used. For whole islet assays, cells were pre-incubated for period of 30 minutes with vehicle (ethanol 0.34% (v/v) final concentration) or 10μM URB597. Conditions were carried out in duplicate or triplicate. Liver homogenates were used at 4mM glucose and employed as a positive control for AEA hydrolysis and URB597 mediated inhibition of FAAH activity. FAAH activity was calculated as the difference in the AEA hydrolytic rates between the control and URB597 pre-treated experimental means. Significant difference between the means (FAAH activity was not included as this was not raw data) was then tested using one-way ANOVA followed by Tukey's *post-hoc* test. All data are expressed as the mean ± SD.

3.3 Results

3.3.1 Glucose- and concentration-dependent effects of anandamide on insulin secretion in isolated islets of Langerhans

In static incubations, rat isolated islets were incubated for one hour in the absence or the presence of 1μM AEA at basal (4mM), intermediate (8mM), and maximal (20mM) levels of glucose-stimulated insulin secretion. AEA did not affect basal or intermediate levels of glucose-stimulated insulin secretion but AEA significantly inhibited insulin secretion at 20mM glucose (Figure 3.1). Islet perifusions were performed to determine the effects of AEA on the kinetics of insulin secretion at 20mM glucose. On exposure of the islets to 20mM glucose (t= 10 minutes), there was a small, transient increase in insulin secretion rates which lasted for ~10 minutes (first phase), immediately followed by a much larger and sustained second phase of insulin secretion (Figure 3.2A). The time-course and biphasic response in insulin secretion concur with other studies using perifused rat islets, however the level of response, especially the first phase, was lower than that typically reported (Section 1.2.2). Area under curve analysis of the compiled perifusion data demonstrated that differences between the vehicle and AEA groups were not statistically significant prior to and during exposure of the islets to 30μM AEA or vehicle (Figure 3.2B).

A



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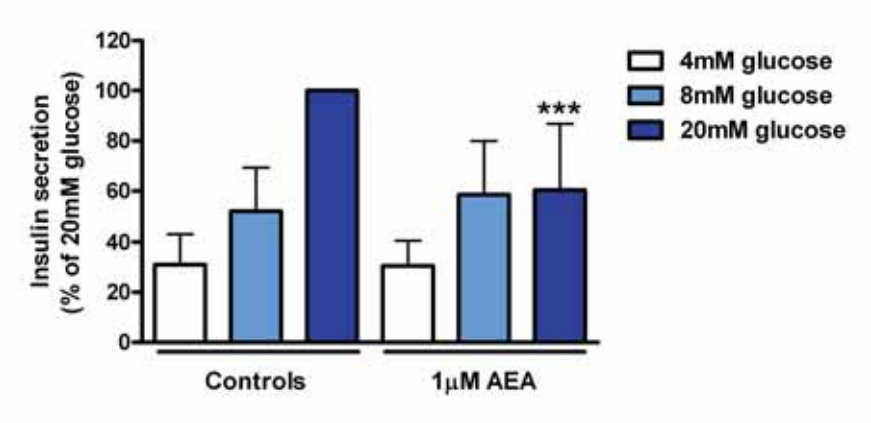

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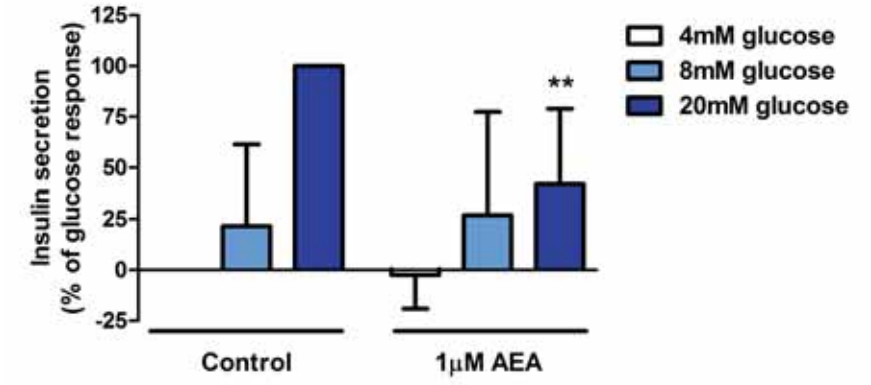
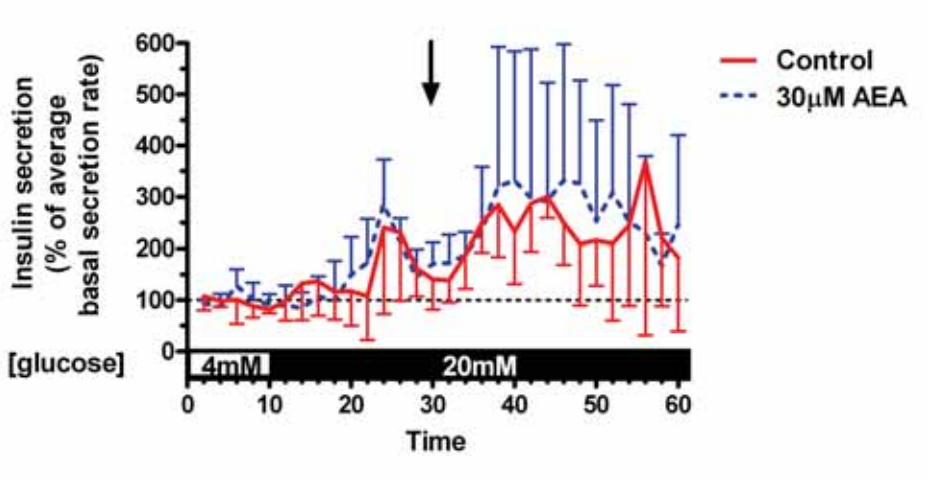


Figure 3.1 The effects of anandamide (AEA) on glucose-dependent insulin secretion. Islets were incubated at 4mM, 8mM or 20mM glucose in the absence (control) or presence of 1μM AEA. All graphs are produced from the same data set that have been normalised according to different criteria. All results are presented as mean insulin secretion rates ± SD (n= 10). A) Insulin secretion rates have not been normalised. ** P< 0.01 *vs.* 20mM glucose control. B) Results from each experiment have been normalised against the 20mM glucose control. *** P< 0.001 *vs.* 20mM glucose control. C) Results from each experiment have been normalised against the difference (glucose response) between the insulin secretion rates observed for the 4mM (0%) and 20mM (100%) glucose controls. ** P< 0.01 *vs.* 20mM glucose control.

A



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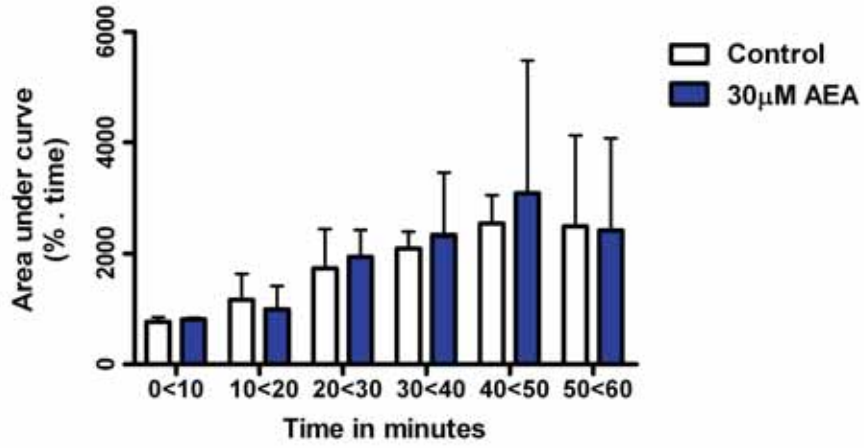
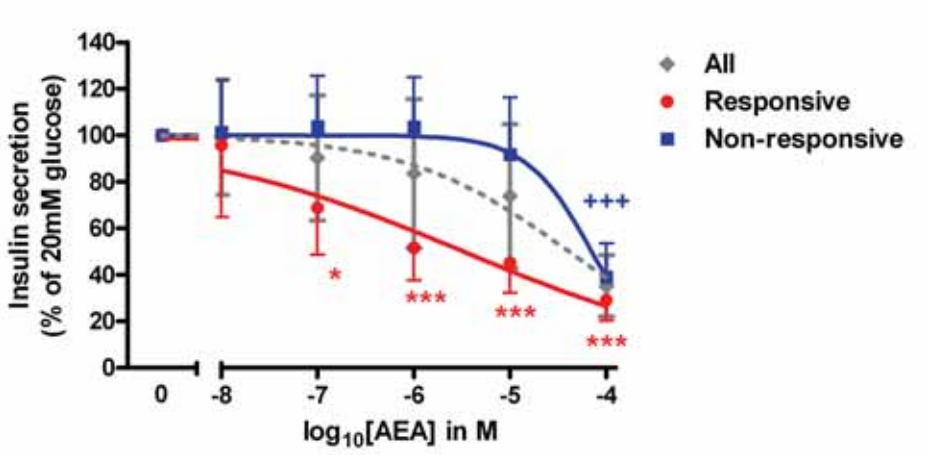


Figure 3.2 The effects of 30μM anandamide (AEA) on insulin secretion at 20mM glucose from perifused islets. A) Islets were perifused at 37ºC in medium containing 4mM glucose for 30 minutes prior to the start of the experiment (at t= 0). Insulin secretion was determined at 2 minute intervals. At t= 10 minutes, 20mM glucose was introduced into the medium. AEA (30μM) or vehicle (control, 0.6% (v/v) final concentration) was infused at t= 30 minutes (indicated by the arrow). Results represent the mean insulin secretion rates ± SD for three individual experiments. B) Area under curve for 10-minute blocks was calculated from the data in Figure A according to the protocol in Section 3.2.3.1.

To ascertain whether the effects of AEA were concentration-dependent, concentration-response experiments were performed at 20mM glucose. It was found that AEA inhibited insulin secretion in a concentration dependent manner with AEA fully inhibiting insulin secretion at 100μM. The mean IC_{50} for 10 of the 13 AEA concentration-response data set (the statistical software was unable to fit the data from 3 experiments to a sigmoidal curve) was calculated as 1.6μM (95% CI: 227nM to 4.0μM; Figure 3.3A). The mean Hill slope was calculated as -2.5 ± 2.6.

A



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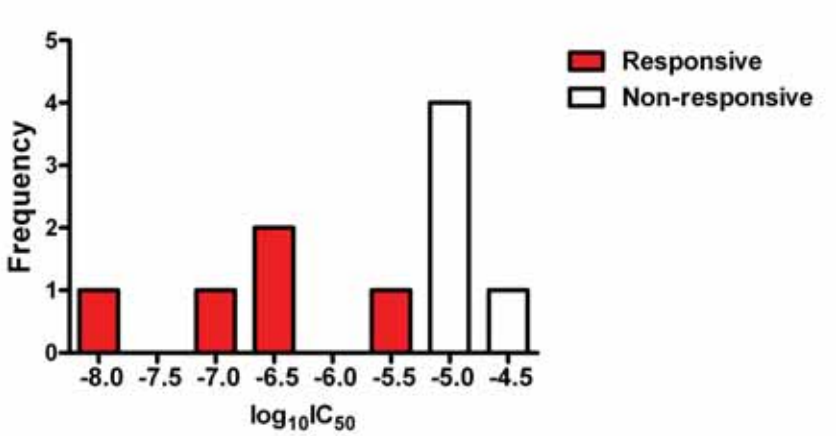


Figure 3.3 The effects of increasing concentrations of anandamide (AEA) on insulin secretion at 20mM glucose. A) Islets were incubated at 20mM glucose with various concentrations of AEA. Basal insulin secretion rates for all (n= 13), responsive (n= 5) and non-responsive groups (n= 8) were 34.7 ± 10.8%, 37.6 ± 7.9% and 32.9 ± 12.4% respectively. Results are presented as mean insulin secretion rates ± SD * P< 0.05, *** P< 0.001, $^{+++}$ P< 0.001 *vs.* 20mM glucose control. B) Distribution frequency of $\log_{10}IC_{50}$ values (calculated by fitting individual AEA concentration-response experimental results to sigmoidal curves; n= 10).

On further analysis of the data, it appeared that in 8 of the 13 experiments, AEA displayed a lack of inhibitory effect on insulin secretion at 1μM. The compiled concentration-response data from these 8 experiments suggest that AEA, at concentrations ≤ 10μM, was unable to cause a substantial (> 25%) reduction in insulin secretion rates. These islets were labelled as being "non-responsive" to AEA and exhibited an IC_{50} of 12.3μM (95% CI: 6.8μM to 19.4μM; n= 5, the statistical software was unable to fit the data to a sigmoidal curve for 3 of the 8 experiments; Figure 3.3A). The mean Hill slope for the non-responsive islets was calculated as -3.6 ± 3.0. The islets from the remaining five experiments were deemed to be responsive to AEA,

with AEA displaying a clear concentration-dependent effect as a marked inhibition of insulin secretion was observed at 100nM with the IC_{50} for the AEA responsive islets calculated as 220nM (95% CI: 21.9nM to 2.2μM). The mean Hill slope for the responsive islets was calculated as -1.3 ± 1.6. In both populations of islets, 100μM AEA was found to fully reduce glucose-stimulated insulin secretion to within basal insulin secretion levels (Figure 3.3A). The distribution of all AEA IC_{50} values was not found to be normally distributed, instead AEA-response and non-responsive islets appear to be distributed about two separate means (Figure 3.3B)

As the effects of 1μM AEA were glucose-dependent the responsiveness of islets may have been linked to how glucose responsive the islets were. Further analysis of all the 1μM AEA at 20mM glucose data did not reveal a significant correlation between the glucose- and AEA-responsive states of the islets (Figure 3.4). The AEA concentration-response data was also re-analysed with the insulin secretion rates normalised against the glucose response (Figure 3.5.A). The distribution of the $\log_{10}IC_{50}$ values for AEA calculated by this method were found to occur independently ($P > 0.05$) of the islets glucose responsiveness as determined by the Spearman rank correlation test (Figure 3.5.B).

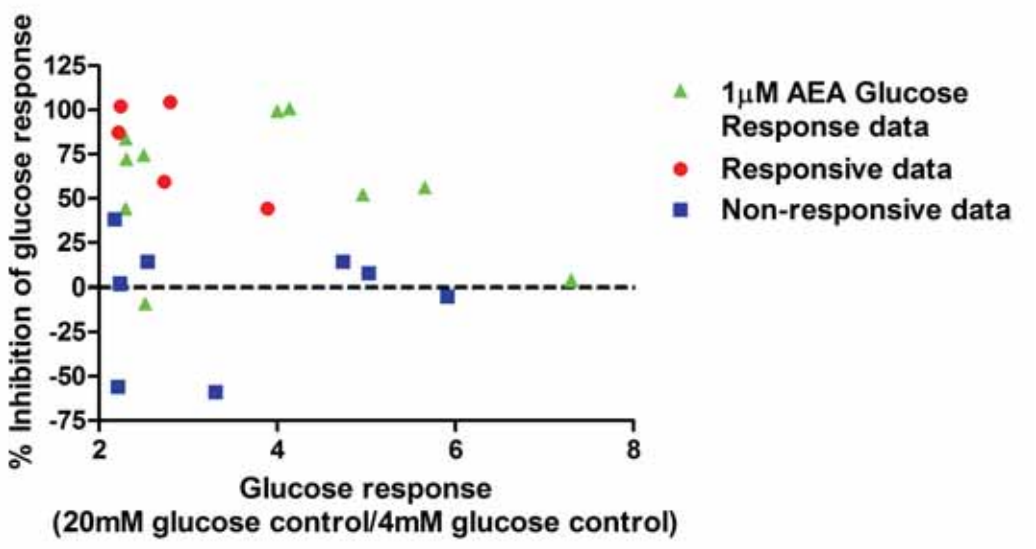
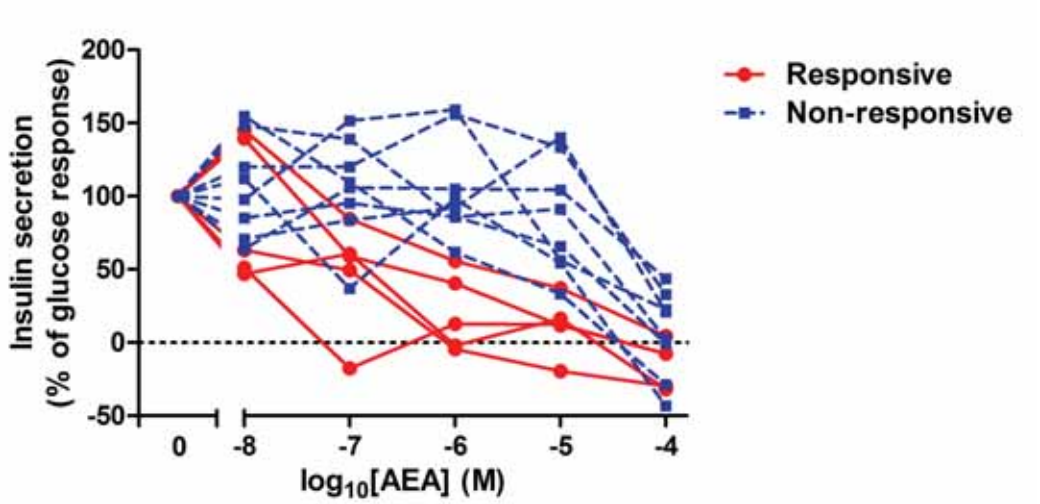


Figure 3.4 The individual experimental effects of 1μM AEA at 20mM glucose plotted against the glucose responsiveness of the islets. Data has been taken from the 1μM AEA glucose response and AEA concentration-response data sets. % inhibition of the glucose response was calculated as 100x (20mM glucose control - 1μM AEA at 20mM glucose)/(20mM glucose control - 4mM glucose control).

A



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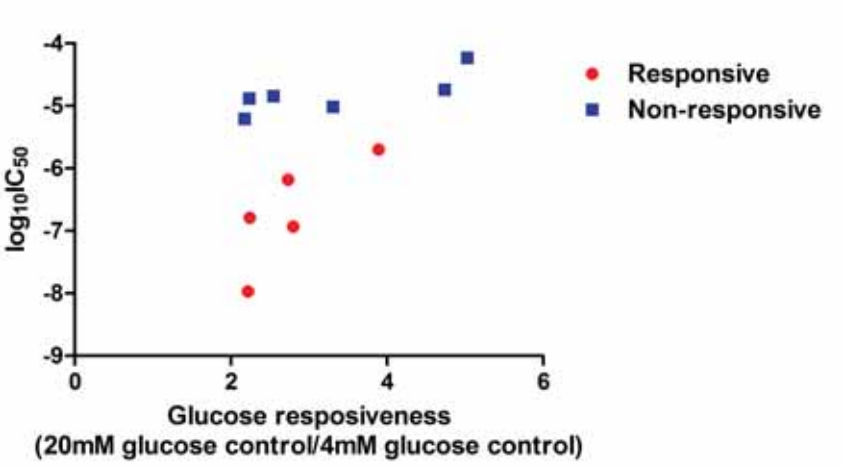


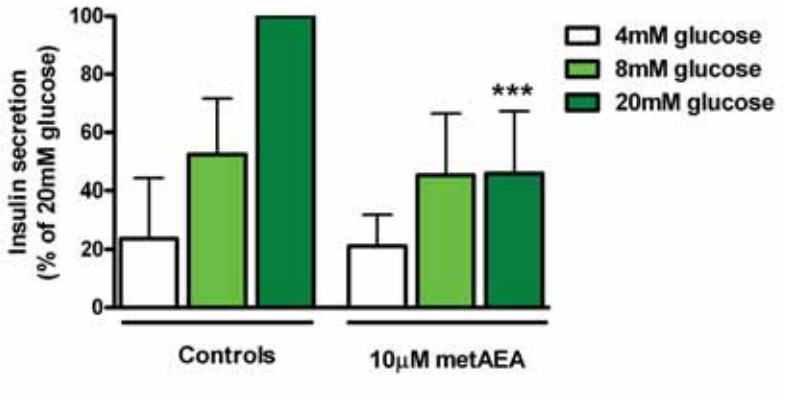
Figure 3.5 The effects of increasing concentrations of anandamide (AEA) on insulin secretion at 20mM glucose expressed as a function of glucose responsiveness. A) Islets were incubated at 20mM glucose with various concentrations of AEA. Results have been normalised against the difference (glucose response) between the insulin secretion rates observed for the 4mM (0%) and 20mM (100%) glucose controls. Results are presented as mean insulin secretion rates (n= 13). B) The AEA responsive state of the islets (represented by the individual $\log_{10}IC_{50}$ values calculated from the data presented in figure A) plotted against the corresponding glucose responsiveness of the islets (n= 10).

3.3.2 N-acylethanolamine metabolism in islets

As described above, there appeared to be two populations of islets based on the level of inhibition caused by 1μM AEA on 20mM GSIS (glucose stimulated insulin secretion). One possible cause of the apparent variability could have been due to variations in local metabolism of anandamide and so additional experiments were carried out with the metabolically stable analogue, methanandamide. Methanandamide (10μM) consistently inhibited insulin secretion at 20mM glucose but

did not affect insulin secretion at basal and intermediate levels of GSIS (Figure 3.6A). The concentration-response curve for methanandamide appeared similar to that of the AEA responsive data as there was a clear concentration-dependent inhibition of insulin secretion. The IC_{50} of methanandamide was 682nM (95% CI: 94.2nM to 4.9μM; Figure 3.6B) and the mean Hill slope was calculated as -3.0 ± 3.2. However, the mean IC_{50} value for methanandamide was not statistically different from the mean IC_{50} values calculated for the either the anandamide responsive or anandamide non-responsive groups.

A



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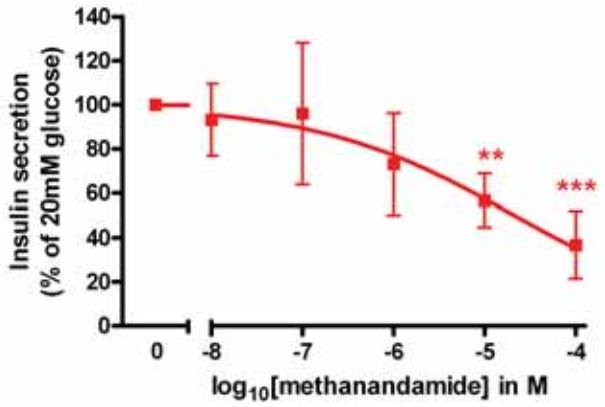

Figure 3.6 The glucose and concentration-dependent effects of methanandamide on insulin secretion. A) Islets were incubated at 4mM, 8mM, or 20mM glucose in the absence (control) or presence of 10μM methanandamide. Results are presented as mean insulin secretion rates ± SD (n= 5) ***P< 0.001 *vs.* 20mM glucose control B) Islets were incubated at 20mM glucose with increasing concentrations of methanandamide. The basal insulin secretion rate for the methanandamide concentration-response curve data was 33.2 ± 18.1 %. Results are presented as mean insulin secretion rates ± SD (n= 5) ** P< 0.01, *** P< 0.001 *vs.* 20mM glucose control.

The fatty acid amide hydrolase (FAAH) specific inhibitor URB597 (10μM) alone did not affect insulin secretion at 4mM or 20mM glucose (Figure 3.7A). Hence, pre-incubation of the islets with 10μM URB597 was deemed appropriate for future use with AEA. It was found that pre-incubation of islets with 10μM URB597 did not affect islet responses to AEA (Figure 3.7B). Upon further analysis of the data in Figure 3.7B, islets where 10μM AEA inhibited insulin secretion, 10μM URB597 also inhibited insulin secretion, whereas if 10μM AEA did not affect insulin secretion, neither did 10μM URB597 (Figure 3.7C). Subsequent re-analysis of the URB597 glucose-response data found there were two experiments where pre-incubation with URB597 inhibited insulin secretion by > 25% (Figure 3.7A).

In light of the URB597 static incubation experiments, endocannabinoid metabolism assays were performed to ascertain the amount of AEA hydrolysis occurring in islets and to determine whether URB597 pre-treatment was effective at blocking AEA hydrolysis. It was found that much larger quantities of homogenised pancreas were required in comparison to homogenised liver to detect measurable levels of AEA hydrolysis (Figure 3.8A). Additionally AEA hydrolytic rates in homogenised pancreatic samples were unaffected by glucose. In total, three endocannabinoid metabolism assays were performed with whole tissue homogenates. The level of AEA hydrolysis, when standardised against the amount (wet weight) of tissue used, in homogenised liver samples was found to be significantly higher in comparison to AEA hydrolytic rates in homogenised pancreatic samples (Figure 3.8B & 3.8C). Pre-incubation of homogenates with 1μM URB597 was found to block fully AEA hydrolysis in liver samples (Figure 3.8B), but only blocked approximately a third of AEA hydrolytic activity in pancreatic samples (Figure 3.8C). Use of whole islets in the endocannabinoid metabolism assays was attempted on several occasions but measurable amounts of hydrolysis were not observed in islets as AEA hydrolytic rates were less than or equal to the AEA hydrolytic rates observed in the non-enzymatic control (Figure 3.9).

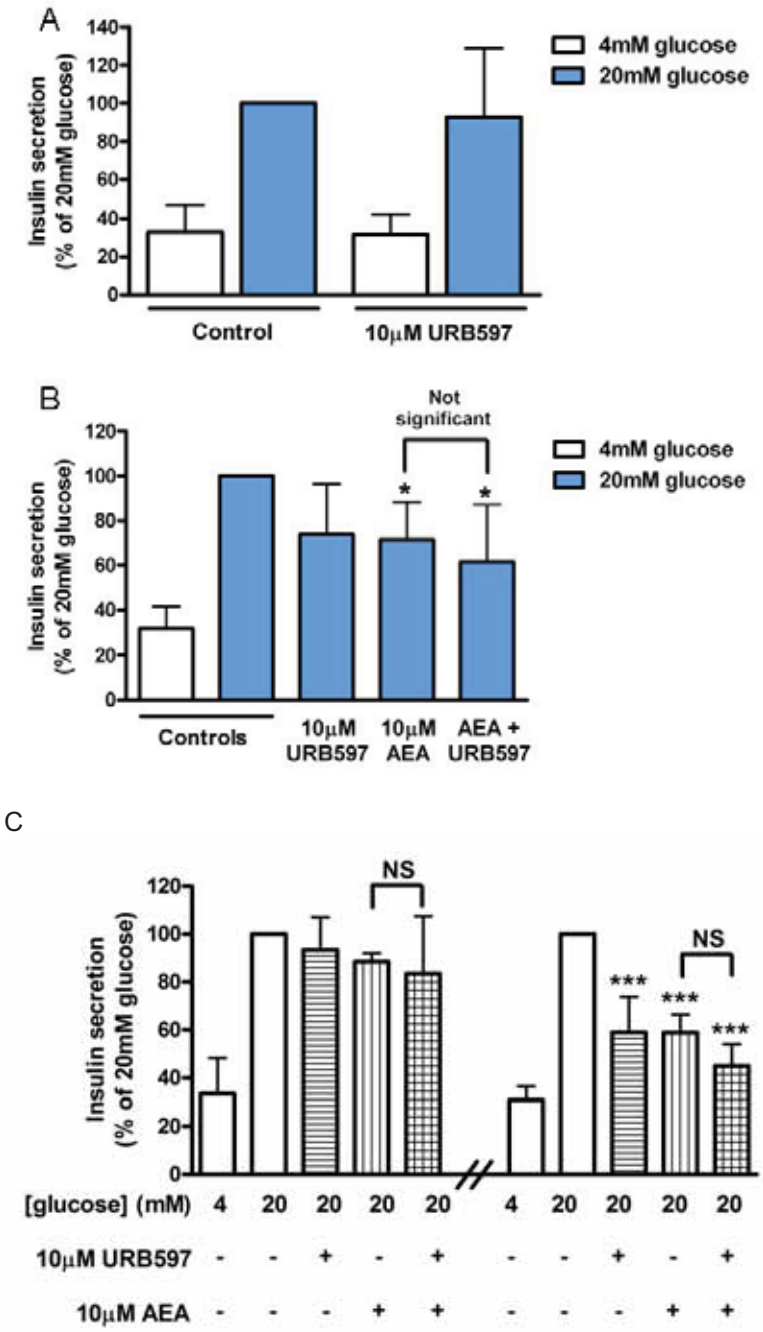

Figure 3.7 The effects of URB597 on AEA induced inhibition of glucose-mediated insulin secretion. A) Islets were pre-incubated with either vehicle (control) or 10μM URB597. Insulin secretion was then determined at either 4mM or 20mM glucose. Results are presented as mean insulin secretion rates ± SD (n= 6). B) Islets were pre-incubated with either vehicle (control) or 10μM URB597. Insulin secretion was then determined at either 4mM or 20mM glucose in the absence or presence of 10μM AEA. Results are presented as mean insulin secretion rates ± SD (n= 7). * P< 0.05 vs. 20mM glucose control. C) Division of the data presented in B, whereby experiments in which URB597 did not inhibit insulin secretion are shown on the left of the X axis break (n= 3) and experiments where URB597 was found to inhibit (> 25%) insulin secretion on the right of the X axis break (n= 4). Islets were pre-incubated with either vehicle (control) or 10μM URB597. Insulin secretion was then determined at either 4mM or 20mM glucose. Results are presented as mean insulin secretion rates ± SD. NS Not significant; *** P< 0.001 vs. 20mM glucose.

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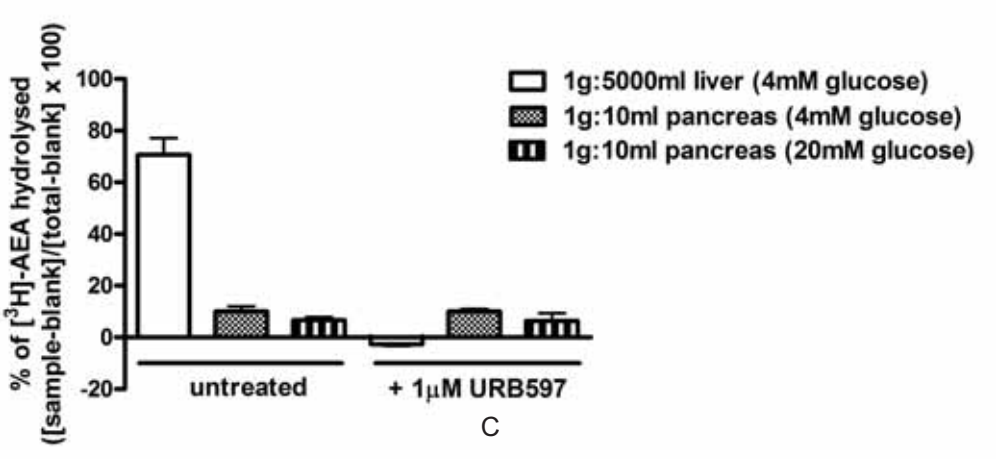

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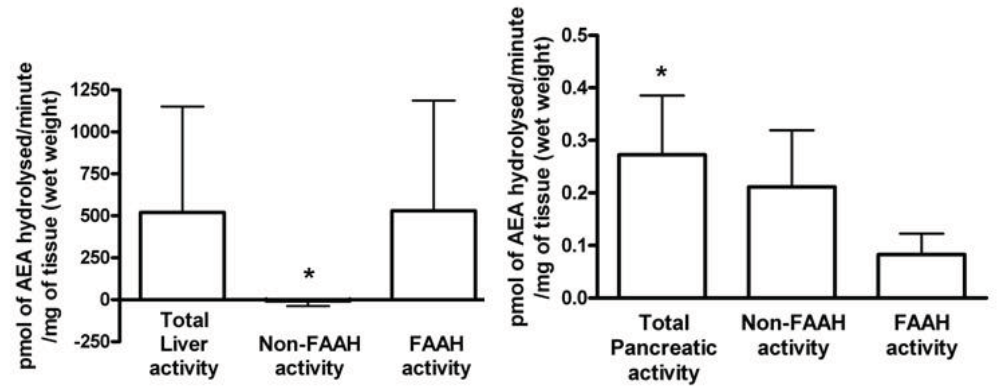


Figure 3.8 The effects of URB597 on AEA hydrolysis in homogenised samples of liver and pancreatic tissues. A) Samples of homogenised tissues were pre-incubated in the absence or presence of 1μM URB597 for 15 minutes prior to an incubation with 2μM [^{3}H]-AEA at 4mM or 20mM glucose for 30 minutes. Each condition was performed in triplicate. AEA hydrolysis is expressed as the % of [^{3}H]-AEA hydrolysed. Data shown are the mean ± SD from one representative experiment. AEA hydrolysis in rat liver (B) and pancreas (C) where AEA hydrolytic activity was standardised according to wet weight of tissue used. Data shown are the mean ± SD (n= 3) * P< 0.05 *vs.* total liver activity.

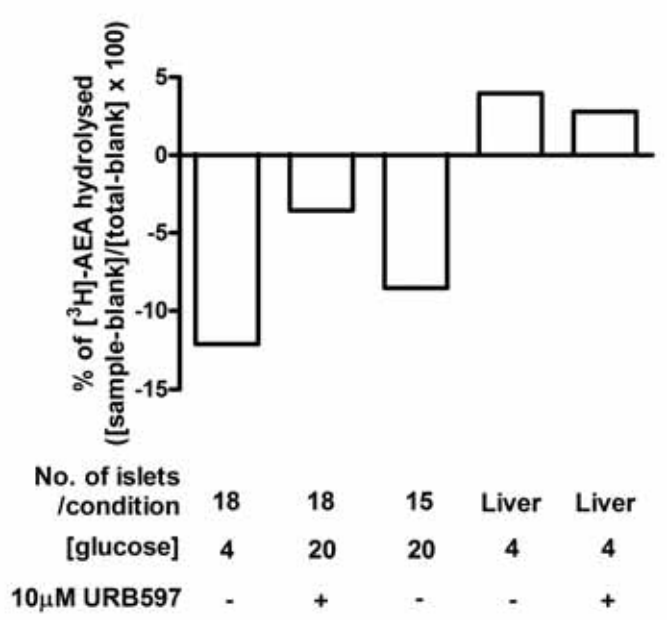


Figure 3.9 The effect of URB597 on AEA hydrolysis in whole islets. Islets were pre-incubated at 4mM glucose in the presence of 10μM URB597 or a vehicle control for 30 minutes prior to a 30 minute incubation with 2μM [^{3}H]-AEA. Each condition was performed in duplicate. AEA hydrolysis is expressed as the % of [^{3}H]-AEA hydrolysed. Data shown are the mean hydrolytic activities from one representative experiment

3.3.3 Effects of clonidine on insulin secretion at 20mM glucose

The potent α_2-adrenoceptor agonist clonidine was used to ascertain whether the responsive state of islets to AEA was due to the loss or degradation of cell surface receptors, which may have occurred during islet isolation. When islets were incubated from 1 hour at either 4mM or 20mM glucose in the absence or presence of 100nM clonidine or 10μM AEA, it was found that clonidine consistently inhibited insulin secretion, whereas 10μM AEA did not (Figure 3.10).

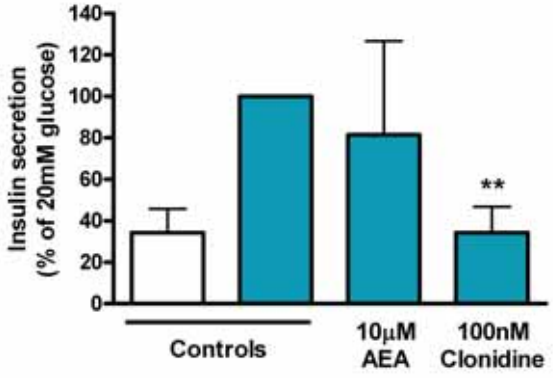


Figure 3.10 The effects of 100nM clonidine and 10μM anandamide (AEA) on insulin secretion at 20mM glucose. Islets were incubated at 4mM or 20mM glucose for 1 hour in the absence (control) or presence of clonidine or anandamide. Results are presented as mean insulin secretion rates ± SD (n= 5) ** P< 0.01 vs. 20mM glucose control.

3.4 Discussion

The endocannabinoid AEA was found to inhibit insulin secretion in a glucose-dependent manner. Further experimentation also found that the effects of AEA on insulin secretion were concentration-dependent. Furthermore, the concentration-dependent effects of AEA on insulin secretion appeared to be governed by the responsive state of the islets as there appeared to be two populations of islets, responsive and non-responsive. In an effort to determine whether islet sensitivity to AEA was due to differing levels of AEA hydrolysis between populations of islets, methanandamide (a non hydrolysable analogue of AEA) was used. Methanandamide was also found to inhibit insulin secretion in a glucose- and concentration-dependent manner similar to AEA in AEA-responsive islets (Figure 3.3A & 3.4B). Pre-incubation of islets with the fatty acid amide hydrolase (FAAH) inhibitor URB597 did not affect islet responsiveness to AEA, but in some experiments URB597 was found to inhibit insulin secretion too. Endocannabinoid metabolism assays were performed to determine whether URB597 pre-treatment was effective in blocking FAAH activity. However, it was found that AEA hydrolysis in control islets was too low for the metabolism assay to detect, therefore, it remains unclear whether URB597 pre-treatment in islets was effective in blocking AEA metabolism.

3.4.1 Effects of anandamide on glucose-stimulated insulin secretion

It was initially found that AEA inhibited GSIS, albeit with tissue variability (Figure 3.1). To date, five studies have examined the effects of AEA on insulin secretion in isolated islets. The studies by Juan-Pico *et al.* (2006) and Nataka & Yada (2008) have reported that AEA (in mouse islets) inhibits GSIS, whereas the study Bermudez-Silva *et al.* (2008) has reported that AEA (in human islets) potentiates insulin secretion. Therefore, the finding that AEA inhibits insulin secretion in this study is in agreement with the studies that have used mouse islets but not in studies that have used human islets, suggesting that AEA signalling in rodent islets is conserved but differs from that

in human islets (Juan-Pico *et al.*, 2006; Bermudez-Silva *et al.*, 2008; Nakata & Yada, 2008). However, the recent study by Vilches-Flores *et al.* (2010) using islets isolated from Wistar rats, found that AEA produced effects similar to that found in Bermudez-Silva *et al.* (2008), and disputes the species-dependent hypothesis. The islets used in this study and the studies by Juan-Pico *et al.* (2006) and Nataka & Yada (2008) were freshly isolated, whereas the islets used in the studies by Vilches-Flores *et al.* (2010) and Bermudez-Silva *et al.* (2008) were cultured at 5.5mM glucose for 16 hours or 3-5 days in culture media, respectively. Differences between endocannabinoid signalling may be due to the activities of stress-related proteins being up-regulated in freshly isolated islets (as a result of the animals' death and/or the process of islet isolation) in comparison to cultured islets (Ihm *et al.*, 2009). Alternatively, islet culture could potentially bring about alterations in endocannabinoid signalling and/or metabolism pathways. Therefore, the differences in AEA signalling could be due methodological differences in the preparation of islets.

Li *et al.* (2010) has recently reported that ACEA, a CB_1 receptor agonist, potentiated insulin secretion from perifused ICR mouse islets at 20mM glucose but not 2mM glucose. Once again, this may be due to the use of cell culture affecting cannabinoid signalling as Li *et al.* (2010) cultured the islets overnight at 11mM glucose whereas, the previously mentioned study by Nataka & Yada (2008) found that ACEA inhibited insulin release from freshly isolated ICR mouse islets. This observation appears to provide further support the hypothesis that cell culture may affect cannabinoid signalling in islets. However, Li *et al.* (2010) measured insulin secretion from perifused islets whereas, Nataka & Yada (2008) used statically incubated the islets, therefore, differences in the affects of ACEA on GSIS may also be due to secondary signalling molecules being washed away before being able to affect β-cell activity. Therefore, to test the hypothesis that cell culture affects cannabinoid signalling in islets, identical experiments should be performed with freshly isolated and cultured islets from the same preparation.

The findings in the present study indicate that AEA inhibits insulin secretion in a glucose-dependent manner which has not been reported in other studies (Juan-Pico *et al.*, 2006; Bermudez-Silva *et al.*, 2008; Nakata & Yada, 2008). However, it is not apparent whether the inhibitory effects of AEA within this study were limited by the glucose concentration at which the islets were incubated or whether inhibition of insulin secretion by AEA occurs once a threshold level of GSIS had been achieved. In this regard, the studies by Juan-Pico *et al.* (2006), Nataka & Yada (2008) and Bermudez-Silva *et al.* (2008) reported that the effects of AEA on insulin secretion rates occurred at the intermediate levels of GSIS. This was consistently not observed in the present study at 8mM glucose.

In continuation to the static incubation studies, the effects of 30μM AEA were examined on insulin secretion from perifused islets. Overall, it did not appear as though AEA had an effect on insulin secretion but this was due to considerable variation. As discussed in Chapter 4, AEA may indirectly affect β cell activity by paracrine signalling (Section 1.2.3) which would depend on the release of secondary signalling molecules (e.g. somatostatin-14) which then bind to their receptors expressed on the β cell surface. Therefore, the variability in the effects of AEA on insulin secretion may have been due to the secondary signalling molecules being washed away before being able to affect β cell activity, as flow rate for the perifusions may have been too high. However, reduction of the flow rate to 0.7ml/minute was not found to to alter the variability.

3.4.2 Characterisation of anandamide responsive and non-responsive islets

In light of the effects of 1μM AEA on glucose-stimulated insulin secretion, further experiments were carried out to determine the concentration-response relationship. In these experiments, only a third of islets were found to be sensitive to 1μM AEA found, determined by a greater than 25% inhibition of insulin secretion. Accordingly, the data were re-analysed and split into AEA responsive and non-responsive groups (Figure

3.3A) and were consistent with two separate islet populations. For consistency the two groups will be referred to as responsive and non-responsive, however, it would be more accurate to refer to the two populations as sensitive and less-sensative, respectively. That there are 2 distinct populations of islets with respect to the effects of anandamide is a novel observation and was not identified in other studies (Juan-Pico *et al.*, 2006; Bermudez-Silva *et al.*, 2008; Nakata & Yada, 2008). Additionally, there is no evidence in the literature that would readily explain this observation. In order to investigate the variability, the effects of the α_2–adrenoceptor agonist clonidine were examined, to assess the viability of G_iPCR signalling (Yamazaki *et al.*, 1982). The results suggest that responsiveness of islets to AEA was not due to wide-spread degradation of plasma membrane receptors or defective G_i protein coupling. This was concluded as the responses to AEA were variable, whereas, clonidine consistently inhibited insulin secretion (Figure 3.10). As the effects of AEA were glucose-dependent and only inhibited insulin secretion at maximal, but not intermediate, levels of GSIS this suggested that AEA may be inhibiting one or more amplification pathways (Section 1.2.2.3). Thus, in less glucose-responsive islets (where the amplification pathways are not as prominent) the effects of AEA may have appeared diminished. Subsequent analysis of the AEA glucose- and concentration-response data (Figures 3.4 & 3.5B) suggest that the sensitivity of islets to AEA does not correlate to how glucose responsive the islets are. Therefore, it appears that the responsiveness of islets to AEA was not a direct result of isolation or handling of the islets but may be due to inherent differences in the islets themselves. For instance, the study by Juan-Pico *et al.* (2006) found that the effects of AEA (but not 2-AG) on glucose-stimulated oscillations in $[Ca^{2+}]_i$ varied between islets, as the effects of AEA appeared to be either CB_1 or CB_2 receptor-mediated. However, the reason why AEA signalling differed between islets when the effects of 2-AG appeared to be consistent in the study by Juan-Pico *et al.* (2006) was not investigated. Additionally, this observation is of limited value as changes in $[Ca^{2+}]_i$ can be linked to insulin granule exocytosis but these changes cannot be readily quantified into insulin secretion rates (Barbosa *et al.,* 1998).

Despite variations in potency, 100μM AEA fully blocked GSIS in all of the AEA concentration response experiments. One possible explanation for these differences in potency could be endocannabinoid uptake and metabolism. In order to assess this, the non-hydrolysable analogue of AEA, methanandamide was used (Abadji *et al.*, 1994; Lin *et al.*, 1998). The concentration-dependent effects of methanandamide were comparable to those of AEA. Specifically, 10μM methanandamide consistently inhibited insulin secretion, even in islets which were found not to respond to 10μM AEA (see later, Figure 4.7B). This suggests that the islet responsiveness may be due to metabolism of AEA or, alternatively, minor differences in AEA and methanandamide pharmacology (e.g. differences in CB_1 and CB_2 receptor affinities) are exacerbated in islets (Lin *et al.*, 1998).

The expression of FAAH, a key enzyme in the degradation of AEA (Section 1.4.2.1), has been reported in mouse, rat and human islets (Starowicz *et al.*, 2008; Tharp *et al.*, 2008). Therefore, if islet responsiveness to AEA was a result of differing levels of FAAH activity then inhibition of this enzyme prior to the application of AEA should overcome the variability. As with methanandamide, 10μM AEA was chosen over the concentration of 1μM, as it was found when reviewing the concentration-response curves for responsive and non-responsive islets that the difference in secretion rates between responsive and non-responsive islets was greatest at 10μM (Figure 3.3A). To inhibit FAAH, islets were pre-incubated with 10μM URB597 as this should inhibit even high levels of FAAH activity (Ahn *et al.*, 2007; Zhang *et al.*, 2007). Initial testing found that pre-treatment of islets with 10μM URB597 did not significantly alter basal or maximal levels of GSIS suggesting that 10μM URB597 was suitable for use with AEA. However, it was noted that the effects of 10μM URB597 at 20mM glucose were variable, so it was unclear whether there was an increased basal endocannabinoid tone (Section 1.4.3) within some islets as a result of URB597 pre-treatment. The variability of 10μM URB597 at 20mM glucose is probably not due to vehicle effects as the islets in the glucose controls were also exposed to an equivalent amount of

ethanol for an identical period time. Subsequently, it was found that pre-treatment with URB597 did not affect islet responsiveness to AEA but it was observed that in these experiments inhibition of insulin secretion by 10μM AEA coincided with 10μM URB597 inhibiting insulin secretion too (Figure 3.7C). It is unclear whether the linkage in the level of inhibition caused by 10μM URB597 and 10μM AEA can be attributed to islet responsiveness, as it was found in three of the AEA non-responsive concentration-response experiments; 10μM AEA caused a < 37% inhibition of insulin secretion at 20mM glucose. It is unclear whether confirmation of the responsive state to AEA would have contributed to a deeper understanding of islet variability without first knowing whether URB597 pre-treatment was blocking FAAH activity.

Data from Bermudez-Silva *et al*. (2008) suggests that 2-AG (and presumably other endocannabinoids) production in human islets is linked to GSIS and so the inhibition caused by URB597 may have been due to a full blockade of FAAH activity resulting a net increase in islet *N*-acylethanolamine levels. An increase in islet-derived AEA levels would lead to the inhibition of insulin secretion, assuming that rat islets produce AEA endogenously. Alternatively, the effects of URB597 could have been occurring as the result of non-specific inhibition of non-FAAH serine hydrolases or other enzymes (Alexander & Cravatt, 2006; Zhang *et al.*, 2007). Therefore, if inhibition of insulin secretion was not observed when islets were pre-treated with URB597 at concentrations ≤ 1μM, this would imply that the effects of 10μM URB597 on insulin secretion were due to non-specific effects. Rather than conducting this experiment, it was chosen to perform endocannabinoid assays instead, as the results from these assays would allow a direct measurement of AEA hydrolysis within islets and establish whether the 10μM URB597 pre-treatment was effective in blocking AEA hydrolysis. The preliminary experiments with whole islets did not produce meaningful data as the protocol was not sufficient to account for islet viability and responsiveness. Firstly, liver samples could only act as a positive control for FAAH activities in the assay but does not control for the treatment of intact islets with URB597 (Figure 3.9). Ideally, additional islets would be needed to run a static incubation in parallel with the

AEA hydrolysis assays, to demonstrate islet viability and to determine the AEA responsive state of the islets, but islet yields at the time were not sufficient. It must also be noted that the endocannabinoid assay only detects metabolism of the [^{3}H]-AEA if the radiolabeled ethanolamine head group is separated from the arachidonic acid moiety. However, if AEA is inhibiting insulin secretion via a plasma membrane receptor's extracellular binding site, then deactivation of AEA could occur by rapid uptake and retention within the islet cells. Therefore, AEA uptake studies may also be useful in probing whether variability in AEA responses are due to non-metabolic means. It must also be considered that if islets were found to have intrinsically low FAAH activity then alternate metabolic pathways may be of increasing importance (Section 1.4.2). For example, islets are thought to express several isoforms of the cyclooxygenase and lipoxygenase enzymes which can oxygenate various endocannabinoids, including AEA, thus making cannabinoid signalling more diverse (Yamamoto *et al.*, 1983; Shannon *et al.*, 1992; Ueda *et al.*, 1995; Han *et al.*, 2002; Kozak *et al.*, 2002; Ross *et al.*, 2002; van der Stelt *et al.*, 2002; Heitmeier *et al.*, 2004; Woodward *et al.*, 2008). Factors such as these may also exacerbate differences in the pharmacology of AEA and methanandamide, which increases the need to characterise AEA signalling (Gardiner *et al.*, 2009). Thus, the status of endocannabinoid metabolic activities within batches of islets may go some way to explaining the variability in islet sensitivity to AEA as observed in the current study.

3.4.3 Conclusions

The data presented in this chapter provide evidence that AEA acutely inhibits insulin secretion from statically-incubated islets, but the effects of AEA on insulin secretion was found to differ when islets were perifused. As the inhibition of insulin secretion caused by AEA is glucose and concentration-dependent, this indicates that the effects of AEA are controlled by receptor-specific signalling pathways. The nature of these signalling pathways were investigated and the findings are presented in Chapter 4.

Intriguingly, the concentration-dependent effects of AEA were variable which has not been documented by other groups. This may suggest that this variability is an artefact of the isolation and/or incubation process; however, attempts to identify the causes were unsuccessful. Hence, it was viewed that the responsive state of the islets may be due to integral rather than induced differences between islets. This led to the role of local metabolism (as the source of islet variability) being investigated using methanandamide and URB597. The data suggest that AEA hydrolysis by FAAH was not the cause of islet un-responsiveness, however, these findings are not conclusive without further evidence. Therefore, it remains unclear as to why the effects of AEA between islet preparations were variable in this study but alternate theories are suggested in later chapters.

4

Characterisation of the signalling pathways involved in the anandamide-mediated acute inhibition of insulin secretion

4.1 Introduction

In Chapter 3, anandamide (AEA) was found to inhibit insulin secretion in a glucose-dependent manner and it was assumed that AEA was acting in a receptor-dependent manner. As discussed in Section 1.4.3., CB_1-, CB_2-, TRPV1-receptors, PPARα, and PPARγ are all receptors linked to endocannabinoid signalling, but there are also data to suggest that endocannabinoids may act through GPR18, GPR55, and GPR119 receptors. Therefore, AEA may be inhibiting insulin secretion via a number of receptors. However, it is uncertain whether all of these receptors are expressed in islets.

It is also uncertain whether AEA is acting directly or indirectly to inhibit insulin secretion, as the expression of CB receptors have been detected on non-β cells which are located in the periphery of the rat islet (Table 4.1). This may indicate that AEA can affect insulin secretion by altering paracrine signalling within islets (Section 1.2.3). Indeed, the study by Bermudez-Silva *et al.* (2008) reported that acute cannabinoid treatment in human islets not only affected the amount of insulin secreted but also affected that of glucagon and somatostatin-14 (sst-14). Therefore, the inhibitory effects of AEA on insulin secretion may have originated from a signalling pathway in non-β islet cells.

The inhibition of insulin release by AEA was glucose- and concentration-dependent. Therefore, the primary aim of this study was to characterise the receptor signalling pathway(s) by which AEA inhibits insulin release. There was also a secondary aim which was to determine whether AEA was inhibiting insulin secretion by paracrine means. In order to achieve these aims, receptor-specific antagonists were used in co-junction with AEA in freshly isolated islets of Langerhans from male Wistar rats.

Table 4.1 Expression of CB_1 and CB_2 receptors in rat islets

Reference	Rat breed	RT-PCR		Immunohistochemistry							
				CB_1				CB_2			
		CB_1	CB_2	α	B	δ	PP	α	β	δ	PP
Bermudez-Silva *et al.* (2007)	Wistar	ND	ND	+?	+	?	?	+?	+	?	?
Starowicz *et al.* (2008)	Wistar	ND	ND	+	-	?	?	+	+	?	?
Vilches-Flores *et al.* (2010)	Wistar	+	ND	+	-	?	?	ND	ND	ND	ND
Tharp *et al.* (2008)	Zucker lean control	+	-	-	-	+	-	-	-	-	-
Bermudez-Silva *et al.* (2009)	Not specified[1]	ND	ND	+	-	?	?	+	+	?	?

RT-PCR was performed using RNA extracted from whole islets. Immunohistochemistry was performed using hormone-specific antibodies and receptor isoform-specific antibodies.[1] Rat strain was not specified by Bermudez-Silva *et al.* (2009) but it is likely that the tissue originated from male Wistar rats, used similarly in their earlier study (Bermudez-Silva *et al.*, 2007). ND not determined; + expressed; **-** not expressed; +? expression in cell type confirmed by basic cell morphology; ? Expression of CB_1 or CB_2 receptors detected on the periphery of the islet but the cell types was not confirmed.

4.2 Methods

4.2.1 Materials

All drugs and buffers were prepared as described in Sections 2.2. The somatostatin-14 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bachem (Bubendorf, Switzerland).

4.2.2 Animals

All procedures were performed using tissues obtained from male Wistar rats with body weights ranging from 230-350g. Animals were housed and killed as described in Section 2.1.

4.2.3 Insulin secretion studies

Islets were isolated from male Wistar rats according to the isolation procedure described in 2.4.2. 48-well plates with 70% confluent INS-1 823/13 cells (passages 86-91) were prepared as described in 2.4.3.

4.2.3.1 Static incubations

Freshly isolated islets were used in 1-hour static incubation studies, as described in 2.4.2. INS-1 823/13 cells were used in either 1- or 2-hour static incubation studies, as described in 2.4.3. The effects of AEA on insulin secretion from INS-1 823/13 cells did vary between 1 or 2 hour incubations. Once the incubations had finished, samples of incubation buffer were taken from each well and the amount of insulin in the samples was determined by RIA as described in 2.6. Analyses of experimental data were carried out as described in 2.5. Where indicated, the Spearman rank (non-parametric) correlation test was used to determine whether there was a significant correlation between two parameters.

4.3 Results

4.3.1 Characterisation of signalling pathways involved in inhibition of insulin release induced by anandamide

In light of the variability in the sensitivity of islets towards AEA treatment (Figure 3.3), a concentration of AEA greater than 10μM was needed in order to cause a reproducible inhibition of insulin secretion. Accordingly, 30μM AEA was found to cause a reproducible and significant inhibition of insulin release at 20mM glucose, and was therefore used for all of the antagonist studies (Figure 4.1).

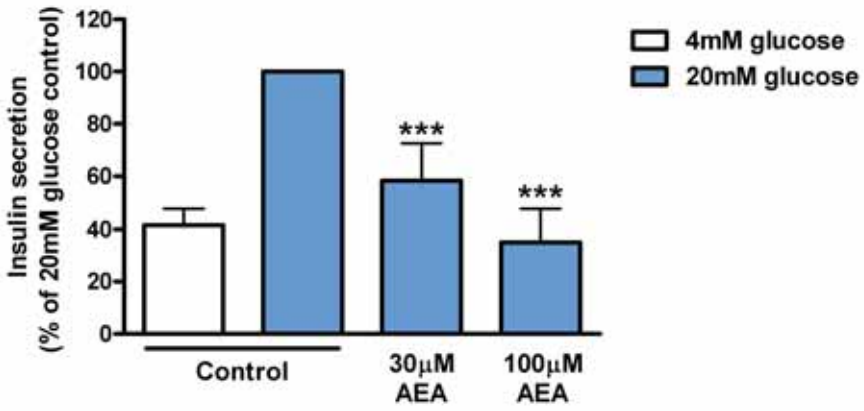


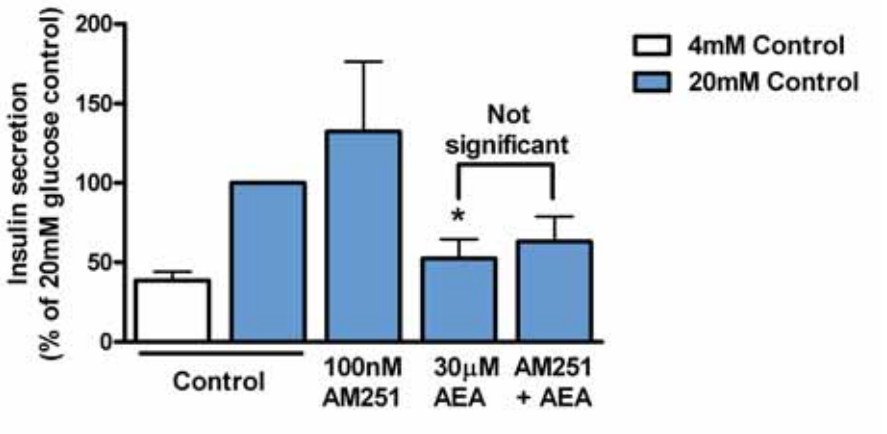
Figure 4.1 The effects of 30μM and 100μM AEA on insulin secretion at 20mM glucose. Islets were incubated at 4mM or 20mM glucose in the absence (control) or presence of 30μM or 100μM AEA. Results are presented as mean insulin secretion rates ± SD (n= 10). *** P< 0.001 *vs.* 20mM glucose control.

4.3.1.1 Effects of CB_1 receptor antagonists, AM251 and O-2050, on responses to anandamide

AM251 is a widely-used CB_1 receptor-specific antagonist/inverse agonist which has been used to investigate CB_1 receptor mediated signalling in both murine and human isolated islets (Savinainen *et al.*, 2003; Juan-Pico *et al.*, 2006; Bermudez-Silva *et al.*, 2008; Xiao *et al.*, 2008). O-2050, another CB_1 receptor-specific antagonist, was also used as a comparison (Martin *et al.*, 2002). Unlike AM251, O-2050 is a relatively new compound that has primarily been used in *in vivo* behavioural studies and so information of its effects *in vitro* are limited. The concentrations of AM251 and O-2050 were restricted to 100nM to limit non-selective effects (Section 5.3.2). AM251 did not

significantly affect insulin secretion at 20mM glucose and did not significantly affect the inhibition of insulin secretion caused by AEA (Figure 4.2A). As observed with AM251, O-2050 alone did not affect maximal levels of insulin secretion and O-2050 did not affect the inhibitory effects of AEA (Figure 4.2B)

A



B

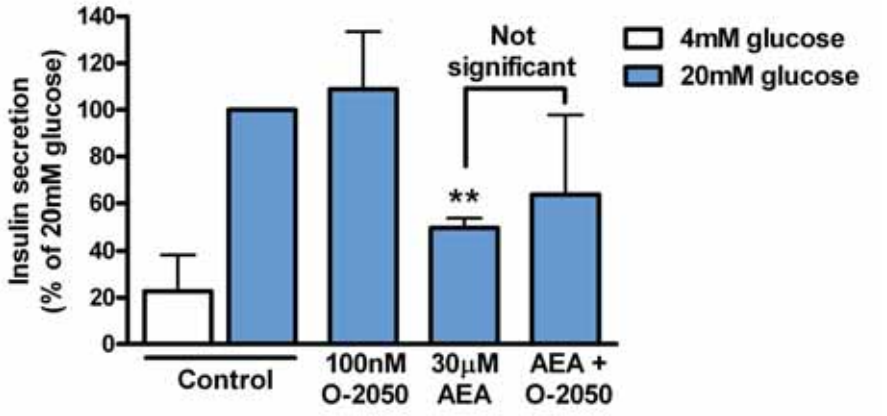


Figure 4.2 The effects of the CB1 receptor antagonists AM251 and O-2050 on responses to AEA. Islets were incubated at either 4mM or 20mM glucose in the absence (control) or presence of 30μM AEA, with or without a CB_1 receptor antagonist. A) Effects of AM251 on responses to AEA. Results are presented as mean insulin secretion rates ± SD (n= 5). * P< 0.05 vs. 20mM glucose control. B) Effects of O-2050 on responses to AEA. Results are displayed as mean insulin secretion rates ± SD (n= 5). ** P> 0.01 vs. 20mM glucose control.

4.3.1.2 Effects of the CB_2 receptor antagonist/inverse agonist AM630 on responses to anandamide

AM630 is described as a CB_2 receptor-selective antagonist/inverse agonist which has been used to attenuate CB_2 receptor signalling in islets without affecting insulin secretion itself (Ross *et al.*, 1999; Mukherjee *et al.*, 2004; Juan-Pico *et al.*, 2006;

Bermudez-Silva *et al.*, 2008). The concentration of AM630 used here was 100nM to limit the possibility of AM630 affecting insulin secretion itself (see Section 5.3.3). In six experiments where AM630 was used in conjunction with AEA, it was found that that AM630 did not significantly affect 20mM glucose-stimulated insulin secretion (Figure 4.3). The compiled results also suggest that AM630 did not affect the inhibition of insulin secretion caused by AEA (Figure 4.3). However, on further analysis, it appears that in four of the six experiments, 100nM AM630 attenuated or blocked the effects of 30μM AEA, whereas in the remaining two experiments, AM630 appeared to potentiate the effects of AEA (Table 4.2).

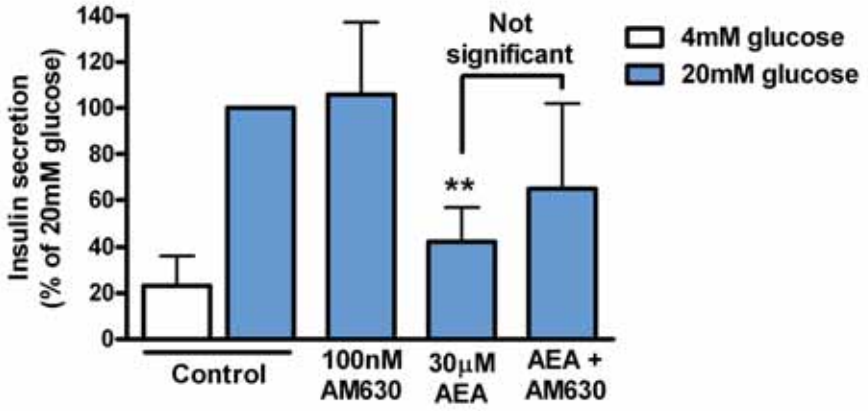


Figure 4.3 The effects of AM630 on responses to AEA. Islets were incubated at either 4mM or 20mM glucose in the absence (control) or presence of 30μM AEA with or without 100nM AM630. Results are presented as mean insulin secretion rates ± SD (n= 6). ** P> 0.01 *vs.* 20mM glucose control

Table 4.2 Mean insulin secretion rates for the six individual experiments examining the effect of AM630 on AEA-induced inhibition of insulin release

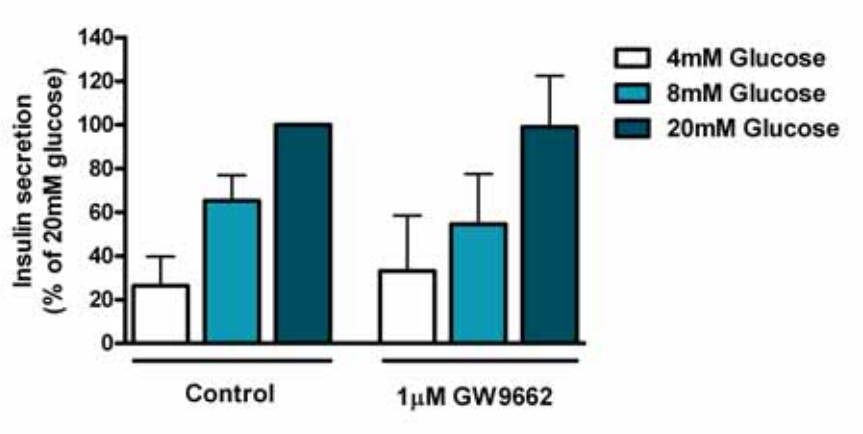
Experiment no	100nM AM630	30μM AEA	30μM AEA + 100nM AM630	Net effect on 30μM AEA
1	100 ± 12.5	52.6 ± 18.5	96.3 ± 18.21	+43.7
2	136 ± 10.0	54.0 ± 12.4	111 ± 7.2	+57
3	148 ± 13.0	39.8 ± 20.6	70.9 ± 29.4	+31.1
4	66 ± 8.6	15.7 ± 2.1	64.9 ± 9.3	+49.2
5	89.7 ± 17.5	39.6 ± 12.5	24.3 ± 7.6	-15.3
6	92 ± 27.7	51.5 ± 12.5	19.8 ± 9.6	-31.7

All values are the mean insulin secretion rates expressed as a percentage of the 20mM glucose control. Net effect on 30μM AEA was calculated as the mean experimental insulin secretion rate for 30μM AEA + 100nM AM630 minus mean experimental insulin secretion rate for.

4.3.1.3 Effects of the PPARγ-antagonist GW9662 on responses to anandamide

GW9662 (1μM) did not affect basal, intermediate or maximal levels of glucose-stimulated insulin secretion (Figure 4.4A). Subsequent testing found that GW9662 did not affect the level of inhibition caused by AEA at 20mM glucose (Figure 4.4B).

A



B

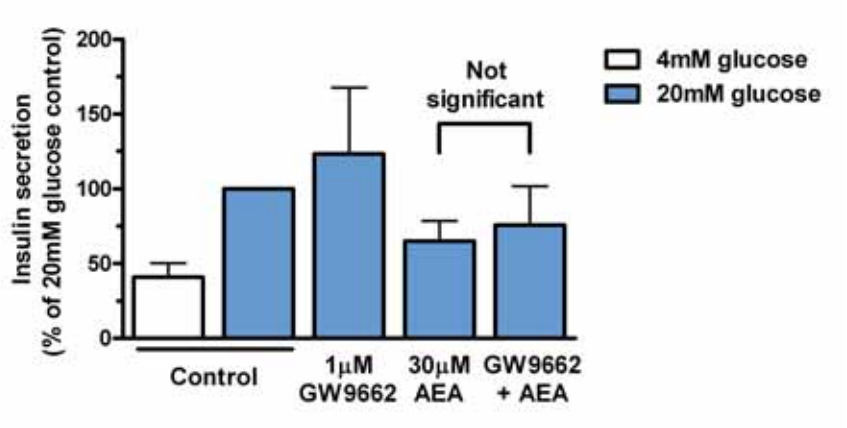


Figure 4.4 The effects of GW9662 on glucose-induced insulin release. A) Islets were incubated at basal (4mM), intermediate (8mM) or maximal (20mM) levels of insulin secretion in the absence (control) or presence of 1μM GW9662. Results are presented as mean insulin secretion rates ± SD (n= 10, except for conditions at 8mM glucose where n= 3). B) Islets were incubated at either 4mM or 20mM glucose in the absence (control) or presence of 30μM AEA with or without 1μM GW9662. Results are displayed as mean insulin secretion rates ± SD (n= 5).

4.3.2 Effects of AEA on insulin secretion from the INS-1 823/13 β-cell line

To determine whether the inhibitory effects of AEA on insulin secretion were caused by a direct effect on β-cell activity or through paracrine signalling insulin secretion experiments were performed in the INS-1 823/13 β-cell line. In compliance with the

previous insulin secretion experiments with AEA in islets, 1μM, 10μM and 30μM AEA were tested at 20mM glucose. AEA did not significantly affect insulin secretion at any concentration tested, Figure 4.5.

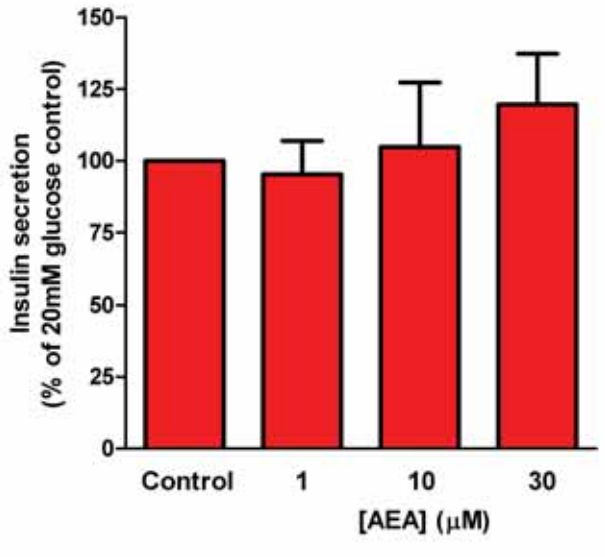


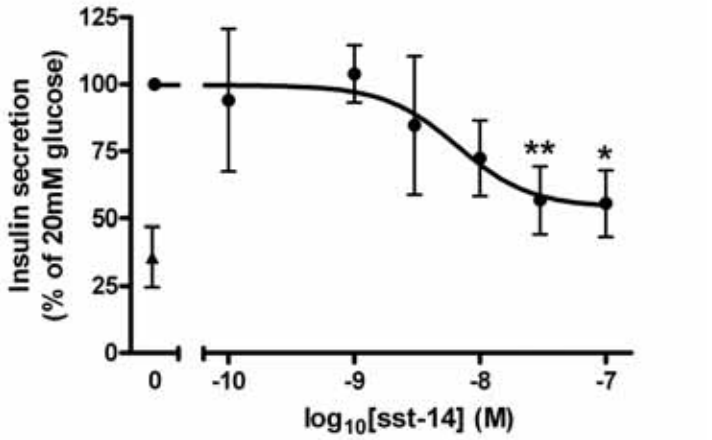
Figure 4.5 The effects of AEA on insulin secretion at 20mM glucose from INS-1 812/13 β-cells. INS 812/13 β-cells were incubated at 20mM glucose in the presence of vehicle (ethanol at a final concentration of 1% (v/v)) or with increasing concentrations of AEA for either 1 or 2 hours. Results are presented as mean insulin secretion rates ± SD (n= 3).

4.3.3 Effects of somatostatin-14 on glucose-stimulated insulin secretion

Next, a series of experiments were performed to investigate whether AEA inhibited insulin secretion via somatostatin-14 (sst-14) signalling within islets. Sst-14 potently inhibited insulin secretion at 20mM glucose in a concentration-dependent fashion, with inhibition occurring at concentrations as low as 10nM. The IC_{50} and Hill slope of sst-14 were calculated as 3.8nM (95% CI: 1.2nM to 11.6nM; Figure 4.6A) and -5.9 ± 9.9, respectively. From the sst-14 concentration-response data (Figure 4.6), it was decided to use sst-14 at concentration of 100nM in future experiments to produce a reproducible inhibition of insulin secretion. Preliminary experiments with the putative SSTR5 antagonist BIM-23056 were unsuccessful as BIM-23056 (at concentration ≤ 30nM) was found to inhibit insulin secretion at 20mM glucose (data not shown).

In a subsequent set of experiments, it was found that the effects of 100nM sst-14 and 10μM AEA on insulin secretion at 20mM glucose were variable without marked overall inhibitory effects as observed previously, whereas 10μM methanandamide caused a consistent inhibition of insulin secretion (Figure 4.7A). Further analysis of the individual experimental results indicated that islet responsiveness to 10μM AEA appeared to coincide with islet responsiveness to 100nM sst-14 but did not reach statistical significance (Figures 4.7B & 4.7C). As with the AEA concentration response data islet sensitivity to 100nM sst-14 and 10μM AEA did not coincide with glucose responsiveness (data not shown).

A



B

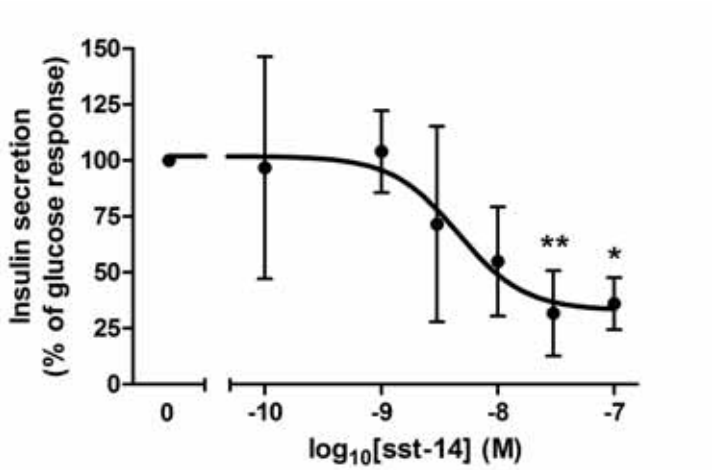
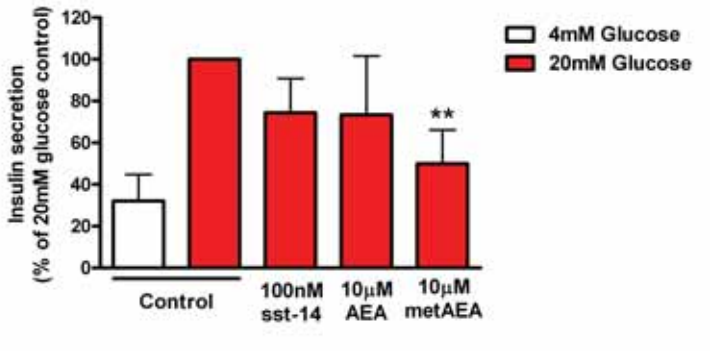
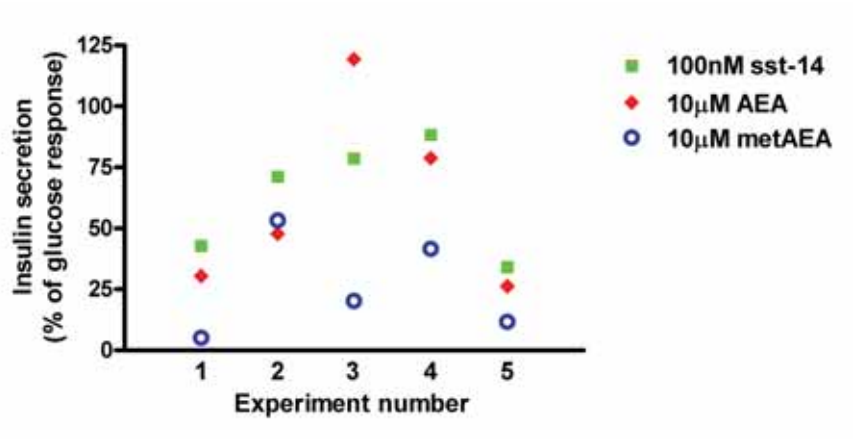


Figure 4.6 The effects of somatostatin-14 (sst-14) on insulin secretion at 20mM glucose. Islets were incubated at 20mM glucose in the presence of somatostatin-14 at increasing concentrations. A) Insulin secretion rates have been normalised against the 20mM glucose control. The mean basal secretion rate is represented as a triangle. B) Insulin secretion rates been normalised against the difference (glucose response) between the insulin secretion rates observed for the 4mM (0%) and 20mM (100%) glucose controls. All results are presented as mean insulin secretion rates ± SD (n= 4). * P< 0.05, ** P< 0.01 *vs.* 20mM glucose control.

A



B



C

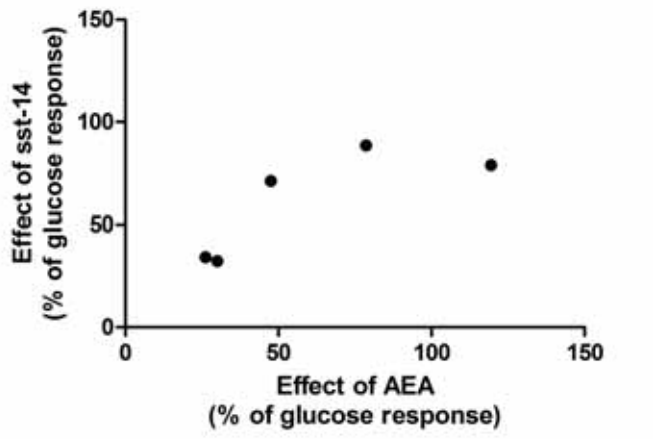
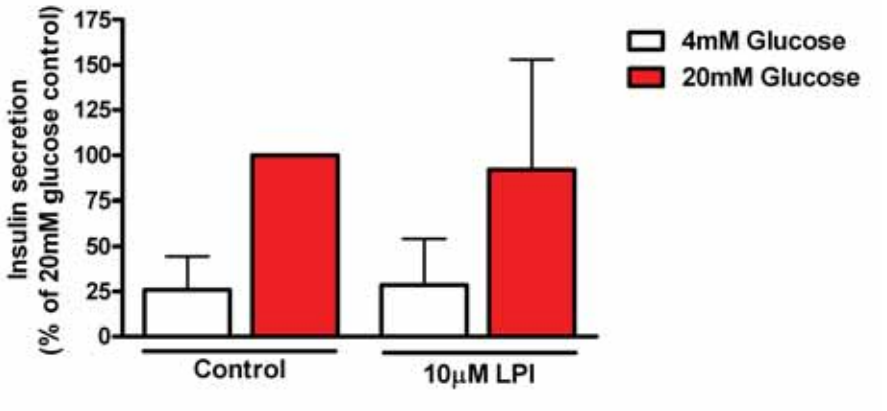


Figure 4.7 The effects of somatostatin-14 (sst-14), AEA and methanandamide (metAEA) on insulin secretion at 20mM glucose. Islets were incubated for 1-hour at 4mM or 20mM glucose in the absence (control) or presence of either 100nM somatostatin-14, 10μM AEA or 10μM metAEA. A) Compiled results which are presented as mean insulin secretion rates ± SD (n= 5). ** P< 0.01 *vs.* 20mM glucose control B) Individual experimental mean insulin secretion rates in the presence of sst-14, metAEA and AEA. Results from each experiment were standardised against the difference between the insulin secretion rates observed for the 4mM (0%) and 20mM (100%) glucose controls. C) The individual experimental mean insulin secretion rates (from figure B) of 10μM AEA plotted against the corresponding data for 100nM sst-14.

4.3.4 Effects of lysophosphatidylinositol (LPI) on glucose-stimulated insulin secretion

LPI (10μM), a putative endogenous GPR55 agonist, was used at basal and maximal levels of GSIS, and it was found that LPI did not significantly affect insulin secretion at 4mM (Figure 4.8A). However, LPI was found to have a variable effect on insulin secretion at 20mM glucose (Figure 4.8B).

A



B

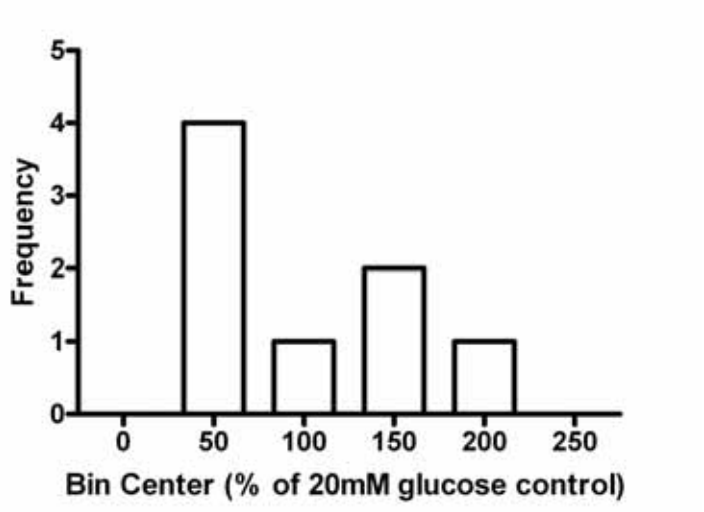


Figure 4.8 The effects of the endogenous GPR55 ligand lysophosphatidylinositol (LPI), on insulin secretion. A) Islets were incubated at 4mM or 20mM glucose for 1 hour in the absence or presence of 10μM LPI. Results are presented as mean insulin secretion rates ± SD (n= 8). B) The effects of 10μM LPI on 20mM glucose-induced insulin secretion expressed as a histogram produced from the 20mM glucose + 10μM LPI data presented in Figure A.

4.4 Discussion

AEA at 30μM caused a consistent and reproducible inhibition of insulin secretion at 20mM glucose and therefore was used in all antagonism studies. Cannabinoid CB_1, CB_2, and PPARγ receptor antagonists did not cause reproducible alterations in islet responses to 30μM AEA. However, on closer examination results from antagonism studies with the CB_2 receptor antagonist AM630 indicated that 30μM AEA may inhibit insulin secretion by more than one inhibitory signalling pathway (including a CB_2 or CB_2-like receptor dependent signalling pathway). Despite attempts to establish a direct link between AEA and sst-14-mediated inhibition of insulin secretion preliminary results found that islet responsiveness to 10μM AEA coincided with islet responsiveness to 100nM sst-14. Additionally preliminary findings with LPI, a putative GPR55 agonist, suggest that the effects of AEA are unlikely to be mediated by GPR55.

4.4.1 Effects of CB_1-, CB_2-, and PPARγ-receptor antagonists on responses to 30μM AEA

Classically, AEA is assumed to act largely in a CB_1 receptor-dependent manner but Juan-Pico et al. (2006) have also indicated significant activity at CB_2 receptors. Due to recent interest in the involvement of PPARγ in cannabinoid actions, as well as the use of PPARγ agonists for the treatment of type 2 diabetes (Section 1.3.3), the role of PPARγ signalling was also tested. Hence, the antagonists described as being CB_1 receptor (AM251 and O-2050), CB_2 receptor (AM630) and PPARγ (GW9662) receptor-specific were used in order to probe the mechanisms of action of AEA. When islets were co-incubated in the presence of AEA and a CB_1, CB_2, or PPARγ receptor antagonist, none of the receptor antagonists examined were found to consistently attenuate the inhibitory effects of AEA. While there is little consensus on the expression patterns of the CB receptors in islet cells, it appears that the expression of the CB receptors within islet to be constant/consistent between lean, obese and type 2 diabetic tissue (Bermudez-Siva *et al.*, 2006; Juan-Pico *et al.*, 2006; Starowicz *et al.*,

2008; Tharp *et al.*, 2008; Bermudez-Silva *et al.*, 2009). Similarly, the expression of PPARγ has been reported to be consistent between islets isolated from lean non-diabetic rodents (Braissant *et al.*, 1996; Patane *et al.*, 2002). Therefore, it was assumed that if AEA was inhibiting insulin secretion through a single receptor type then use of the correct type of receptor specific antagonist should consistently attenuate or block the effects of AEA. Thus, the results presented in this chapter suggest that 30μM AEA inhibits insulin via a mechanism that appears to be independent of CB_1, CB_2, and PPARγ signalling pathway. This conclusion appears to be verified by initial observations in Chapter 5, which found that use of 1μM ACEA (CB_1 receptor-specific agonist) and 1μM JWH133 (CB_2 receptor-specific agonist) did not consistently reproduce the inhibitory effects of AEA.

Although the present study would appear to exclude the involvement of 'classical' cannabinoid and PPARγ receptors, this is based on the individual application of antagonists. It is conceivable that AEA might have a multiple sites of action and so future experiments might be designed with combinations of antagonists to address this possibility.

Recent evidence from Vilches-Flores *et al.* (2010) suggests that CB_1 receptor expression in islets (determined by RT-PCR and immunohistochemistry) is glucose-dependent, as higher expression levels were described in islets isolated from Wistar rats that were fasted prior to death. It is unclear whether CB_2 receptor expression within islets is influenced by the fasting state of the animal too. As the Wistar rats used in this study were fed *ad libitum* then it may be possible that CB receptor expression within islets also varied between preparations. Hence, it may be more reasonable to re-assess the experimental results from the antagonism studies on an individual islet batch basis as cannabinoid receptor signalling may differ between islets. When the data are viewed in this context, then it appears that AEA inhibited insulin secretion by a CB_2 receptor-dependent signalling pathway, as in the majority of islets co-incubation with AEA and AM630 attenuated the inhibitory effects of AEA

(Table 4.2). Interestingly, in the remaining two experiments of this type, co-incubation of islets with AEA and AM630 resulted in a greater inhibition of insulin secretion (Table 4.2). Therefore, the effects of 30μM AEA may be restricted to a CB_2 (or CB_2-like) receptor signalling pathway in some islets, but in other islets AEA at 30μM may act by other signalling pathways. It may also be that the responsive state of islets to AEA could be due to the presence or absence of a specific signalling pathway. As such, in non-responsive islets, 30μM AEA may be able to cause significant activation of signalling pathways that concentrations of AEA ≤ 10μM would not be able to stimulate. Therefore, future antagonism experiments should be designed around a complex, rather than a simplistic, pharmacological model of cannabinoid signalling within islets.

4.4.2 Effects of lysophosphatidylinositol on glucose-stimulated insulin secretion

LPI has been reported to be an endogenous ligand for the GPR55 receptor by several *in vitro* studies using transfected HEK293 cell lines and primary cells (Section 1.4.9). A concentration of 10μM LPI was chosen as it has been reported to induce maximal or near maximal responses in GPR55 expressing cells at this concentration (Oka *et al.*, 2007; Lauckner *et al.*, 2008; Henstridge *et al.*, 2009; Oka *et al.*, 2009; Yin *et al.*, 2009). LPI did not affect the basal insulin secretion rate but the effect of LPI at 20mM glucose varied. In some experiments, it was found that LPI caused significant potentiation (> 25%) of insulin secretion, whereas in others LPI potently inhibited insulin secretion.

At present, there are no published data regarding the expression of GPR55 in islets or the pancreas as a whole. Hence, it is unknown whether any of the effects of LPI on insulin secretion were GPR55-mediated. As mentioned in Section 1.4.9, experimental evidence suggests that activation of GPR55 leads to activation of RhoA-ROCK signalling and/or PLC via $G_{\alpha 12/13}$- or G_q-coupled receptor signalling, respectively. Information regarding RhoA-ROCK signalling in islets is limited but information from a

study using dispersed primary β-cells, from male Wistar rat islets, suggests that activation of this signalling pathway inhibits GSIS by stabilisation of the F-actin cytoskeleton (Hammar *et al.*, 2009). Based on the activation of other G_qPCR receptors in β-cells, it would be expected that if GPR55 were signalling via Gq signalling pathways, then this would result in the potentiation of insulin secretion (Sawaki *et al.*, 1993; Iismaa *et al.*, 2000; Duttaroy *et al.*, 2004; Waldeck-Weiermair *et al.*, 2008; Henstridge *et al.*, 2009). Therefore, if both effects of LPI were GPR55-mediated then this would suggest that AEA was inhibiting by a GPR55 receptor-independent signalling pathway.

Alternatively, the inhibitory and stimulatory effects of LPI on insulin secretion (Figure 4.8D) could be due to alternate signalling pathways as 10μM LPI has been reported to potentiate TRPV2 (transient receptor potential vallinoid-type 2, divalent ion channel) and TREK-2 (TWIK-related K^+ channel 2) activity in transfected cell lines (Lesage *et al.*, 2000; Monet *et al.*, 2009). TRPV2 expression in mouse islets is believed to be restricted to β-cells and information gathered from primary islet β-cells and MIN6-cells suggests that activation of TRPV2 channels leads to the influx of extracellular Ca^{2+}, which may also explain the potentiation of insulin secretion by LPI (Hisanaga *et al.*, 2009). TREK-2 has been described as a slightly inward-rectifying K^+ channel in both transfected COS cell and natively-expressing MIN6 cells (Lesage *et al.*, 2000; Kang *et al.*, 2004). Lesage *et al.* (2000) postulated that activation of TREK-2 in β-cells may lead to a reduction in GSIS by re-stabilisation of the resting (polarised) state as the channel was more active in the depolarised state than the hyperpolarised state (Lesage *et al.*, 2000). Therefore, further work is needed to characterise LPI signalling within islets to ascertain whether either effect of LPI was GPR55-mediated. It may first be appropriate to determine whether islets express GPR55 mRNA by RT-PCR before characterising GPR55 signalling by pharmacological means. This could be further examined by determining whether islets express GPR55 receptors under experimental conditions by western blotting and/or immunohistochemistry too.

4.4.3 AEA as a paracrine mediator of islet activity

As shown in Table 4.1, expression of cannabinoid receptors has been detected in rat islets on the surfaces of non-β cells, therefore endocannabinoids may mediate inhibition of insulin secretion by activating CB receptors on the β-cells or through paracrine signalling within islets. To determine whether the effects of AEA were due to cannabinoid signalling in β-cells, the INS-1 823/13 β-cell line was used (Hohmeier *et al.*, 2000). The primary advantage of this approach over the use of purified β-cells was that INS-1 823/13 β-cells can be rapidly grown, whereas there was insufficient primary tissue available to produce the required amounts of primary β-cells required for insulin secretion studies. Hence, the effects of AEA, at micromolar concentrations, on insulin secretion at 20mM glucose in INS-1 823/13 were tested but it was found that AEA did not significantly affect insulin secretion in these cells. The lack of effect of AEA in these cells may have been caused by de-differentiation of the INS-1 823/13 cells (through successive passages) which leads to the progressive loss of the β-cell phenotype in β-cell lines (Hohmeier *et al.*, 2000; Nakashima *et al.*, 2009). This could have occurred as the INS-1 823/13 were not found to be glucose-repsonsive. The results may also have been indicative that the inhibition of insulin secretion (caused by AEA in islets) was due to alterations in paracrine signalling, therefore, this was investigated further.

Based on the information in Section 1.2.3, it was viewed that the most likely paracrine mechanism by which AEA could be inhibiting insulin secretion was through potentiation of sst-14 secretion from δ-cells. The general consensus is that sst-14 inhibits insulin secretion by a SSTR5-mediated signalling pathway, but, as shown previously in Table 1.2, there is also evidence that inhibition may occur via SSTR2-mediated signalling pathway (Rossowski & Coy, 1994; Atiya *et al.*, 1997; Zambre *et al.*, 1999; Strowski *et al.*, 2000; Cejvan *et al.*, 2003; Strowski *et al.*, 2003; Wang *et al.*, 2004; Yao *et al.*, 2005; Singh *et al.*, 2007). In accordance with the literature, sst-14 was found to inhibit insulin secretion at 20mM glucose in a concentration-dependent manner with high potency (Figure 4.6) and, from these data, it was decided that sst-14 would be used at 100nM to produce a reproducible and consistent inhibition of insulin

secretion (Schuit *et al.*, 1989; Hurst & Morgan, 1990; Rossowski & Coy, 1994; Wang *et al.*, 2004). It was planned that SSTR antagonists would be used to verify the involvement of endogenous sst-14 in the mechanism of AEA-mediated inhibition of insulin secretion. However it was found that BIM-23056, a putative SSTR5 selective antagonist, inhibited insulin secretion itself. Hence, it was not possible to directly test whether AEA was inhibiting insulin secretion by a sst-14 mediated mechanism by pharmacological means.

During the testing of BIM-23056, it was found that islet responsiveness to 100nM sst-14, in common with AEA, was variable with islets appearing to either be responsive or non-responsive to sst-14. The data presented in Figure 4.7B suggest that AEA responsive islets are also responsive to sst-14, implying that at concentrations ≤ 10μM, AEA may inhibit insulin secretion by an sst-14-dependent mechanism. However, there was not a significant correlation between the inhibitory effects of sst-14 and AEA but this may be due to the limited number of experiments in this data set. Therefore, additional experiments should be performed to verify whether the observation that inhibition of insulin secretion by 10μM AEA is mediated by sst-14. If AEA inhibited insulin secretion by an sst-14 dependent signalling mechanism then variability in AEA responsiveness may be dependent on the δ-cell content of the islets. The composition of islets (ratio of β: α: δ cells) is known to differ between different regions of the pancreas (Tasaka *et al.*, 1989). Therefore, the variation in islet responses to AEA and sst-14 may have inadvertently been pre-determined by the islet isolation step as a larger number of islets may have isolated from a certain region of the pancreas despite whole pancreata being used (especially as islet preparations are considered, at best, to be 50% efficient; van Suylichem *et al.* (1992)). Alternatively the SSTR receptors may have been degraded (Turcot-Lemay *et al.*, 1975) or δ-cells lost (Hauge-Evans *et al.*, 2009) during the collagenase isolation step. Thus, the responsiveness of islets to AEA may be linked to the number of functional SSTR receptors left on the plasma membranes of the β-cells after the process of islet isolation. Therefore, use of islet culture may be beneficial to allow the β-cells to

synthesis new somatostatin receptors but, as mentioned in Chapter 3, the use of islet culture may also affect CB signalling in islets. However, both theories are confounded with the observation that 10μM methanandamide-mediated inhibited of insulin secretion consistently. Alternatively, 10μM methanandamide might be able to inhibit insulin secretion through several different signalling pathways in a manner similar to 30μM AEA (Section 4.4.1).

Measurement of sst-14 and insulin secretion by RIA in parallel could be used in future studies to determine whether alterations in somatostatin secretion coincide with the inhibition of insulin secretion. This was attempted in the current study but too few islets (5 islets/well) were used to reliably detect sst-14 secretion by ELISA. As described in Section 1.2.3, glucagon secretion may also contribute to maximal rates of insulin secretion. Therefore, determination of glucagon secretion by RIA may also be beneficial. This too could be expanded to determine if cannabinoid signalling may alter glucagon secretion at low glucose concentrations. This information would also develop a more complete understanding of the physiological role that cannabinoid signalling has in islets.

On reviewing the literature regarding somatostatin signalling in islets, two studies found that binding of radiolabeled sst-14 increased in proportion to the external glucose concentration (Mehler *et al.*, 1980; Draznin *et al.*, 1985). Hence, AEA may be able to increase somatostatin signalling independently or in parallel to potentiating somatostatin secretion. Therefore, alternate approaches aside from measuring somatostatin secretion may be needed to confirm negative findings. Alternate approaches may include expansion of the INS-1 823/13 β-cell work by co-culturing β-cells with δ-cell lines (and possibly α-cell lines) to confirm the whether the effects in islets can be recreated in mixed islet-cell line populations. Finally, alternate SSTR antagonists could be tested to assess whether AEA-mediated inhibition of insulin secretion is dependent on SSTR5 and/or other SSTR receptor signalling.

4.4.4 Conclusions

The experiments performed within this chapter were designed with the notion that cannabinoid signalling was conserved in islets and that variation in the responsiveness of islets to AEA (as observed in Chapter 3) was the result of local metabolism. Based on such a hypothesis, it was found that AEA inhibited insulin secretion in a CB_1, CB_2, and PPARγ receptor independent manner. However, data from the AEA antagonism studies and the sst-14 studies suggests that AEA signalling within islets was more complex than originally thought. For instance, islet responsiveness to AEA at concentrations ≤ 10μM may be solely dependent on a sst-14 mediated signalling pathway, whereas at higher concentrations AEA may be able to inhibit insulin secretion by a sst-14 mediated signalling pathway and/or by a CB_2 (or CB_2-like) receptor signalling pathway and/or other unidentified pathway(s). Intriguingly, such a scenario may not only be dependent on the concentration of AEA used but may be influenced by the islet cell composition and fasting state of the animal prior to tissue collection. Unfortunately, the data within this chapter is not sufficient to verify such a model.

5

The acute effects of cannabinoid receptor ligands on insulin secretion from Wistar rat isolated pancreatic islets

5.1 Introduction

As discussed in Chapter 4, the expression of the cannabinoid (CB) receptors (CB_1 and CB_2) have been detected in pancreatic islets isolated from rats. However, the specific roles of the CB_1 and CB_2 receptors in the control of insulin secretion have not been characterised. This information may clarify the signalling pathways involved in AEA-mediated inhibition of insulin release. Therefore, the principal aim of this chapter was to characterise the acute effects of CB_1- and CB_2-receptor activation on insulin secretion. Hence, CB_1 and CB_2 receptor agonists and antagonists were used in freshly isolated islets of Langerhans from male Wistar rats.

5.2 Methods

5.2.1 Materials

All drugs and buffers were prepared as described in Sections 2.2.

5.2.2 Animals

All procedures were performed using tissues obtained from male Wistar rats with body weight ranging from 230-350g. Animals were housed and killed as described in Section 2.1.

5.2.3 Insulin secretion studies

Islets were isolated from male Wistar rats according to the isolation procedure described in 2.4.1.1.

5.2.3.1 Static incubations

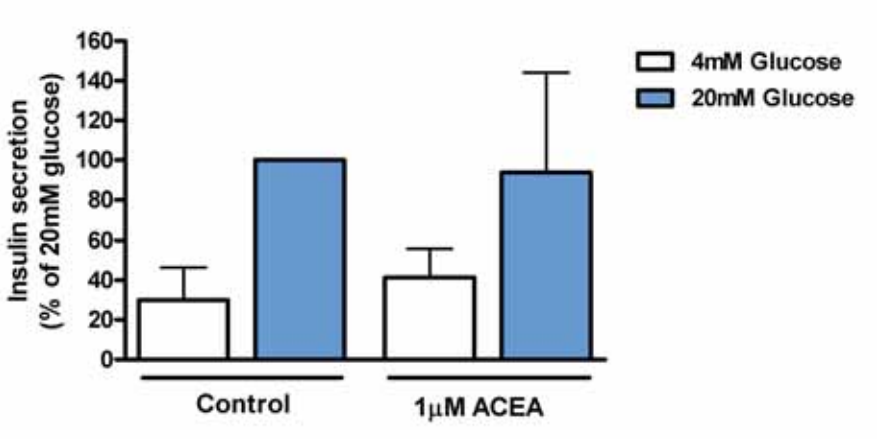
Freshly isolated islets were used in static incubation studies, as described in 2.4.2. Once the one hour incubation had finished, samples of incubation buffer were taken from each well and the amount of insulin in the samples was determined by RIA as described in 2.6. Analysis of experimental data was carried out as described in 2.4.5.

5.3 Results

5.3.1 Effects of arachidonyl-2-chloroethylamide (ACEA), a CB_1 receptor agonist, on glucose-dependent insulin secretion

ACEA, a CB_1 receptor agonist, was chosen to investigate CB_1 receptor signalling as it is reported to have a high affinity, efficacy, and selectivity for the CB_1 receptor (Hillard *et al.*, 1999). ACEA has been used in a number of cell types and tissues, with 1μM ACEA typically reported in most studies to induce substantial CB_1 receptor specific signalling events (Sterin-Borda *et al.*, 2005; Mato *et al.*, 2009; Cencioni *et al., 2010*). In isolated islets ACEA (1μM) did not affect basal rates of insulin secretion and failed to consistently affect 20mM glucose stimulated insulin secretion, with a large degree of variability in the secretory response at 20mM glucose (Figure 5.1A). Subsequent experiments with 10μM and 30μM ACEA also appeared not to affect insulin secretion at 20mM glucose either but again there was wide variability (Figure 5.1B).

A



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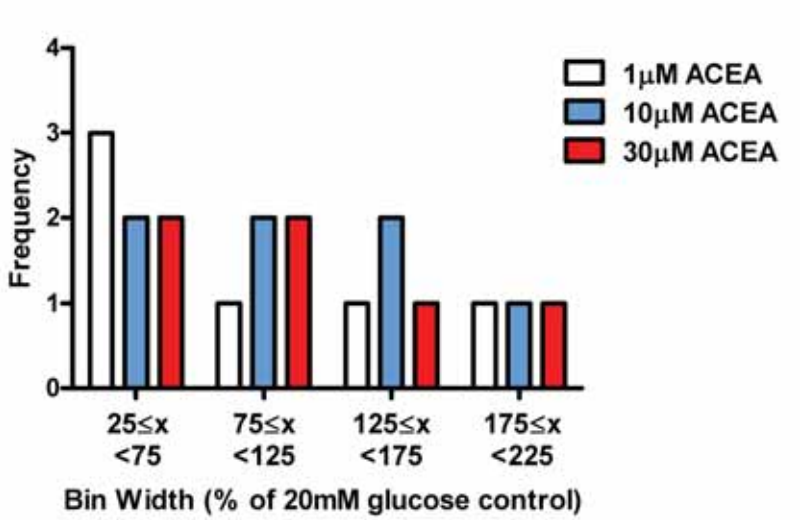


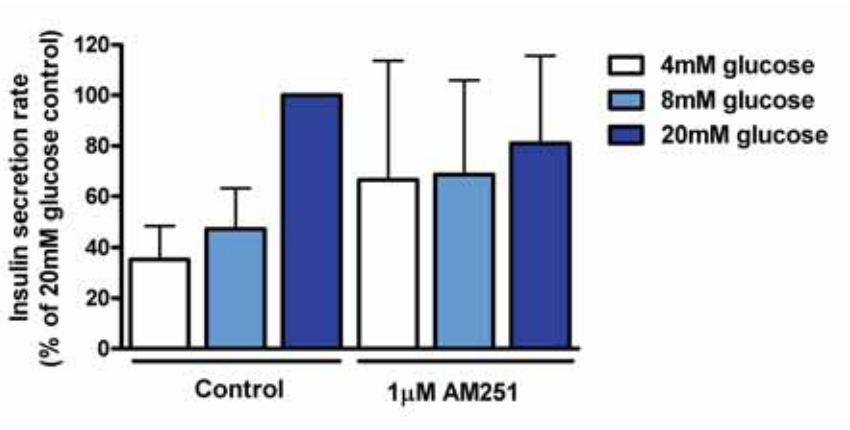
Figure 5.1 The effects of ACEA, a CB_1 receptor agonist, on glucose-dependent insulin secretion. A) Islets were incubated at 4mM or 20mM glucose in the absence (control) or presence of 1μM ACEA for 1 hour. Results are presented as mean insulin secretion rates ± SD (n= 6). B) A distribution frequency presenting all the observed effects of ACEA at 1μM (n= 6), 10μM (n= 7) and 30μM (n=6) on insulin secretion at 20mM glucose.

5.3.2 Effects of CB_1 receptor antagonists, AM251 and O-2050, on glucose-dependent insulin secretion

AM251 (a CB_1 receptor antagonist) was tested at a concentration of 1μM as it has been reported that 1μM AM251 did not affect glucose stimulated oscillations in $[Ca^{2+}]_i$ or GSIS from murine islets, which may occur if higher concentrations of AM251 were used (Savinainen *et al.*, 2003; Juan-Pico *et al.*, 2006; Price *et al.*, 2007). The effects of 1μM AM251 were found to be highly variable with no consistent effect on insulin secretion being observed at any glucose concentration tested (Figure 5.2A). As the

observed effects of AM251 may have been due to non-specific concentration-dependent effects, concentration-response experiments with AM251 were performed at 20mM glucose (Figure 5.2B). At concentrations > 100nM, AM251 inhibited insulin release more frequently as the concentration increased. At concentrations > 1μM, AM251 consistently inhibited insulin secretion with the maximal inhibitory effects of AM251 being observed at 10μM. The IC_{50} of AM251 for inhibition of 20mM GSIS was 1.6μM (95% CI: 507nM to 3.3μM). The mean Hill slope was calculated as -3.1 ± 5.4.

A



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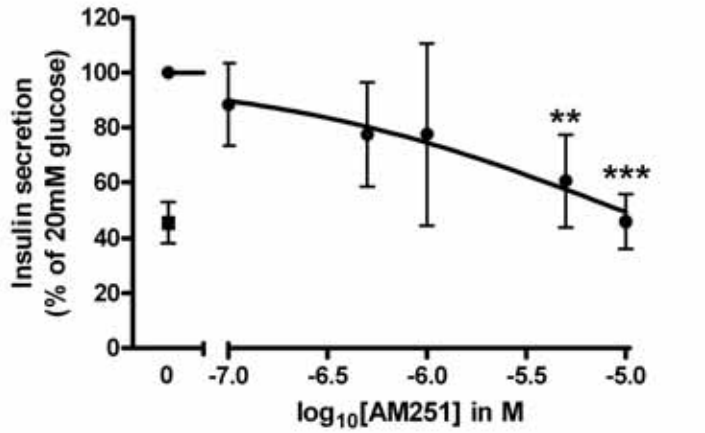


Figure 5.2 The effects of AM251, a CB_1 receptor antagonist, on glucose-dependent insulin release. A) Islets were incubated at 4mM, 8mM or 20mM in the absence (control) or presence of 1μM AM251. Results are presented as mean insulin secretion rates ± SD (n= 9). B) Islets were incubated at 20mM glucose with increasing concentrations of AM251. The mean basal insulin secretion rate is represented as a square. Results shown as mean insulin secretion rates ± SD (n= 6). ** P< 0.01, *** P< 0.001 *vs.* 20mM glucose control.

AM251 when used at a concentration of 10μM, caused a marked inhibition of insulin secretion at 20mM glucose, but in islets incubated at 4mM and 8mM glucose, AM251 did not significantly affect insulin release (Figure 5.3). The effects of 10μM AM251

may have been due to PPARγ activation (O'Sullivan *et al.*, 2007) or inverse agonism at the CB_1 receptor (Section 1.4.3). Therefore, 10μM AM251 was used in antagonist studies with GW9662, a PPARγ antagonist, and the CB_1 receptor antagonist O-2050. Use of 1μM GW9662 did not affect islet responses to AM251 (Figure 5.4A). O-2050 (100nM), which is structurally dissimilar to AM251, did not affect islet responses to 20mM glucose and failed to modify the inhibitory effect of 10μM AM251 (Figure 5.4B). At the higher concentration of 1μM, O-2050 did not affect insulin secretion at 20mM glucose but significantly attenuated the inhibitory effects of 10μM AM251 on insulin secretion (Figure 5.5A). Again, there appeared to be some variability in the responses to O-2050 alone since subsequent sub-analysis revealed that O-2050 at 1μM caused a slight inhibition of insulin release insulin secretion, whereas 100nM O-2050 did not (Figure 5.5B).

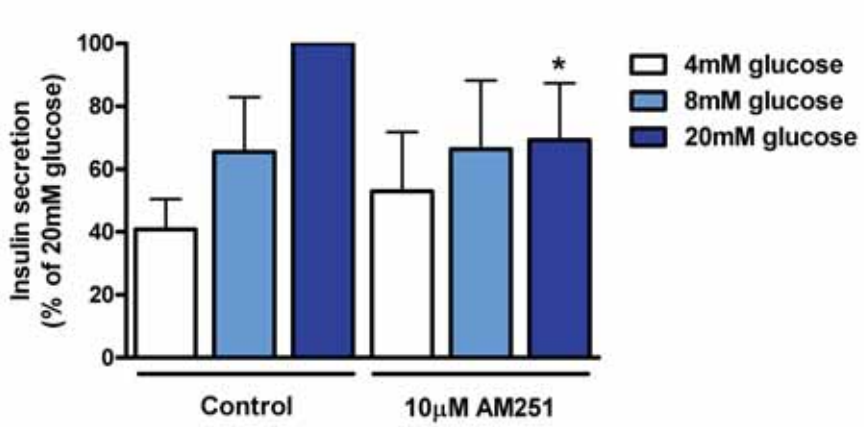
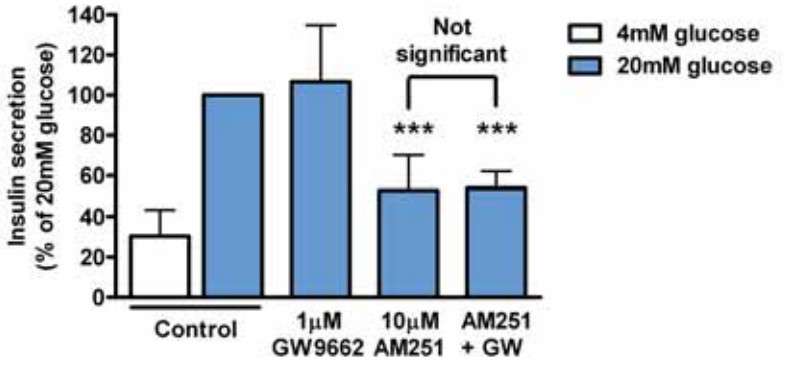


Figure 5.3 The effects of 10μM AM251 on glucose-dependent insulin secretion. Islets were incubated at 4mM, 8mM or 20mM in the absence (control) or presence of 10μM AM251. Results are presented as mean insulin secretion rates ± SD (n= 7). * P< 0.05 *vs.* 20mM glucose control.

A



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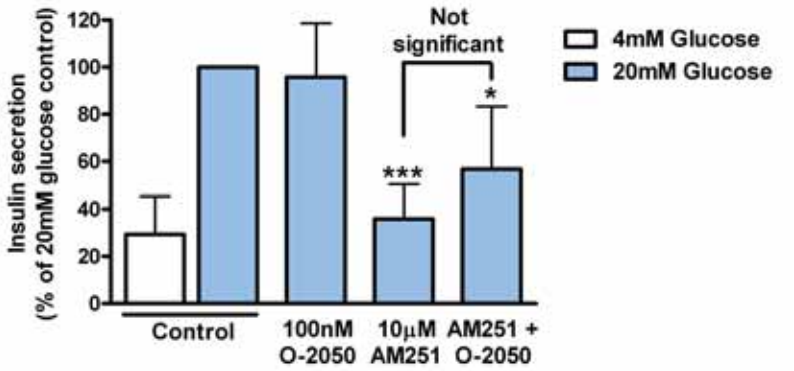
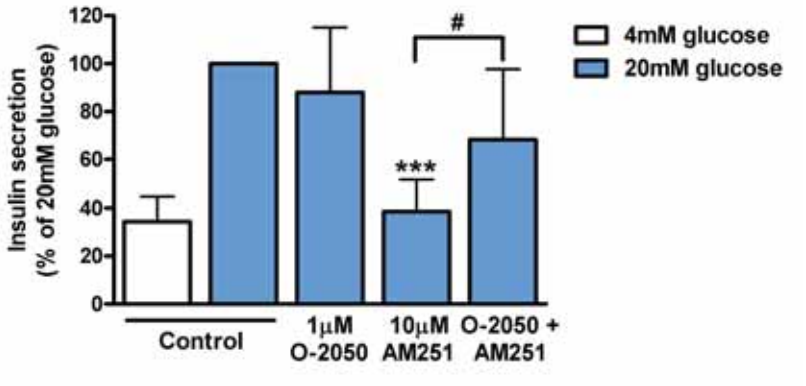


Figure 5.4 The effects of the PPARγ antagonist GW9662 and the CB_1 receptor antagonist O-2050 on islets responses to 10μM AM251. A) Islets were incubated for 1 hour at 4mM or 20mM glucose in the absence (control), or presence of 10μM AM251, with or without 1μM GW9662 (GW). Results are presented as mean insulin secretion rates ± SD (n= 6). *** P< 0.001 *vs.* 20mM glucose control B) Islets were incubated for 1 hour at 4mM or 20mM glucose in the absence or presence of 10μM AM251 with or without 100nM O-2050. Results shown are mean insulin secretion rates ± SD (n= 5) * P< 0.05, *** P< 0.001 *vs.* 20mM glucose control.

A



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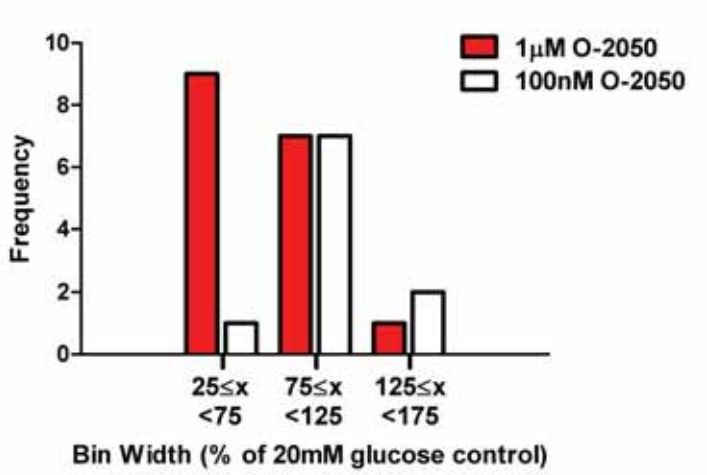
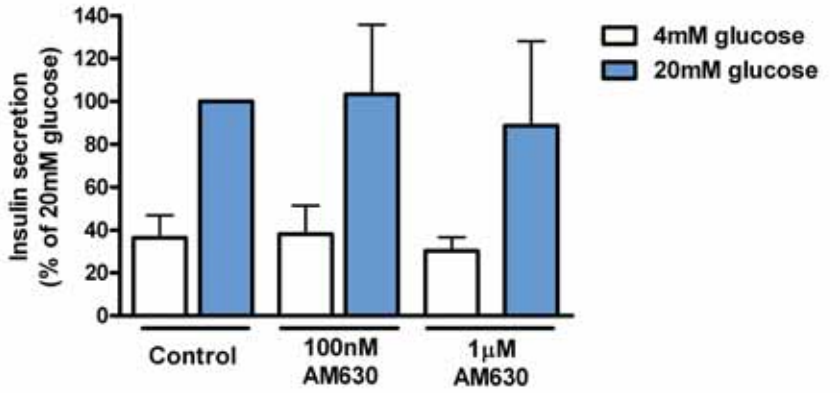


Figure 5.5 The effects of 100nM and 1μM O-2050 (O-2) on insulin secretion at 20mM glucose and islet responses to 10μM AM251. A) Islets were incubated for 1 hour at 4mM or 20mM glucose in the absence or presence of 10μM AM251 with or without 1μM O-2050. Results are presented as mean insulin secretion rates ± SD (n= 7). ***P< 0.001 *vs.* 20mM glucose control; # P< 0.05. B) Distribution frequency of all the observed effects of 100nM and 1μM O-2050 on insulin secretion at 20mM glucose. 1μM O-2050 n= 17; 100nM O-2050 n= 10.

5.3.3 The effects of CB_2 receptor ligands on glucose-dependent insulin secretion

AM630 is a CB_2 receptor antagonist but at concentrations ≥ 1μM AM630 may also act as a CB_1 receptor agonist in CB_1 receptor-transfected Chinese hamster overy cells (Ross *et al.*, 1999). Therefore, AM630 was not only tested at 100nM but at 1μM too. Neither 100nM nor 1μM AM630 significantly affected insulin release at 4mM or 20mM glucose (Figure 4.3 & 5.6A). JWH-133 has been reported to be a potent and selective (at concentrations ≤ 1μM) CB_2 receptor agonist and has been used in numerous studies performed in islets and other tissues to investigate the effects of CB_2 receptor signalling (Huffman *et al.*, 1999; Juan-Pico *et al.*, 2006; Mule *et al.*, 2007; Bermudez-Silva *et al.*, 2008). JWH-133 (1μM) failed to significantly alter insulin release at basal or maximal levels of GSIS (Figure 5.6B). However, on further experimentation, when used at 10μM, JWH-133 inhibited insulin secretion induced by 20mM glucose (by > 25%) in the majority of experiments (Table 5.1). In the same set of experiments, islets were also co-incubated with 100nM AM630 and 10μM JWH-133 to determine whether JWH-133 was acting in a CB_2 receptor-specific manner. Due to a large degree of variation in the effects of 100nM AM630, when used alone, it could not be verified whether the effects of 10μM JWH-133 were CB_2 receptor-specific (Table 5.1).

A



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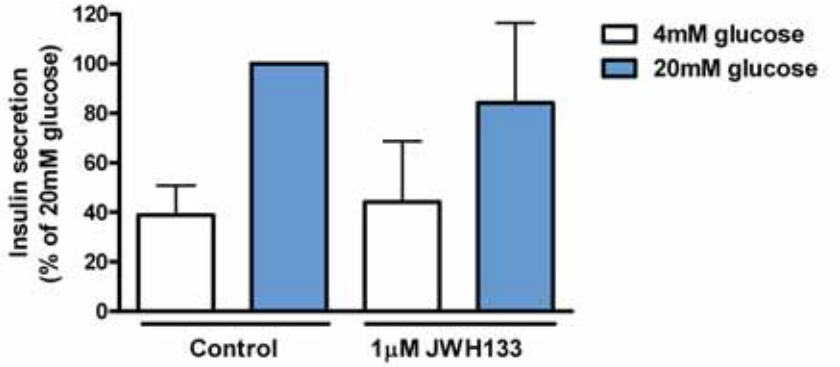


Figure 5.6 The effects of JWH-133 (a CB_2 receptor agonist) and AM630 (a CB_2 receptor antagonist) on basal and maximal levels of glucose-stimulated insulin secretion. A) Islets were incubated at 4mM or 20mM glucose for 1 hour in absence (control) or presence of AM630 at either 100nM or 1μM. Results are presented as mean insulin secretion rates ± SD (n= 7, except for 100nM and 1μM AM630 at 4mM glucose where n= 3). B) Islets were incubated at 4mM or 20mM glucose for 1 hour in the absence (control) or presence of 1μM JWH-133. Results are shown as mean insulin secretion rates ± SD (n= 9).

Table 5.1 The mean insulin secretion rates from 6 individual experiments detailing the effects of AM630 on islet responses to JWH-133.

Experiment no.	100nM AM630	10μM JWH-133	10μM JWH-133 + 100nM AM630	Net effect of 100nM AM630 on 10μM JWH-133
1	36.3 ± 3.4	36.3 ± 7.9	38.5 ± 7.1	+2.2
2	148 ± 13	32.2 ± 9.4	82.3 ± 23.3	+50.1
3	89.7 ± 6.8	32.7 ± 10.4	72 ± 14.3	+39.3
4	92 ± 27.7	49.5 ± 9.2	37.5 ± 6.1	-12.0
5	133 ± 23.4	66.8 ± 17.1	93.7 ± 7.6	+26.9
6	174 ± 44.8	105 ± 42.3	50.3 ± 12.1	-54.7

Islets were incubated for 1 hour at 4mM or 20mM glucose in the absence or presence of 10μM JWH-133 (a CB_2 receptor agonist) with or without 100nM AM630 (a CB_2 receptor antagonist). All values are the mean insulin secretion rate (expressed as a percentage of the 20mM glucose control) ± SD for n= 3-5 replicates per condition. The net effect of 100nM AM630 on 10μM JWH-133 was calculated as the mean insulin secretion rate for 30μM AEA + 100nM AM630 minus the mean insulin secretion rate for 10μM JWH-133.

5.4 Discussion

In this chapter, ligands which have been described as cannabinoid receptor-specific, were used to examine the involvement of CB_1 and CB_2 receptors in the regulation of insulin secretion. The CB_1 receptor agonist ACEA did not affect basal insulin secretion. In addition, ACEA did not produce a clear-cut effect on maximal levels of GSIS despite being tested at several micromolar concentrations. Similarly, JWH-133, a CB_2 receptor agonist, at 1μM did not affect basal or maximal levels of GSIS, but at 10μM, it inhibited insulin secretion at 20mM glucose. It is uncertain whether the effects of 10μM JWH-133 were CB_2 receptor-specific. In conjunction with testing of CB receptor agonists, the effects of CB receptor antagonists on insulin release were examined. AM251 (CB_1 receptor antagonist) inhibited insulin secretion in a glucose- and concentration-dependent manner at concentrations greater than 100nM. The inhibition of insulin secretion caused by 10μM AM251 was attenuated by another CB_1 receptor antagonist O-2050 (1μM) but not by the PPARγ antagonist GW9662. However, when used at 1μM, O-2050 behaved in a similar manner to AM251, causing an inhibition of GSIS in 53% of experiments in which it was used.

5.4.1 Effects of ACEA on glucose-dependent insulin secretion

In the present study, the CB_1 receptor agonist ACEA was found to have an inconsistent effect on 20mM glucose, as ACEA potentiated insulin secretion in some islets but inhibited insulin release in others (Figure 5.1B). To date, ACEA has been used at 100nM in two other islet studies, and it has been shown to inhibit insulin secretion in mouse islets but to potentiate insulin secretion in human islets (Bermudez-Silva *et al.*, 2008; Nakata & Yada, 2008). This may appear to suggest that there are species-dependent differences in the effects of ACEA between rodent and human islets. However, as discussed in Chapter 3, the difference in effect of ACEA could be due to how the islets were prepared, as Nakata & Yada (2008) using freshly isolated islets, whereas Bermudez-Silva *et al.* (2008) used cultured islets. Regardless of the effects of ACEA on insulin secretion, neither study reported ACEA to have

mixed effects on insulin secretion whereas, the effects of 1, 10 and 30μM ACEA in this study were variable (Figure 5.1B). CB_1-receptors has been described to be expressed along the periphery of the islets, in α-cells (Bermudez-Silva *et al.*, 2007; Starowicz *et al.*, 2008; Tharp *et al.*, 2008; Bermudez-Silva *et al.*, 2009; Vilches-Flores *et al., 2010*) and in δ-cells (Tharp *et al.*, 2008). As shown in Figure 5.7, the potentiation and inhibition of insulin secretion by ACEA could be explained by the hypothesis that CB_1 receptors, expressed on α- and δ-cells, are postively linked to the secretion of glucagon and somatostatin, respectively. However, CB_1 receptor-mediated potentiation of glucagon secretion (and the resultant increase in insulin release) would only be apparent if sst-14 signalling was somehow blocked in islets. As discussed in Section 4.4.3, islet respones to 100nM sst-14 were found to be variable suggesting that sst-14 signalling could be blocked in some islets, therefore, both scenarios appear to be feasible. As suggested previously for AEA (see Chapter 4), several approaches could be used to test whether certain effects of ACEA are linked to alterations in paracrine signalling events.

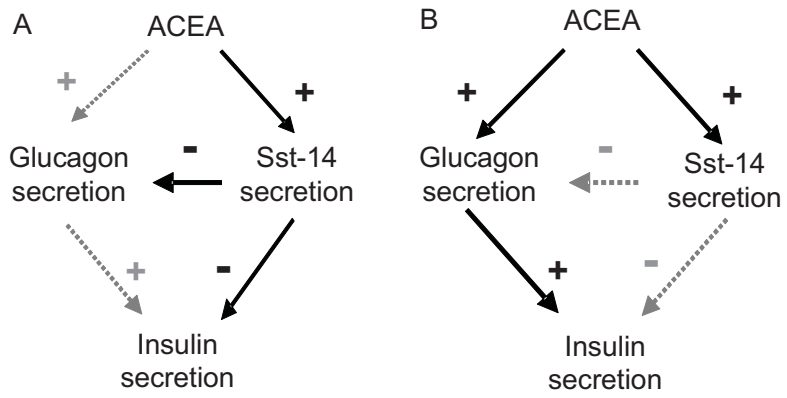


Figure 5.7 Two schematics of ACEA signalling in islets at 20mM glucose based on the assumption that CB_1 receptor signalling potentiates the secretion of glucagon, from α-cells, and sst-14, from δ-cells. A) ACEA-mediated potentiation of somatostatin-14 (sst-14) release inhibits the secretion of glucagon and insulin. B) ACEA potentiates the release of sst-14 but sst-14 signalling on the α- and β-cells is somehow blocked. Thus, allowing increased glucagon to be secreted from the α-cells, which in turn potentiates insulin release from the β-cells. Bold block arrows represent dominant signalling pathways and faint broken arrows signify recessive (A) or non-functioning (B) signalling pathways. + and - represent positive and negative effects on hormone secretion.

The differential effects of ACEA may be due to the occurrence of CB_1 receptor signalling in some islets, with non-CB_1 receptor signalling in other islets, despite

ACEA being described as CB_1 receptor-specific at 1μM (Hillard *et al.*, 1999; Mule *et al.*, 2007). The use of CB_1 and CB_2 receptor antagonists with ACEA would determine the specificity of ACEA signalling in islets. This would also help to clarify which effects of ACEA were associated with which CB receptor. However, as observed in this chapter, the availability of truly "neutral" antagonists at CB receptors which bind without affecting insulin secretion may be an issue. It should also be considered whether ACEA may be affecting insulin secretion via non-CB receptor(s). For instance, ACEA may be acting at GPR55 and/or a CB_2-like receptor as LPI (Chapter 4) and AM630 (discussed below) were the two only compounds, aside from ACEA, used in this study which were found to sporadically potentiate insulin secretion. The potentiation of insulin secretion by ACEA may have also been caused by activation of β-cell TRPV1 channels but currently the literature suggests that primary β-cells do not express TRPV1 channels (Akiba *et al.*, 2004; Price *et al.*, 2004; Razavi *et al.*, 2006; Gram *et al.*, 2007).

5.4.2 Effects of JWH-133 on glucose-dependent insulin secretion

In the literature, JWH-133 is described to act in a CB_2 receptor-dependent manner, with nanomolar concentrations of JWH-133 being reported to significantly inhibit insulin secretion in islets (human and mouse) but potentiate insulin secretion in RIN-m5F (β-cell line) cells (Juan-Pico *et al.*, 2006; De Petrocellis *et al.*, 2007; Mule *et al.*, 2007; Bermudez-Silva *et al.*, 2008). However, in the present study, JWH-133, used at a concentration of 1μM (several fold higher than that used in the studies described above) failed to affect insulin secretion in rat islets (Figure 5.6B). However, when used at 10μM, JWH-133 was found to inhibit insulin secretion in 83% of experiments. This is in accordance with earlier experiments, in which 30μM AEA was used in the presence of 100nM AM630, which lead to the suggestion that CB_2 (or CB_2-like receptors) may be negatively coupled to insulin secretion (Chapter 4). Assuming that CB_2 receptor signalling directly affects β-cell activity, the low potency of JWH-133 in islets may be due to the number of islet cells which express the CB_2 receptor. Indeed, immunohistological data presented in Bermudez-Silva *et al.* (2009) suggest that CB_2

receptors are not uniformly expressed by rat β-cells. Therefore, it may be beneficial in future work to determine whether the potency of JWH-133 is linked to the ratio of CB_2 receptor expressing β cells to non-CB_2 receptor expressing β-cells. Information from studies performed in other tissues suggests that at 10μM, JWH-133 can also act via the CB_1 receptor (Baldassano *et al.*, 2008). Hence, the specificity of 10μM JWH-133 should not only be examined in the context of CB_2 receptor signalling on insulin secretion but CB_1 receptor signalling too. As discussed with ACEA, this may not be easily implemented as it is uncertain whether other CB_1 or CB_2 antagonists would be able to block CB receptor signalling without affecting insulin secretion. It is also feasible that the effects of 10μM JWH-133 occurred independently of either cannabinoid receptor.

5.4.3 Effects of AM251 and O-2050 on glucose-dependent insulin secretion

Initial testing of the CB_1 receptor antagonist AM251 began with use of 1μM AM251 at basal, intermediate and maximal levels of GSIS. Despite 1μM AM251 being reported by Juan-Pico *et al.* (2006) not to affect glucose-stimulated oscillations in $[Ca^{2+}]_i$ in mouse islets, it was found in the current study that the effects of 1μM AM251 on GSIS were variable (Figure 5.2A). In subsequent experiments, AM251 displayed clear concentration-dependent effects on insulin secretion at 20mM glucose with 100nM AM251 not affecting insulin secretion, while 10μM AM251 fully inhibited insulin secretion. The study by Bermudez-Silva *et al.* (2008) confirms the finding that 100nM AM251 does not affect insulin secretion but the effects of AM251 at concentrations > 1μM have not been reported in islets. At low micromolar concentrations, AM251 and its structural analogue rimonabant, are either known or thought to act as inhibitors of adenosine A_1 receptors, dopamine transporters, voltage-gated L-type Ca^{2+} channels, K_{ATP} channels and large conductance Ca^{2+}-activated K^+ channels (White & Hiley, 1998; Savinainen *et al.*, 2003; Price *et al.*, 2007). Therefore, the inhibition of insulin secretion caused by AM251 may have been CB_1 receptor-independent. Subsequent experiments found that 10μM AM251 inhibited insulin secretion in a glucose-

dependent manner (Figure 5.3). Similar findings have also been reported with 1μM rimonabant on insulin secretion from Zucker Fatty rat islets but the results from the study by Getty-Kaushik *et al.* (2009) contrast with data presented in Chapter 6. This may be due to the use of overnight-cultured, instead of freshly isolated, Zucker Fatty rat isolated islets (Getty-Kaushik *et al.*, 2009). Similarly, 1μM BAR-1, a novel analogue of AM251, was reported by Vilches-Flores *et al.* (2010) to potentiate insulin secretion at 3mM and 16mM glucose in islets from Wistar rats, which is again contrary to the present findings with AM251. The differences in the effects of the CB_1 receptor antagonists on insulin secretion may be due to the use of freshly isolated islets in this study and the use of cultured islets in the study by Vilches-Flores *et al.* (2010). However, the contrasting findings between Vilches-Flores *et al.* (2010) and this study may be because AM251 and BAR-1 were affecting insulin secretion by different non-CB_1 receptor-mediated signalling events.

As the effects of AM251 may have been caused by PPARγ agonism, islets were co-incubated with GW9662, a PPARγ receptor antagonist (O'Sullivan *et al.*, 2007). The results from these experiments suggest that 10μM AM251 inhibited insulin secretion by a PPARγ receptor-independent signalling pathway. Based on the study by Chaytor *et al.* (1999), an alternative mechanism by which 10μM AM251 may inhibit insulin secretion is by the blockade of gap junctions (Bertuzzi *et al.*, 1999; Calabrese *et al.*, 2003; Rocheleau *et al.*, 2006). The synchronised release of insulin from islets is mediated by the activity of gap junctions which electrically couple the β-cells, allowing the co-ordinated release of insulin secretion from β-cells within an islet (Ravier *et al.*, 2005). This can be observed using calcium imaging techniques, as GSIS is Ca^{2+} dependent (Section 1.2.2). Therefore, in islets where gap junction function is inhibited, oscillations in cytosolic Ca^{2+} levels will no longer be synchronised between all the β-cells within an islet (Ravier *et al.*, 2005). Hence, Ca^{2+} imaging techniques could be used to determine whether 10μM AM251 was inhibiting gap junction functionality in islets. However, the study by Juan-Pico *et al.* (2006) has already investigated the affects of 1μM AM251 on glucose-stimulated Ca^{2+} oscillations and reported that

AM251 had no effect on glucose induced $[Ca^{2+}]_i$ oscillations in mouse islets. Additionally, Ravier *et al.* (2005) found that genetic knock-out of Connexin36 in mice abolished gap-junction function in β-cells. The resultant loss of gap junction function in islets was only found to significantly potentiate basal insulin secretion rates of insulin secretion but did not significantly affect GSIS (Ravier *et al.*, 2005). Therefore, inhibition of gap junction function does not appear to account for 10μM AM251 mediated inhibition of insulin release at 20mM glucose.

O-2050, another CB_1 antagonist, was used to determine whether the effects of AM251 were CB_1-receptor mediated, as CB_1 receptor signalling may potentiate insulin secretion in islets. In such a model, CB_1 receptors in islets may be constitutively-active (Section 1.4.3). Therefore, the inhibitory effects of AM251 on insulin release could be explained by inverse agonism (Xiao *et al.*, 2008). However, the CB_1 receptors may be constitutively activated by islet-derived endocannabinoids that are produced in a glucose-dependent manner (Konrad *et al.*, 1994; Bermudez-Silva *et al.*, 2008). Therefore, the apparent inverse agonistic activty of AM251 may also be due to blockade of an endocannabinoid tone in islets. O-2050 was chosen over other CB_1 antagonists despite being a relatively poorly-characterised CB_1 receptor antagonist, as O-2050, at the time of use, was reported to be devoid of inverse agonistic behaviour. Additionally, O-2050 is structurally dissimilar to AM251, therefore, it is less probable that the two CB_1 receptor antagonists would have the same non-CB_1 receptor mediated effects. Use of 100nM O-2050 did not consistently attenuate the effects of 10μM AM251. Therefore, the experiments were repeated with 1μM O-2050 and it was found that 1μM O-2050 did not significantly affect insulin secretion but consistently attenuated the inhibitory effects of 10μM on AM251 on insulin secretion. On further analysis, 1μM O-2050 was found to inhibit insulin secretion (by > 25%) in approximately half of the experiments (Figure 5.5B). As mentioned above, the effects of AM251 and O-2050 could be rationalised by the blockade of an islet-derived endocannabinoid mediated CB_1-receptor tone in islet. As ACEA did not consistently potentiate insulin secretion, the hypothesis that CB_1 receptor signalling potentiates

then seems unlikely. This suggests that 1μM O-2050 was inhibiting insulin secretion by non-CB_1 receptor-mediated signalling. This has also been suggested in the study by Gardner & Mallet (2006) which reported that O-2050 induced behavioural changes *in vivo* that were not associated with "classical" CB_1 receptor signalling. So, O-2050 may act similarly to AM251, with both molecules interacting through a common binding site at a CB_1-like receptor. The results suggest that O-2050 (at concentrations greater than 100nM) has a similar or higher affinity for the CB_1-like receptor compared to AM251 but is a partial agonist.

The CB_1-like receptor site could be GPR55, as AM251 is frequently reported to be a GPR55 agonist in GPR55 transfected cell lines and natively expressing primary cells (Janiak *et al.*, 2007; Getty-Kaushik *et al.*, 2009; Kapur *et al.*, 2009; Yin *et al.*, 2009; Henstridge *et al.*, 2010a). The study by Janiak *et al.* (2007) reported that daily ingestion of rimonabant had beneficial effects on Zucker fatty islet morphology and function, which was believed to be due to blockade of CB_1 receptor signalling. However, if some of the positive effects of rimonabant treatment were mediated by signalling via the CB_1-like receptor site, then its identification should be considered in future studies.

5.4.4 Effects of AM630 on glucose-dependent insulin secretion

Neither 100nM nor 1μM AM630 significantly affected basal or maximal levels of insulin secretion, which is in accordance with other studies performed in islets (Juan-Pico *et al.*, 2006; Bermudez-Silva *et al.*, 2008). There was a large variability in the secretory rates when islets were treated with AM630, with either potentiation or inhibition insulin release at 20mM glucose. Based on the finding by Bermudez-Silva *et al.* (2008), that 2-AG production is positively linked to extracellular glucose concentration, there may be a constitutive endocannabinoid tone in islets at 20mM glucose. The constitutive endocannabinoid tone may then negatively affect GSIS via CB_2 receptor signalling. If the theory is correct, then this may explain the instances in which AM630 potentiates insulin secretion. Yet, this theory does not explain the instances where AM630 inhibits

insulin secretion unless CB_2 receptor signalling is compromised in some islets. In these instances, AM630 may block "non-functional" CB_2 receptor sites allowing endocannabinoids to inhibit insulin secretion by non-CB_2 receptor mediated means. It may also be that AM630 is affecting insulin secretion by CB_1 receptors mediated signalling (Ross *et al.,* 1999). Therefore, future experiments should establish whether endocannabinoid production is linked to GSIS. If there is an endogenous endocannabinoid tone in islets, it should then be determined what endocannabinoids (or endocannabinoid-like molecules) are produced by islets and what their effects on insulin secretion are.

5.4.5 Conclusions

The results from the present study suggest that CB or CB-like receptor signalling does occur within islets. Additionally, the effects of 10μM JWH-133 appeared to corroborate previous findings in Chapter 4, that a large CB_2 or CB_2-like receptor mediated signalling event can lead to an inhibition of insulin secretion in a majority of islets.

6

Acute effects of cannabinoids and fatty acid amide hydrolase enzyme inhibition on insulin secretion in a rat model of obesity and type 2 diabetes

6.1 Introduction

As described in Section 1.3.1, several genetic and environmental risk factors have been identified or have been linked to the development of type 2 diabetes mellitus. The interactions between the different genetic and environmental risk factors are complex, and deliberate induction of diabetes in humans to assess these interactions would be unethical. Hence, a wide variety of animal models are used to study the progression of islet dysfunction and/or insulin resistance, as well as for the testing of treatments for the control or prevention of type 2 diabetes, many of which have been reviewed by Srinivasan & Ramarao (2007) and Franconi *et al.* (2008). As discussed in these reviews, animal models used for the study of type 2 diabetes range from strains that were observed to become diabetic (either spontaneously or induced by high fat diet), had specific alterations made to their genome, through to those who had diabetes induced by surgery or drug treatment.

6.1.1 The Zucker rat strain

In the present study, Zucker Fatty (ZF) and Diabetic Fatty (ZDF) rats were used as models for obesity and obesity-induced type 2 diabetes. Zucker Fatty rats, in common with obese humans, have insulin resistant peripheral tissue, hyperinsulinaemia, high plasma triglycerides (TG) levels, hypertension (increases proportionately with body weight) and high plasma levels of non-esterified free fatty acids (Kurtz *et al.*, 1989; Sreenan *et al.*, 1996; Despres *et al.*, 2005; Janiak *et al.*, 2007; Di Nardo *et al.*, 2009). Despite these factors, they do not develop diabetes and their plasma glucose levels remain similar to those of their lean littermates (Sreenan *et al.*, 1996; Nolan *et al.*, 2006). ZDF rats, up to seven weeks of age, are considered to be pre-diabetic as their plasma glucose levels are similar to age-matched Zucker Lean Control (ZLC) rats and are phenotypically similar to age-matched ZF rats (Pick *et al.*, 1998; Harmon *et al.*, 1999; Harmon *et al.*, 2001; Nolan *et al.*, 2006). After seven weeks of age, diabetes is known to occur spontaneously (without any special dietary requirements) in male ZDF rats, and by 12 weeks of age all untreated male ZDF rats have type 2 diabetes (Orci

et al., 1990). While the diabetic ZDF rats are still obese compared to age-matched ZLC rats, they are usually lighter than age-matched ZF rats (Pick *et al.*, 1998). Unlike their male counterparts, obese female ZDF rats do not become spontaneously diabetic but diabetes can be induced by a high fat diet (Clark *et al.*, 1983; Pick *et al.*, 1998; Corsetti *et al.*, 2000). As discussed in the review by Franconi *et al.* (2008), the prevalence of type 2 diabetes between genders in humans is roughly equal, therefore this is a limitation of ZDF rat as a model of human type 2 diabetes.

6.1.1.1 Establishment and maintenance of the Zucker rat strain

The Zucker rat strain was originally derived from cross breeding Merck Stock M and Sherman rats, with the rats either having lean or spontaneously obese phenotypes (Bray, 1977). It was found that obesity was inherited following a simple Mendelian pattern, in which obesity was found to be caused by a single recessive allele of an unknown gene (Bray, 1977). The lean littermates were called Zucker Lean Controls (ZLC) and the genetically obese rats were called Zucker Fatty rats.

Several years after the establishment of the Zucker rat strain, it was observed in a small colony of Zucker rats that some of the ZF animals became spontaneously hyperglycaemic (Clark *et al.*, 1983). Subsequent testing established that the diabetic trait was only manifested in the genetically obese rats and diabetes could not be induced in the ZLC rats by a high fat and sucrose diet or by pregnancy (Clark *et al.*, 1983). The majority of the ZF rats which were hyperglycaemic were male, with only two of the rats being female (Clark *et al.*, 1983). From this colony, an inbred line of Zucker rats was created consisting of ZLC rats and the diabetic ZF rats, which were renamed Zucker Diabetic Fatty rats. It was later found that the genetic cause for the obesity seen in the ZF and ZDF rat strains was due to a mutated, non-functional form of the leptin receptor which results in the blockade of leptin signalling (Wang *et al.*, 1998). The ZLC rats for ZF and ZDF rat strains are either homozygous for the wild type receptor (+/+) or are heterozygous for the mutated leptin receptor (*fa*/+), while the ZF and ZDF rats are homozygous with mutated form of the leptin receptor (*fa*/*fa*).

ZF rat colonies are maintained by outbreeding ZF (*fa*/*fa*) males with ZLC (*fa*/+) females, while ZDF rats colonies are maintained by inbreeding ZLC males (*fa*/+) with (*fa*/+) females (Charles River Laboratories International, 2010b, a).

6.1.1.2 Islet dysfunction in the Zucker diabetic fatty rats

Progression of diabetes in the ZDF rat brings about marked effects on islet morphology. Typically, the most notable change is increased islet size and subsequent loss of a regular spherical shape, which are the result of multiple factors reported to primarily affect β-cells. A leading cause of islet size increase is the accumulation of triglycerides, which is common to islets isolated from ZF and ZDF rats (Nolan *et al.*, 2006). Similarly, both ZF and ZDF islets are reported to have higher β-cell replication rate than their ZLC littermates, which also contributes to the increase in islet size (Tokuyama *et al.*, 1995; Pick *et al.*, 1998; Li *et al.*, 2006; Larsen *et al.*, 2008). Similarly, β-cell apoptosis rates are increased too, but in ZF and pre-diabetic ZDF animals the apoptotic rate is exceeded by the rate of β-cell proliferation (Pick *et al.*, 1998). As the ZDF animals become diabetic, β-cell mass decreases; this is thought to be due to a shift in the β-cell proliferation: apoptosis ratio, in the favour of apoptosis (Pick *et al.*, 1998). Islet cell organisation is also affected in ZF and ZDF islets as it has been noted that the other non-β cells (normally found on the periphery) are partially interspersed throughout the islet (Pick *et al.*, 1998).

It has also been observed in people with type 2 diabetes that their islets have lost their organised morphology (Tokuyama *et al.*, 1995; Iki & Pour, 2007). In islets from human type 2 diabetics, the ratio of β-cells to non-β-cells is decreased, with respect to islets from weight- and age-matched non-diabetic controls (Yoon *et al.*, 2003; Iki & Pour, 2007). As with ZDF rats, reductions in β-cell mass in islets isolated from obese people with type 2 diabetes is also believed to be caused by increased rates of apoptosis (Butler *et al.*, 2003).

The alterations in islet morphology also coincide with changes in glucose-stimulated insulin secretion responses, as the islets from ZF and ZDF rats adapt to meet the increased need for insulin due to increasing peripheral insulin resistance. This need for additional insulin is achieved by the islets by increases to both basal and maximal rates of insulin release (Sreenan *et al.*, 1996; Harmon *et al.*, 1999; Zhou *et al.*, 1999). In ZF islets, it has been noted that there is a leftward shift in islet glucose responsiveness, particularly in the larger islets, as well as an increase in basal insulin secretion rates in comparison to islets isolated from ZLC rats (Chan *et al.*, 1998; Zhou *et al.*, 1999). As with ZF rats, pre-diabetic ZDF rats show similar changes in GSIS, with an increased basal secretion rate and a leftward shift in GSIS (Pick *et al.*, 1998). When the ZDF rats develop diabetes, their islets no longer secrete insulin with a distinct increase in insulin secretion rates in response to increasing glucose concentrations (Sreenan *et al.*, 1996; Zhou *et al.*, 1999). The loss of glucose responsiveness by the ZDF islets is thought to be due to maximal levels of GSIS decreasing with increased rates of basal insulin secretion (Sreenan *et al.*, 1996; Harmon *et al.*, 1999; Zhou *et al.*, 1999). In humans, the loss of the glucose-stimulated insulin release is thought to be due to the loss of a distinct first phase of insulin secretion rather than alterations in basal secretion rates, as observed in the Zucker rats (Bogardus & Tataranni, 2002; Festa *et al.*, 2008; Nijpels *et al.*, 2008).

6.1.1.3 Secondary complications in the Zucker diabetic fatty rat

As stated in Section 1.3, people with type 2 diabetes are at an increased risk of developing various secondary complications, many of which can be modelled in the ZDF rats. ZDF rats are employed as models of impaired wound healing, retinopathy, cardiac dysfunction, vascular dysfunction, nephropathy and neuropathy in type 2 diabetes (Vrabec, 1998; Chatham & Seymour, 2002; Chander *et al.*, 2004; Behl *et al.*, 2008; Brussee *et al.*, 2008; Romanovsky *et al.*, 2008; Sugimoto *et al.*, 2008; Oltman *et al.*, 2009). However, there are limitations in the relevance of ZDF rats as a model for secondary complications in type 2 diabetes. The study by Marsh *et al.* (2007) suggests that the Zucker rats (ZLC, ZF and ZDF) are susceptible to renal

abnormalities independent of phenotype. Therefore, increased risk in renal abnormalities may increase the incidence of cardiac dysfunction, especially in ZDF rats.

6.2 Zucker Fatty and Diabetic Fatty rats as models of human obesity and type 2 diabetes

As stated above, obesity in the Zucker strain is due to a defect in the leptin receptor, while obesity in humans is mainly attributed to hyper calorific diets (Wang *et al.*, 1998). As ZF rats display several characteristics associated with obesity in humans (Section 6.1), they are thought to be a good model of obesity. Metabolic syndrome is primarily based on the observation that people with central obesity and insulin resistance are at highest risk of developing type 2 diabetes and/or cardiovascular disease (Alberti *et al.*, 2006). The 2006 World Health Organisation definition of metabolic syndrome is the occurrence of central obesity with at least two of the following symptoms- raised triglycerides, low or reduced levels of HDL-cholesterol, raised blood pressure and IFG (or IGT, section 1.3.1; Alberti *et al.*, 2006). Therefore, ZF rats can be used as models of metabolic syndrome as they posses central obesity, have impaired glucose tolerance and raised blood pressure (Section 6.1). However, as ZF rats are not considered to become diabetic and do not appear to be used as models of cardiovascular disease, their use in studying the progression of the syndrome is therefore limited. However, as ZDF rats become diabetic (Section 6.1.1) and develop cardiovascular disease (Section 6.1.1.3), they appear to be a good animal model for studying metabolic syndrome.

With regard to ZDF rats as a model of type 2 diabetes in humans, they have several limitations with respect to β-cell dysfunction (such as differences in the loss of GSIS), which have already been described in Section 6.1.1.2. There is also evidence to suggest that islet amyloid polypeptide (otherwise known as amylin) may be linked to the loss of β-cell mass in type 2 diabetes in humans, as Butler *et al.* (2003) reported high incidence of amyloid plaque formation in islets obtained from people with type 2

diabetes. As rat amylin does not undergo fibrosis (Betsholtz *et al.*, 1989), this would suggest that this is another limitation in the ZDF rat as a model of type 2 diabetes, but Yoon *et al.* (2003) who also studied amyloid plaque formation in pancreata from human type 2 diabetic did not reproduce the observations made by Butler *et al.* (2003). This may emphasise the ethnicity linked risk factor in the development type 2 diabetes, as Yoon *et al.* (2003) used South Korean donors, whereas 98% of donors for the study by Butler *et al.* (2003) were Caucasian American of north European descent.

Using islets from ZF and ZDF rats, the aim of this study was to assess whether any potential differences in cannabinoid signalling in islets may have occurred as a result of obesity or type 2 diabetes. Due to the limited availability of tissue, 10μM methanandamide and 10μM AM251 were used due their consistent inhibition of insulin secretion from normal Wistar rat islets (see Chapters 3 and 5). Additionally, the effects of fatty acid amide hydrolase inhibition, by 30 minute pre-incubation with 10μM URB597, was also used to assess whether any potential changes in endocannabinoid metabolism and/or endocannabinoid tone had occurred.

6.2 Methods

6.2.1 Materials

All drugs and buffers were prepared as described in Sections 2.2.

6.2.2 Animals

All procedures using tissues obtained from male Wistar rats were obtained from rats with body weight ranging from 230-350g. Six-week old male Zucker Fatty and Zucker Diabetic Fatty rats (Charles River, UK) were kept in-house until 9-11 weeks of age when they were used for experimentation. All animals were housed and killed as described in Section 2.1. Blood samples were taken and blood glucose levels were measured using a blood glucose meter (Medisense, Optuim). Unfortunately, ZLC rats were not available.

6.2.3 Insulin secretion studies

Islets isolated from male Wistar rats were produced according to the isolation procedure described in 2.4.1. Zucker rat isolated islets were prepared from a single pancreas and were isolated using an adapted version of the isolation procedure described in 2.4.1. The islets isolated from the ZF and ZDF rats varied in size, shape, and appeared grey in colour when examined on a dark background. This meant that the islets isolated from the Zucker rats could not be easily distinguished from exocrine tissue. Therefore, to identify islets, the digested tissue was stained with the zinc-chelating substance dithizone (diphenylthiocarbazone; Sigma, Dorset, UK) at the end of the digestion. This allowed islets to be identified by their pink/red colour when viewed against a white background.

Briefly, 50mg DTZ was dissolved in 2ml DMSO/3ml ethanol to produce a 10mg/ml stock solution. At the time of use, the 10mg/ml DTZ stock was then diluted 10-fold in 70% (v/v) ethanol to produce a 1mg/ml solution. The collagenase-digested tissue was

resuspended in 10ml of buffer, to which 100µl of the 1mg/ml DTZ solution was added and then left to stand for 60 seconds. Once this time period had elapsed, the DTZ-stained digest was briefly centrifuged at 3000rpm for 3 seconds. The supernatant was then poured off and the pellet resuspended in 10ml buffer. The islets (now pink in colour) were then handpicked in a clear Petri dish on a white background.

6.2.3.1 Static incubations

Incubation studies used freshly isolated islets with batches of 5 ZF islets or 10 ZDF islets. Islets were incubated in 0.5ml of Gey and Gey buffer (Section 2.3.1) with test reagents in 1.5ml micro-centrifuge tubes and incorporated the URB597 pre-incubation protocol described in Chapter 3. Briefly, islets were pre-incubated for 30 minutes at 4mM glucose with either 10µM URB597 or 0.7% (v/v) ethanol. Following the pre-incubation period, test reagents were added, the 1.5ml micro centrifuge were agitated, briefly re-gassed, recapped and incubated for a further hour.

Once the hour incubation had finished, the tubes were inverted and centrifuged at 2000g (Hermle Z233MK-2) at room temperature for 3 minutes. Samples of the incubation media were removed for determination of insulin secretion rates by RIA (see section 2.6). The rest of the supernatant was then carefully removed from the tubes taking care not to disturb the pellet. The islets were then homogenised, using an Ultra Turrax T8 (Ika, Staufen, Germany), for 30 seconds (on setting 3) in 0.5ml acidic ethanol (ethanol: 0.8M HCl, 3:1). Following a brief centrifugation step, samples of supernatant were taken to determine islet insulin content by RIA (see section 2.6). Insulin secretion for each replicate was determined as the amount of insulin present in incubation media and expressed as a percentage of the total insulin (islet insulin content plus secreted insulin). Insulin secretion was then standardised against the 4mM glucose control.

6.2.3.2 Comparison of basal insulin secretion rates

The mean basal insulin secretion rates for Wistar rat islets was averaged from static incubation studies performed 1 week before and after the experiments when the Zucker islet studies were performed.

6.2.4 Data analysis

All data are presented as the mean ± SD. ZDF rat physiological parameters were compared against the corresponding ZF rat parameters using the unpaired two-tailed Student's t-test. Analysis of insulin secretion data were carried out as described in 2.4.5. Basal insulin secretion rates from Wistar, ZF and ZDF islets were compared using one-way ANOVA analysis. For all statistical comparisons, a P value < 0.05 was considered significant.

6.3 Results

The ZDF rats had reduced body weight, were hyperglycaemic and had a lower islet insulin content compared to age-matched ZF rats (Table 6.1). Islets obtained from ZF rats had an approximate two-fold difference in the insulin secretion rates between 4mM (basal secretion) and 20mM (maximal levels of secretion) glucose-stimulated insulin secretion (Figure 6.1A). The ZF islet insulin content was found to be ~2 fold higher than the ZDF islet insulin content (Table 6.1). Meanwhile, the ZDF islets showed minimal glucose responsiveness, as insulin secretion rates at 20mM glucose were only ~40% higher than basal release rates (Figure 6.1B). The mean basal insulin secretion rates for ZF and ZDF islet data sets were calculated as 0.56 ± 0.21 and 0.47 ± 0.30 ng of insulin secreted/islet/hour, respectively. In addition, the mean basal insulin secretion rate for Wistar rat isolated islets was determined as 0.34 ± 0.15 ng of insulin secreted/islet/hour (n=9). The mean basal insulin secretion rates for Wistar, ZF and ZDF did not significantly differ.

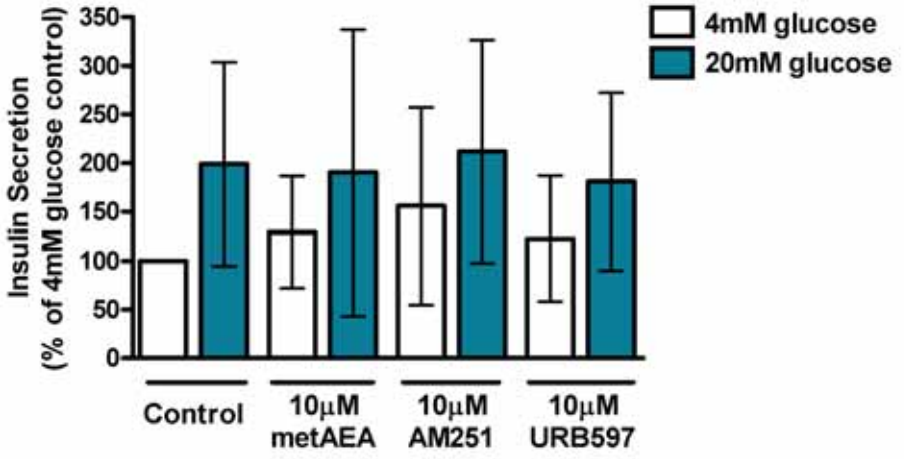
The use of methanandamide, AM251 or URB597 (at 10μM) did not significantly affect insulin secretion rates at 4mM or 20mM glucose in either ZF or ZDF islets. The same drug stocks were used in experiments with Wistar rat isolated islets (preceding these experiments) and it was found that the drugs performed in accordance with previous observations made in Wistar rat islets. Thus, the lack of drug effect in the Zucker islets, in comparison to Wistar islets, was due to differences in cannabinoid signalling pathways (rather than drug degradation).

Table 6.1 Physiological variables, including islet insulin content, of the ZF and ZDF rats used in the study

Zucker rat type	Zucker Fatty	Zucker Diabetic Fatty
Number of rats	8	8
Age in weeks	9-11	9-11
Body weight (g)	362 ± 16.4	325 ± 18.9***
Blood glucose (mM)	10.2 ± 1.7	18.3 ± 4.2***
Islet insulin content (ng of insulin/islet)	15.8 ± 2.0	8.8 ± 0.9***

Data are presented as the mean ± SD (n= 8 except for ZDF rat blood glucose where n=6). *** p< 0.001 *vs.* corresponding ZF condition

A Zucker Fatty islets



B Zucker Diabetic Fatty islets

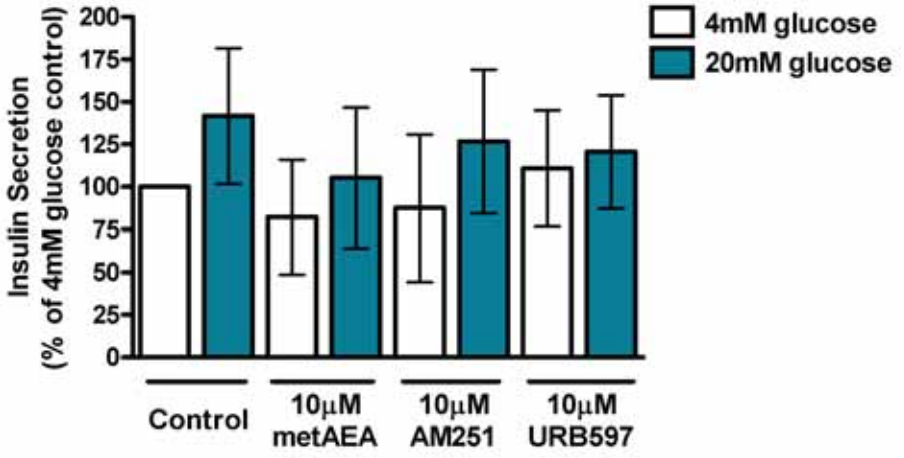


Figure 6.1 The effects of cannabinoid treatments in insulin secretion from isolated Zucker Fatty (A) and Zucker Diabetic Fatty (B) rats. Islets were pre-incubated with either vehicle (control) or 10μM URB597. Insulin secretion was then determined at either 4mM or 20mM glucose in the absence or presence of 10μM methanandamide (metAEA) or 10μM AM251. Results are displayed as mean insulin secretion rates ± SD (n= 8).

6.4 Discussion

ZDF rats had lower body weights but had significantly higher blood glucose levels than age-matched ZF rats. Unlike ZDF islets, ZF islets were found to be glucose responsive, and had higher insulin contents. Neither 10μM methanandamide, 10μM AM251 nor 10μM URB597 significantly affected insulin secretion from ZF or ZDF islets.

6.4.1 Pathology of the Zucker Fatty and Zucker Diabetic Fatty rats

It was found that the ZDF rats weighed less than the age-matched ZF rats which is in accordance with observations made by Pick *et al.* (1998). The weights of the ZF and ZDF rats are also in agreement with other studies which used ZF and/or ZDF rats of a similar age range (Tokuyama *et al.*, 1995; Chan *et al.*, 1999; Liu *et al.*, 2002). The blood glucose levels of the ZF rats were found to be similar to that typically reported (~8mM) by other groups (Pick *et al.*, 1998; Li *et al.*, 2006). As the average ZF rat plasma glucose concentration was 10.2mM, this may suggest that the ZF rats had raised plasma glucose levels which could be due to impaired glucose tolerance (Section 1.3.1). It was also found that the ZDF rats had blood glucose levels > 11.0mM glucose which would suggest that these animals were diabetic (Section 1.3.1). As discussed in the Introduction for this chapter, diabetes can occur spontaneously in ZDF rats past seven weeks of age, therefore, the occurrence of diabetes in the ZDF animals was expected.

The islets isolated from the Zucker rats, especially the ZDF islets, were typically larger, irregularly shaped and more grey in colour which made it more difficult to distinguish between islet and exocrine tissues than experienced with age-matched Wistar rat islet. As discussed in the Introduction, these changes in islet morphology are typical of those reported for islets from ZF and ZDF. Therefore, DZT was used to identify the islets as β-cells contain considerably higher levels of Zn^{2+} ions than any other cell type in the pancreas (Section 6.2). In addition to morphological changes, it

was observed that the ZF islets were glucose responsive, whereas, the ZDF islets had all but lost their glucose responsiveness. Both these observations were in line with other studies that have used similarly aged ZF and diabetic ZDF rats (Lee *et al.*, 1994; Sreenan *et al.*, 1996; Pick *et al.*, 1998). The insulin contents of ZF and ZDF islets were lower than that reported by other group's, but this anomaly may be due to the varying precisions in each groups insulin assay (Tokuyama *et al.*, 1995; Zhou *et al.*, 1999; Harmon *et al.*, 2001; Liu *et al.*, 2002). The insulin content of the ZDF islets was approximately half that of the ZF islets. Pick *et al.* (1998) reported a 50% reduction in ZDF islet β cell mass in comparison to age-matched islets from ZF rats, which may explain the difference in islet insulin content. A similar deficit (~4 fold) in the insulin content of ZDF islets, in comparison to the insulin content of ZF islets, has also been reported in the study by Zhou *et al.* (1999). However, Zhou *et al.* (1999) reported that the ~4 fold deficit in ZDF islet insulin content was not due to alterations in insulin mRNA levels or alterations in β-cell mass, and may be due, instead, to reductions in the level of insulin mRNA translation (Fred & Welsh, 2009).

6.4.2 Effects of methanandamide, AM251 and URB597 on insulin secretion in ZF and ZDF islets

Within this study, it was found that 10μM methanandamide, 10μM AM251 and pre-incubation with 10μM URB597 did not affect basal or maximal levels of GSIS in ZF or ZDF islets. It was reported by Getty-Kaushik *et al.* (2009) that acute (30 minute) exposure of ZLC and ZF rat isolated islets to 1μM rimonabant (a structural analogue of AM251) resulted in a lowering of insulin release at 16mM glucose. If the effects of AM251 were due to the blockade of islet-derived endocannabinoid CB_1 receptor signalling or inverse agonism at the CB_1 receptor then it would be expected that the results in ZF rat islets presented here would have been similar to those reported for rimonabant in Getty-Kaushik *et al.* (2009). As this was not the same, then the differences between AM251 and rimonabant may be due to differences in pharmacology, use of freshly picked *vs.* 24 hour cultured islets or variations between ZF rat colonies.

Despite methanandamide, AM251 and URB597 not significantly affecting insulin secretion, it appeared that all three compounds caused a slight increase in the basal insulin secretion rate in the ZF islets. Further examination of the data also indicates that methanandamide inhibited basal secretion in the majority of ZDF islets, but this was not observed in ZF islets. This suggests a cannabinoid signalling pathway may have become dysfunctional as insulin secretion at 4mM appeared to be affected by methanandamide, an effect not observed with methanandamide in Wistar rat islets (Chapter 3). Additionally, methanandamide also appeared to inhibit insulin secretion at 20mM glucose in the majority of experiments performed with ZF and ZDF islets. This may indicate that one or more pathways by which methanandamide could inhibit insulin release at 20mM glucose in Wistar islets may have been affected by obesity. Whether these findings indicate that cannabinoid signalling differs between obese and obese type 2 diabetic states is uncertain without a greater number of experiments being performed.

Pre-incubation of islets with 10μM URB597 did not significantly affect insulin secretion from ZF or ZDF islets at either 4mM or 20mM glucose. As discussed in Section 3.4.2, further work is needed to confirm the level of FAAH activity and specificity of URB597 pre-incubation protocol within these islets before the affects of FAAH inhibition can be discussed with confidence. In Wistar rat islets, both 10μM methanandamide (Chapter 3) and 10μM AM251 (Chapter 5) consistently inhibited insulin secretion. This would primarily suggest that the receptor signalling pathways through which these cannabinoids affected insulin release in the lean (Wistar) islets are altered as a result of obesity and/or type 2 diabetes in the Zucker rat islets. Yet without similar information from ZLC rats, it cannot be ascertained whether the differences in cannabinoid signalling in islets is primarily affected by the physiological state of the animal, a lack of leptin signalling, or differences between Zucker and Wistar strains. Indeed, future experiments must include islets from ZLC rats (that are age-matched to the Wistar rats), to establish whether islet responses to cannabinoids are comparable

between the rat strains before it can be determined whether endocannabinoid signalling is altered in diabetes. Future experiments should also include comparisons of islet responses between diet-induced obese ZLC and ZF rats to establish whether the lack of leptin signalling, rather than obesity, effects cannabinoid signalling within Zucker islets.

6.4.3 Conclusions

Methanandamide and AM251 have been demonstrated to inhibit insulin secretion from Wistar islets but failed to significantly affect insulin secretion rates from either ZF or ZDF islets. The results from this study suggest that cannabinoid signalling in islets is affected by obesity but it is unclear whether cannabinoid signalling is altered further when islets become dysfunctional at the onset of type 2 diabetes. However, as islets from ZLC rats were not used, it is uncertain whether cannabinoid signalling in islets fundamentally differs between Wistar and Zucker islets. It is also unclear whether the lack of leptin signalling also has an effect on cannabinoid signalling. Therefore, future experiments first need to determine whether the Zucker rat is a suitable model to study any potential cannabinoid dysfunction that may occur as a result of obesity and/or type 2 diabetes. If these studies confirm that Zucker islets are suitable then further work can then proceed to characterise whether cannabinoid signalling plays a causative role in the development of islet dysfunction in type 2 diabetes.

7

General Discussion

The principal aim of this programme of research was to characterise the effects of cannabinoid signalling on insulin secretion from islets. A variety of ligands which are reported to interact with cannabinoid receptors, together with inhibition of endocannabinoid metabolising enzymes were used in secretion studies. Figure 7.1 presents a schematic summary of cannabinoid signalling in islets based on results from Chapters 4 and 5, which suggest that CB_1-receptor signalling in some islets can potentiate insulin secretion but inhibit insulin secretion in others, whereas CB_2-receptor signalling can only inhibit insulin secretion. These conclusions are based on the effects of the endogenous cannabinoid AEA (Chapter 4) and the synthetic cannabinoids, ACEA and JWH-133 (which are described as CB_1- and CB_2-receptor specific agonists; Chapter 5) on insulin secretion. These conclusions assume that both the potentiation and inhibition of insulin secretion caused by ACEA, in separate islet preparations, were CB_1-receptor specific and as discussed in section 5.4.1 are due to differences in sst-14 signalling. These conclusions also assume JWH-133 was inhibiting insulin release in a CB_2-receptor specific manner and that AEA, as implicated in cell lines and other tissues, displays a higher affinity for CB_1 receptors over CB_2 receptors (McPartland *et al.*, 2007). These data have then been fitted to the expression of CB-receptor on specific cell types and is based on data from Table 4.1. The schematic also assumes that CB_1 and CB_2 receptors are expressed on δ-cells. However, this schematic diagram does not factor in the possibility that other receptor signalling events (e.g. CB-like and GPR55) may also be occurring. It also does not account for the source of the endocannabinoids nor the influence that different endocannabinoid metabolising pathways may have.

Due to the variability of islet responses to the different cannabinoids used, it still remains unclear whether there is an archetypal endocannabinoid signalling system in islets, especially if cannabinoid responses are dependent on the fasting state of the animal, islet cell composition, expression of endocannabinoid metabolising enzymes and CB/CB-like receptor expression patterns. If cannabinoid signalling in islets were to be affected by these factors then it may explain why a consensus has not been

reached yet within this field. To address this question, it may be appropriate to perform immunohistological or *in situ* hybridization studies with whole pancreata. Ideally, such a study would address whether factors such as islet size, pancreatic location and fasting state influence the expression and/or activity of receptors and metabolising enzymes of the endocannabinoid system in islets.

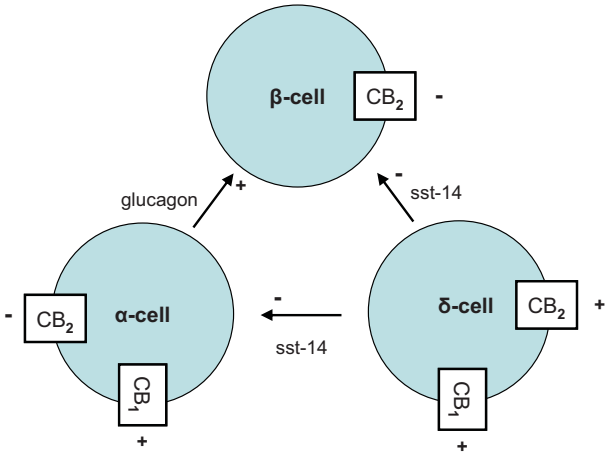


Figure 7.1 Schematic diagram of possible CB_1- and CB_2-receptor signalling events that may occur in islets at maximal levels of glucose-stimulated insulin secretion. Within the islet, glucagon secreted by α-cells act on insulin-secreting β-cells in a positive manner, while somatostatin (sst-14) secreted by δ-cells inhibit hormone release from both α- and β-cells. Activation of cannabinoid receptors (CB_1 and CB_2) expressed on islet cell potentiate (+) or inhibit (-) hormone secretion from the cell type indicated.

In addition, the influence of endocannabinoid tone on islet function should also be assessed. As discussed in Chapters 3 and 5, endocannabinoid tone in islets may have also been a confounding factor when interpreting the effects of CB receptor antagonists (AM251, O-2050 and AM630) and the FAAH inhibitor URB597. Therefore, it should be determined which endocannabinoids (including endocannabinoid-like molecules) are produced by islets and whether endocannabinoid production is linked to GSIS. This could be accomplished using techniques such as liquid chromatography-tandem electrospray ionization mass spectrometry which can be used to detect and measure the levels of a number of different endocannabinoids in parallel (Richardson *et al.*, 2007). This work could then be followed by RT-PCR (reverse transcriptase polymerase chain reaction) and Western blotting to explicitly characterise the expression of endocannabinoid synthetic machinery within islets. As

shown in Table 1.4, this has been done to a limited extent by other groups but these studies have not accounted for alternate biosynthetic pathways which may be important in the production of *N*-acylethanolamines (Section 1.4.1.1). Once pathways have been identified then genetic alteration in the expression of key enzymes (e.g. MAGL knock-out, overexpression of MAGL or use of siRNA to reduce MAGL expression) could be used to examine how endocannabinoid tone affects islets function, especially if enzymes inhibitors were not available or were themselves found to affect insulin secretion. As discussed in Section 5.4, it is possible that the effects of the CB receptor agonists and antagonists on insulin secretion, observed within this study, could have been due to a reduced specificity at the concentrations used and/or previously uncharacterised activites at non-CB receptors. Therefore, the use of CB receptor knock-out animals or siRNA techniques could also be used to study cannabinoid signalling in islets in future studies instead of the pharmacological approaches.

7.1 Physiological role of the endocannabinoid system in islets

It was also found in this study that the effects of the cannabinoids were limited to maximal levels of insulin secretion. Assuming the results are physiologically-relevant, they suggest that endocannabinoid signalling does not play a major regulatory role on insulin secretion within physiological ranges of plasma glucose levels. If endocannabinoid signalling in islets were restricted to supraphysiological plasma glucose levels, it could suggest that the endocannabinoid system in islets plays a protective role in hyperglycaemic states. For example, endocannabinoid signalling may limit over activation of signalling pathways in islets which could lead to a longer-term desensitisation of these pathways which could attenuate future insulin secretory responses. Yet, at physiological ranges of plasma glucose levels the role of endocannabinoid signalling has not been investigated with regards to other aspects of islet function. For example, cannabinoid signalling currently has not been investigated with regard to insulin secretion in response to other secretagogues, such as free fatty acids, or whether cannabinoid signalling may affect other receptor signalling pathways

(Selley *et al.*, 2004; Cinar *et al.*, 2008; Kim *et al.*, 2008b). It must also be considered that islet-derived endocannabinoids may affect islet endothelial cell function or islet-associated neuronal function (by pre-synaptic inhibition).

In addition to affecting islet function, the endocannabinoid system may also affect islet cell viability, which may have important implications in pathological states. As discussed in Section 1.3.3, the preservation of β-cell function and mass is of key interest in the treatment and prevention of type 2 diabetes. Studies performed with islets and β-cell lines have implicated various factors contributing to the fate of β-cells in pathological states. Firstly, activation by phosphorylation of the serine/threonine protein kinase Akt through insulin receptor signalling promotes β cell survival (Srinivasan *et al.*, 2002; Gonzalez-Pertusa *et al.*, 2010; Natalicchio *et al.*, 2010). Activation of mitogen-activated protein kinase (MAPK) signalling (by p38 and/or JNK signalling) promotes β-cell death (Bachar *et al.*, 2010; Ma *et al.*, 2010). Cytokines, such as interleukin-1β (IL-1β), tumour necrosis factor α (TNFα) and interferon γ (IFNγ), also promote increased rates of β-cell apoptosis and reductions in β-cell replication rates (Heitmeier *et al.*, 2004; Donath *et al.*, 2010; Ma *et al.*, 2010). This is achieved by increased reactive oxygen species (ROS) generation (through expression of inducible nitric oxide synthase) and MAPK signalling, but may also involve other mechanisms, such as increased lipoxygenase-12 (LOX-12) activity (Heitmeier *et al.*, 2004; Donath *et al.*, 2010; Ma *et al.*, 2010; Natalicchio *et al.*, 2010). Endoplasmic reticulum stress (excessive protein production and protein misfolding) is another factor contributing to β-cell apoptosis, as reviewed in Fonseca *et al.* (2009).

CB_2-receptor agonism (confirmed by a lack of effect in CB_2-receptor knock-out mice) has been found to reduce the incidence of apoptosis in several *in vivo studies*, each examining a different model of apoptosis in different tissues (Defer *et al.*, 2009; Viscomi *et al.*, 2009; Mukhopadhyay *et al.*, 2010). The anti-apoptotic effects of CB_2-receptor signalling were linked to reduced ROS generation, reduced cytokine expression and increased Akt signalling (Defer *et al.*, 2009; Viscomi *et al.*, 2009; Mukhopadhyay *et al.*, 2010). As rat β-cells express CB_2 receptors (Table 4.2) and CB

receptor expression patterns in islets have not been reported to in differ type 2 diabetes, then CB_2 receptor agonism in physiological and pathological states may have beneficial effects on β-cell function and survival (Srinivasan *et al.*, 2002; Tharp *et al.*, 2008; Natalicchio *et al.*, 2010). Use of thiazolidinediones (TZDs) in genetically obese animals has been observed to preserve islet mass, insulin content and glucose responsiveness of the β-cells (Shimabukuro *et al.*, 1998; Ishida *et al.*, 2004; Kawasaki *et al.*, 2005). Additionally, *in vitro* testing in human islets found that rosiglitazone reduced rates of β-cell apoptosis and β-cell stress induced by islet amyloid protein and palmitate (Lin *et al.*, 2005; Vandewalle *et al.*, 2008). As mentioned in Section 1.4.5, endocannabinoids also possess agonistic activities at the PPARγ receptor, thus in lipotoxic conditions, endocannabinoids may also reproduce the protective effects of TZDs, through PPAR agonism, on β-cell survival and function. Yet, endocannabinoid signalling may also have pro-apoptotic effects in β-cells. For instance, chronic activation of pro-survival signalling pathways by CB_2 signalling may make β-cells susceptible to free fatty acid (FFA) induced apoptosis (Gonzalez-Pertusa *et al.*, 2010). Furthermore, CB_2 signalling (involving TRPV1 signalling) has been reported to reduce mononuclear cell survival, which may involve increased ER stress (Saunders *et al.*, 2009). Meanwhile, CB_1 receptor signalling may negatively affect β-cell survival indirectly by upregulating cytokine production, leading to the promotion of apoptosis in β-cells (Heitmeier *et al.*, 2004; Donath *et al.*, 2010; Ma *et al.*, 2010; Mukhopadhyay *et al.*, 2010). Cannabinoids have also been found to negatively affect mitochondrial activity which could also contribute to β-cell dysfunction and may trigger apoptotic signalling through cytochrome c release (Athanasiou *et al.*, 2007; Catanzaro *et al.*, 2009). Finally, in islets the phytocannabinoid Δ^9-tetrahydrocannabindiol was found to increase arachidonic acid oxidation by LOX-12 (Laychock *et al.*, 1986). As stated above, increased LOX-12 activity is linked to increased rates of apoptosis through MAPK signalling, which again may suggest that the endocannabinoid system upregulates apoptotic signalling in β-cells (Ma *et al.*, 2010). Altogether, the literature suggests that the endocannabinoid system may play a role in the development and

progression of type 2 diabetes by directly affecting β-cell viability and therefore warrants further investigation.

Long-term incubation studies, where islets are co-incubated with cannabinoids in the absence or presence of hyperglycaemic and/or lipotoxic conditions, are a feasible means to assess the effects of prolonged CB-receptor signalling on islet function and insulin secretion. These types of experiments were originally planned at the start of this study with THC and rosiglitazone, to examine whether THC could exert effects similar to rosiglitazone through PPARγ receptor agonism. However, there was not sufficient time to perform these experiments but they still appear to be viable and should also included in future studies. In addtion to THC and rosiglitazone the use CB_1 and CB_2 receptor agonists/antagonists should also be tested in order to verify the predictions in the previous paragraph.

7.2 Establishing the role of the endocannabinoid system in glucose homeostasis

As described previously (Section 1.1), the regulation of blood glucose levels is achieved by the coordinated release of islet hormones which then affect peripheral tissue function. Hence, cannabinoid signalling may not only affect the release of insulin, and other islet hormones, but may also affect insulin-mediated glucose uptake too. Research into the role of the endocannabinoid system in peripheral tissue over recent years suggests that prolonged exposure to endocannabinoids promotes insulin resistant states (Esposito *et al.*, 2008; Motaghedi & McGraw, 2008). This research field has primarily focused on the pathological state induced by chronic cannabinoid exposure and the benefits of global CB_1 receptor blockade. As introduced in Section 3.1, CB_1 receptor antagonism may become a future treatment for type 2 diabetes as it promotes weight loss and increases glycaemic control. However, a viable CB_1-receptor antagonist for the treatment of type 2 diabetes may never be developed due to adverse side-effects, such as depression and risk of suicide (Johansson *et al.*, 2009). Therefore, more work needs to be performed to define and understand how

cannabinoid signalling affects insulin release from islets and insulin signalling in peripheral tissue (liver, skeletal muscle and adipose tissue). Such information would also be of wider use as research into the manipulation of the endocannabinoid system is being carried outside of the fields of obesity and type 2 diabetes. Hence, if treatments were used that affected the endocannabinoid system globally then other systems may also be affected promoting dysfunction in other systems. For instance, there is interest in the use endocannabinoid metabolising enzyme inhibitors and CB_1 receptor agonists for the treatment of inflammatory disorders (Cluny *et al., 2010;* Sagar *et al.*, 2009). Therefore, therapeutic potential in one area might be negated by adverse effects on glucose homeostasis.

As discussed in Chapter 3, AEA appears to inhibit insulin secretion from freshly-isolated islets (which were used in this study) but potentiate insulin secretion when islets have been cultured. Therefore, it needs to be determined whether freshly-isolated or cultured islets reproduce the affects of cannabinoid signalling on islet function *in vivo*. A similar *in vivo* approach to that used in Bermudez-Siva *et al.* (2006) could be used but changes in plasma insulin and glucagon levels should be measured alongside changes in plasma glucose levels. This will help to determine whether alterations in glucose tolerance were due to changes in islet function and/or insulin signalling (Section 1.1). This information could then be used to address the role of the endocannabinoid system in glucose-stimulated insulin secretion and its wider role in glucose homeostasis.

As described in Section 1.4.10, chronic studies suggest that the endocannabinoid system appears to play a role in peripheral tissue dysfunction in dietary-induced obesity and affects several processes involved in glucose homeostasis. Thus, in co-junction with the possible alterations in islet activity, other parameters should be measured to gain a greater understanding of acute cannabinoid signalling in other peripheral tissues. As discussed in Sections 1.2.5 and 1.3.3, the incretin hormones have an important effect on insulin secretion in response to orally ingested glucose

and also promote β-cell survival but whether cannabinoid signalling affects the release of the incretin hormones appears to be unknown. Therefore, changes in the plasma levels of the incretin hormones should be measured as this would affect islet function. If cannabinoid signalling in the gut was also involved in glucose homeostasis, it may be informative to compare the effects of cannabinoid signalling when glucose was loaded intra-peritoneally or intra-venously to oral ingestion. Another aspect would be to measure the acute effects of cannabinoid signalling and endocannabinoid metabolism on insulin-mediated glucose uptake in hepatic, adipose and skeletal muscle tissues. For instance, this could be tested *in vitro* and/or *in vivo* (Nogueiras *et al.*, 2008) by measuring radiolabeled 2-deoxyglucose uptake. Chronic rimonabant dosing has also been demonstrated to affect hepatic glucose production, therefore, this parameter should also be measured in response to acute treatment (Nogueiras *et al.*, 2008).

The discussion, thus far, has focused on the role of endocannabinoid signalling once plasma glucose levels were high enough to induce GSIS but endocannabinoid signalling may be important during fasting states. Studies performed in the gut suggest that CB_1 receptor expression is upregulated during extended periods of fasting (Burdyga *et al.*, 2004). It has also been noted that the circulating levels of *N*-acylethanolamines are highest during periods of fasting and in physiological states decrease once feeding has occurred (Matias *et al.*, 2006; Matias *et al.*, 2007). With regard to islet function the obvious question is, does cannanabinoid exposure during periods of fasting affect insulin secretory responses when glucose levels increase sufficiently to induce GSIS? This could be tested *in vitro* by culturing islets for increasing periods of time (e.g. 0.5, 1, 2, 4, 8 and 12 hours) in the absence or presence of endocannabinoids at non-stimulatory concentrations of glucose (≤ 4mM glucose) followed by acute secretion studies. In initial tests, comparisons between vehicle and endocannabinoid-treated islets could then assess whether β-cell glucose responsiveness, islet content and/or insulin secretion kinetics had been altered. The initial tests could also examine whether cannabinoid treatment had altered α- and/or

δ-cell secretory activity and hormone content too. Experiments in islets could also be performed to assess whether the expression and/or activity of the cannabinoid receptors, endocannabinoid synthesising and metabolising enzymes are affected by different periods of β-cell activity and inactivity.

As mentioned above, the endocannabinoid system appears to be most active during periods of fasting (Burdyga *et al.*, 2004; Matias *et al.*, 2006; Matias *et al.*, 2007). Thus, the physiological role of the endocannabinoid system in the periphery may be to promote effective food assimilation. This could be achieved by promoting the upregulation of lipogenic pathways in the periphery as well as increasing nutrient absorbtion and increasing the amount of time food is in the gut (Carr *et al.*, 2008; Duncan *et al.*, 2008; Quarta *et al.*, 2010; Zhao *et al., 2010*). Therefore, by understanding how the endocannabinoid system adapts in response to different physiological states in peripheral tissues, it would then help determine if and why endocannabinoid signalling may become dysfunctional in the periphery during obesity and/or type 2 diabetes.

7.3 Conclusions

While this PhD has not provided full characterisation of the endocannabinoid system within islets, it has highlighted that cannabinoid signalling within islets is more complex than has been reported by other research groups. For example, islet responsiveness to AEA at concentrations ≤ 10μM varied between islet preparations in a FAAH-independent manner (Chapter 3). Results from Chapters 4 and 5 suggest that CB-receptor signalling pathways may differ between islets and may account for the variations in the potency of AEA between islet preparations. In addition, putative CB-receptor specific ligands, as discussed in Chapter 5, may possess extra non-CB-receptor mediated activities within islets. It is hoped that the information obtained from these studies will aid future investigations in this field to gain a more complete understanding of the role of endocannabinoid system in glucose homeostasis.

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