

**Glucagon and GLP-1 receptor
dual agonism:
a therapeutic approach for the
treatment of obesity**

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ABSTRACT

Obesity is a growing global epidemic and current medical therapies have proven inadequate. Endogenous satiety hormones provide an attractive target for the development of drugs which aim to cause effective weight loss with minimal side effects. Two related peptide hormones, glucagon and glucagon-like peptide 1 (GLP-1), are the subject of this thesis. Both have been found to reduce appetite and cause weight loss. Additionally, glucagon increases energy expenditure. GLP-1 also improves glucose homeostasis, whereas glucagon causes undesirable hyperglycaemia. It is proposed that co-administration of both peptides will have an additive effect on appetite reduction, while GLP-1 will protect against the hyperglycaemic effect of glucagon.

In this thesis, I have investigated the effects of co-administration of glucagon and GLP-1 on energy balance. Co-infusion of glucagon and GLP-1 in humans reduces food intake, increases energy expenditure and improves glucose homeostasis. This supports the notion that dual agonism at the glucagon and GLP-1 receptors is a rational approach to the development of a new therapy for obesity and diabetes.

To further understand the cellular mechanisms by which glucagon activates the glucagon receptor, I have investigated the interaction of the glucagon receptor with RAMP2, a member of the family of Receptor Activity Modifying Proteins. This work suggests that RAMP2 interacts with the glucagon receptor modifying the agonist activity of glucagon, GLP-1 and related peptides. This work improves our fundamental understanding of the glucagon receptor's physiological function and how this is modified by RAMP2. It could potentially suggest new therapeutic avenues for obesity and diabetes. For example, it may inform the construction of new peptide analogues with selective agonist activities, incorporating therapeutically desirable properties such as appetite suppression and increase in energy expenditure, without undesired properties such as increasing hepatic glucose output and provoking hyperglycaemia.

DECLARATION OF CONTRIBUTORS

The majority of the work described in this thesis was undertaken by the author. All collaborations and assistance are detailed below:

Chapter 2: Human studies were performed in collaboration with Dr Rachel Troke, Dr Katherine McCullough, Dr George Tharakan, Dr Ben Jones and Prof Tricia Tan, with assistance from Dr Julia Kenkre, Dr Nassim Parvizi, Dr Mohammed Hussein and Dr Chung Thong Lim.

Chapter 3: Human studies were performed in collaboration with Dr Rachel Troke, Dr Katherine McCullough, Dr George Tharakan, Dr Ben Jones and Prof Tricia Tan.

Chapter 4: Cell work and assays were performed in collaboration with Dr Ben Jones, with assistance from Dr Tom Marjot.

In-house radioimmunoassays (RIA) used in this thesis were established and maintained by Professor Mohammad Ghatel.

All glucagon and GLP-1 analogue custom-made peptides were designed by Professor Steve Bloom. All intellectual property relating to these peptides belong to Imperial Innovations.

A major part of the work presented in chapter 2 has been published and the manuscript is appended (see Appendix E):

Cegla J, Troke RC, Jones B, Tharakan G, Kenkre J, McCullough KA, Lim CT, Parvizi N, Hussein M, Chambers ES, Minnion J, Cuenco J, Ghatel MA, Meeran K, Tan TM, Bloom SR. Coinfusion of low-dose GLP-1 and glucagon in man results in a reduction in food intake Diabetes 2014 Nov; 63 (11):3711-20.

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ABBREVIATIONS

AgRP	Agouti-related peptide
AM	Adrenomedullin
AMPK	5'-adenosine monophosphate-activated protein kinase
AMY	Amylin
AP	Area postrema
ARC	Arcuate nucleus
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
BMR	Basal Metabolic Rate
cAMP	Cyclic adenosine 3',5'-monophosphate
CART	Cocaine and amphetamine-regulated transcript
CaSR	Calcium-sensing receptor
CFP	Cyan fluorescent protein
CGRP	Calcitonin gene related peptide
CHO-K1	Chinese hamster ovarian cells
CNS	Central nervous system
CRLR	Calcitonin receptor like receptor
CT	Calcitonin
CTR	Calcitonin receptor
DEB-Q	Dutch Eating Behaviour Questionnaire
DPP-4	Dipeptidyl peptidase-4
EE	Energy expenditure
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FDA	Food and Drugs Administration
FFA	Free fatty acid
FSIVGTT	Frequently sampled intravenous glucose tolerance test

GCG	Glucagon
GCGR	Glucagon receptor
GDP	Guanosine diphosphate
GDW	Glass-distilled water
GFP	Green fluorescent protein
GHRH	Growth hormone releasing hormone
GIP	glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLP-2	Glucagon-like peptide-2
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GRPP	Glicentin-related pancreatic peptide
HPLC	High performance liquid chromatography
IFG	Impaired fasting glucose
InsP ₃	Inositol triphosphate
IP	Intervening peptide
LB	Liquid Broth
MAPK	Mitogen-activated protein kinase
MPGF	Major proglucagon fragment
NPY	Neuropeptide Y
NTS	Nucleus of the tractus solitarius
OXM	Oxyntomodulin
PACAP	Pituitary adenylyl cyclase-activating peptide
PBS	Phosphate buffer solution
PEPCK	Phosphoenolpyruvate carboxykinase
PFA	Paraformaldehyde
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PC	Prohormone convertase
PEI	Polyethylenimine
PP	Pancreatic Polypeptide
PTH1R	Parathyroid hormone-1 receptor

PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
qPCR	Quantitative polymerase chain reaction
RAMP	Receptor Activity Modifying Protein
RFU	Relative fluorescence units
RIA	Radioimmunoassay
RQ	Respiratory quotient
RYGB	Roux-en-Y Gastric Bypass
s.d.	Standard deviation
s.e.m.	Standard error of the mean
SP-2	Spacer peptide
SPPS	Solid phase peptide synthesis
T2DM	Type 2 Diabetes Mellitus
TFB	Transformation buffer
VAS	Visual analogue scale
VIP	Vasoactive intestinal peptide
VMN	Ventromedial nucleus

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Obesity is a growing global epidemic. The Health Survey for England and Wales reports that 24% of adults are obese and a further 41% of men and 32% of women are classified as overweight (The NHS information Centre, 2009). The World Health Organisation estimates that over one billion adults and over forty-two million children under the age of five are overweight worldwide (World Health Organisation, 2002). The serious health consequences of obesity are well documented, and include type 2 diabetes, hypertension and hyperlipidaemia, all of which are independent risk factors for cardiovascular disease (Chan et al., 1994, Nieves et al., 2003, Dyer et al., 1990). Furthermore, obesity confers an increased risk of sleep apnoea, infertility, arthritis and cancer (Dhaliwal and Welborn, 2009).

Surgical treatments for obesity are currently the most effective means of achieving significant and sustained weight loss and improving morbidity and mortality (Sjostrom, 2008). These procedures, however, are irreversible and confer a perioperative risk of mortality of 0.5% (Marsk et al., 2008). Non-surgical treatments for obesity have included the medications orlistat, sibutramine and rimonabant. Orlistat causes modest weight loss and its utility is limited due to poor tolerance (Rucker et al., 2007). Sibutramine and rimonabant were recently withdrawn from the market due to an associated increase in cardiovascular events and depression respectively (Christensen et al., 2007, Rucker et al., 2007, Sharma et al., 2009). In the past three years, three new centrally-acting anti-obesity agents have been approved by the FDA, however, each has its own safety concerns. The selective serotonin 2C receptor agonist, lorcaserin, was approved by the FDA in June 2012, however, concerns over development of cancer with lorcaserin have overshadowed its use (Heal et al., 2012). The combination of phentermine and topiramate has also raised safety concerns such as the risk of teratogenicity and therefore this drug may not be the ideal choice for women of child-bearing age (Gadde et al., 2011). Thirdly, the combination of naltrexone and bupropion has elicited worries regarding psychiatric disturbance (Ornellas and Chavez, 2011). None of these centrally-acting drugs have been approved within the European Union.

Therefore it is clear that new strategies are urgently needed to tackle obesity. The endogenous hormones involved in appetite regulation are an attractive target for the

development of drugs which aim to cause effective weight loss with minimal side effects.

1.2 Regulation of energy balance

Despite fluctuations in food intake and physical activity, healthy adults maintain a relatively constant weight over decades. However, consistent with the laws of thermodynamics, if less energy is expended than consumed then the excess energy will be stored. It is calculated that an average North American man will increase his weight by 9.1 kg between 25 and 35 years of age, as a consequence of a mere 0.3% imbalance between energy consumed and energy expended over this period (Rosenbaum et al., 1997).

The control of food intake is complex and depends on neural and hormonal signals passing between the gut and central nervous system (CNS). Additionally, hormones such as leptin indicate 'long term energy reserves' within adipose tissue and are strongly implicated in modifying feeding behaviour and energy expenditure (EE). The brain interprets signals from the periphery to modify feeding behaviour depending on energy requirements and the 'desire' to eat. Even before eating, gut hormones are released and begin the process of meal termination, by inducing feelings of satiety. Gut hormones act on key brain areas such as the hypothalamus and brainstem which contain intricate neuronal networks and connections related to energy homeostasis, regulation of food intake and glucose homeostasis.

On the other side of the 'energy balance' scale is energy expenditure (EE), defined as the amount of work required by the body to sustain vital functions such as thermogenesis, physical activity, cardiac function and respiration. Total EE can be divided into three main components. First is the basal metabolic rate (BMR) which is the minimum energy required by the body at rest to ensure the function of cells and tissues. This represents approximately 60-75% of total EE and depends on body weight and composition. Second, physical activity on average accounts for 15-30% of total daily EE. Finally, energy is expended through adaptive thermogenesis in response to cold, food and physical activity. All three components of EE are tightly regulated, by central circuits in

the brainstem and hypothalamus and by peripheral tissues including skeletal muscle and brown adipose tissue (BAT).

1.2.1 Central regulation of energy balance

This system is centred around the hypothalamus and brainstem, which have reciprocal neuronal connections (Murphy and Bloom, 2006). Specifically, two discrete groups of neurones in the arcuate nucleus (ARC) of the hypothalamus appear to be critical: orexigenic neurones expressing neuropeptide Y (NPY) and agouti related peptide (AgRP), and anorexigenic neurones expressing pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART). The resulting change in appetite impacts energy intake and expenditure, and therefore overall energy homeostasis (Schwartz et al., 2000). Furthermore, the ARC influences neuronal activity in the paraventricular nucleus (PVN) of the hypothalamus, which has a role in responsiveness to alterations in energy expenditure such as basal metabolic rate and sympathetic regulation (Gardiner et al., 2008).

The crucial inputs to this system include firstly, short-term signals which denote meal ingestion, a function mostly handled by the 'gut-brain axis'. Secondly, long-term energy stores are signalled to the brain principally by leptin, an adipose-derived factor (Morris and Rui, 2009). Thirdly, circadian rhythms can also influence appetite, and recent data suggests that prokineticin-2, which is highly expressed in the 'master circadian oscillator' or the suprachiasmatic nucleus of the hypothalamus, may mediate this by directly influencing the ARC and other hypothalamic nuclei (Gardiner et al., 2010). The following discussion will concentrate on the 'gut-brain axis'.

1.2.2 Peripheral regulation of energy balance

1.2.2.1 The gut-brain axis

The gastrointestinal tract, together with the pancreas, forms the largest endocrine organ in the body (Ahlman and Nilsson, 2001). The 'gut-brain axis' transmits information on the state of energy balance from the gastrointestinal tract to the hypothalamus and brainstem, via gut hormones and the vagus nerve. These gut

hormones involved in appetite control can be either orexigenic (stimulating appetite) or anorexigenic (reducing appetite). Physiologically, ghrelin is the only orexigenic hormone to have been found thus far while several anorexigenic hormones have been discovered. These include glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM), peptide tyrosine-tyrosine (PYY), pancreatic polypeptide (PP) and cholecystokinin. These peptides appear to act as meal initiators and terminators, thus impacting short-term energy intake (Chaudhri et al., 2008).

Circulating gut hormones influence neuronal activity in the central nervous system (CNS) either 1) directly by penetrating the blood-brain barrier at circumventricular organs, such as the median eminence of the hypothalamus and the area postrema (AP) of the brainstem, or 2) indirectly via receptors on the afferent vagus, in conjunction with stretch receptors and nutrient chemoreceptors. These signals converge in the nucleus of the tractus solitarius (NTS) in the brainstem (Koda et al., 2005) and are integrated with information from higher brain centres, such as drive and reward, to coordinate appetite regulation via the ARC (see figure. 1.1).

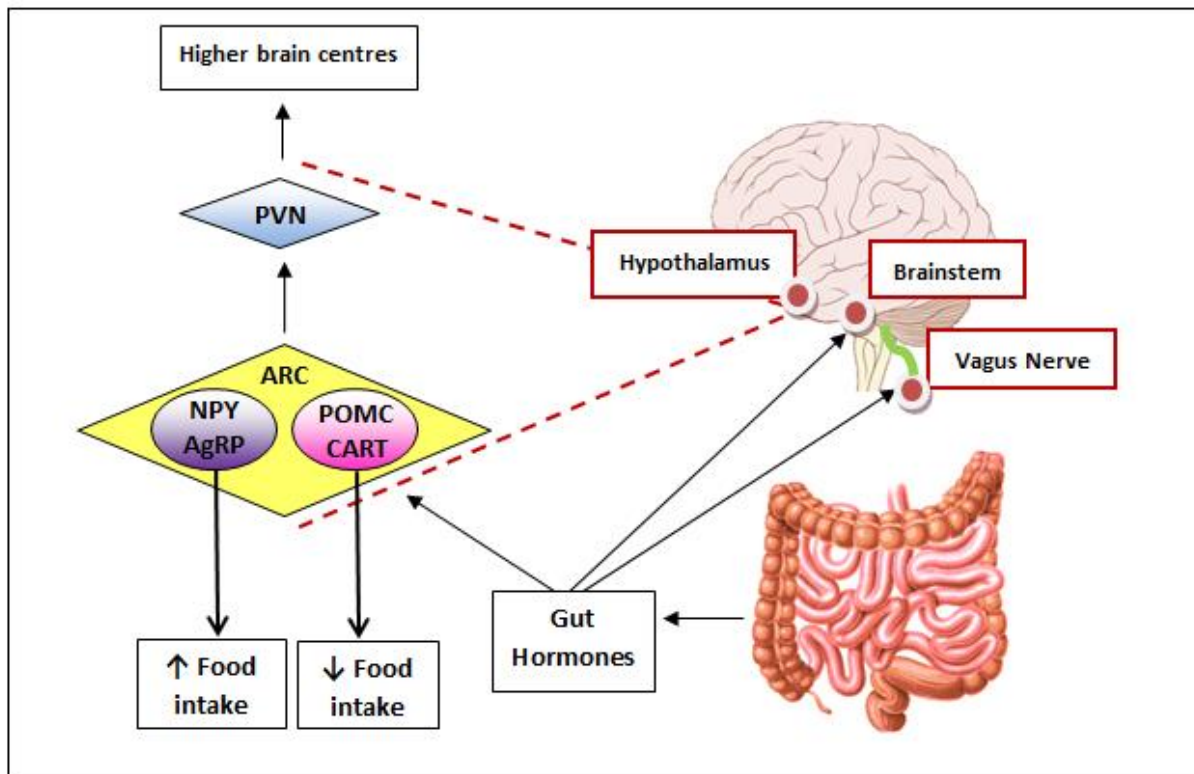


Figure 1.1. Mechanisms of gut hormone action. Gut hormones released from the gut are proposed to act at three sites to influence food intake: 1) the vagus nerve, 2) the brainstem and 3) the hypothalamus. PVN paraventricular nucleus, ARC arcuate nucleus, NPY neuropeptide Y, AgRP agouti-related peptide, POMC pro-opiomelanocortin, CART cocaine and amphetamine-regulated transcript.

1.2.2.2 Secretin family peptides

The secretin family comprises glucagon, GLP-1, GLP-2, glucose-dependent insulinotropic peptide (GIP), growth hormone releasing hormone (GHRH), secretin, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase-activating peptide (PACAP). These hormones are expressed in endocrine cells of the pancreas, gastrointestinal epithelium and neurones in the brain. Their biological functions include regulation of food intake, glucose homeostasis, gut transit and digestion, EE and growth (Mayo et al., 2003).

These peptides are structurally related (see figure 1.2) and their active site is contained within the N terminal amino acids. Their actions are exerted through the evolutionarily distinct G protein coupled receptor (GPCR) 'class B' or 'secretin' family. These receptors comprise a large N-terminal extracellular domain, which has an important role in ligand binding, and seven transmembrane helices with intra and extracellular loops followed by an intracellular C terminal domain.

Following binding of secretin family peptides to the GPCR class B family of receptors, these hormones activate adenylyl cyclase and increase cyclic adenosine 3',5'-monophosphate (cAMP) through downstream signalling pathways (Hill et al., 2010). Although each hormone binds to its own respective receptor, at high doses, they can bind to other receptors within the family. For example, PACAP binds to several GPCRs including PAC1 (highest affinity to PACAP), VPAC1 and VPAC2 (VIP receptors) but can also bind to the secretin receptor at high doses (Felley et al., 1992). Due to the structural similarity within the secretin family, these hormones exert similar biological effects in the gut including inhibition of gastric acid secretion, increased insulin production and delayed gastric emptying.

This thesis focuses on two members of the secretin family, glucagon and GLP-1, and the regulation of food intake, EE and glucose homeostasis.

	1				5					10				15				20			25				30				35			40											
GLUCAGON	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr															
GLP-1(7-36)	His	Ala	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly	Gln	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg														
GLP-2	His	Ala	Asp	Gly	Ser	Phe	Ser	Asp	Glu	Met	Asn	Thr	Ile	Leu	Asp	Asn	Leu	Ala	Ala	Arg	Asp	Phe	Ile	Asn	Trp	Leu	Ile	Gln	Thr	Lys	Ile	Thr	Asp	Arg									
GIP	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ile	Ala	Met	Asp	Lys	Ile	His	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala	Gln	Lys	Gly	Lys	Thr	Asp	Arg								
GHRH	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly	Glu	Ser	Asn	Asp	Trp	Lys	His	Asn	Ile	Thr	Gln	
SECRETIN	His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg	Glu	Gly	Ala	Arg	Leu	Gln	Arg	Leu	Gln	Gly	Leu	Val																	
VIP	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn															
PACAP	His	Ser	Asp	Gly	Ile	Phe	Thr	Asp	Ser	Tyr	Ser	Arg	Tyr	Arg	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr	Leu	Ala	Ala	Val	Leu	Gly	Lys	Arg	Tyr	Lys	Gln	Arg	Val	Lys	Asn	Lys					

Figure 1.2. Amino acid sequences of the secretin family peptide hormones. Fully conserved regions are depicted in pink and regions of similar residue polarity in green. There is high sequence homology at the N-terminal region which contains the active site. Abbreviations: glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glucose-dependent insulintropic peptide (GIP), growth hormone releasing hormone (GHRH), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP). Amino acids: alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), tyrosine (Tyr).

1.2.2.3 Glucagon

Glucagon is a 29 amino acid peptide hormone, released by pancreatic alpha cells and was first discovered by Kimball and Murlin in the 1920s (Kimball and Murlin, 1923). The peptide is derived from proglucagon, which undergoes post-translational cleavage in a tissue-dependent manner under the action of different prohormone convertase (PC) enzymes (Rehfeld, 1998). In alpha cells of the pancreas, PC2 produces glucagon whereas in the intestinal L cells (Dey et al., 2005) and CNS, PC1/3 produce GLP-1, GLP-2 and oxyntomodulin (Mojsov et al., 1986) (figure 1.3).

Glucagon is best known as a counter-regulatory hormone that is secreted at high levels during hypoglycaemia and fasting, promoting glycogenolysis and gluconeogenesis, as well as hepatic fatty acid β -oxidation and ketogenesis (Cryer, 2012). Glucagon release is also stimulated by low circulating levels of certain free fatty acids (FFAs) and amino acids (Kuhara et al., 1991, Olofsson et al., 2004). In contrast, insulin, GLP-1 and somatostatin have an inhibitory effect on glucagon secretion (Akesson et al., 2003, Franklin et al., 2005, Fehmann et al., 1995, Wendt et al., 2004). Hypoglycaemia causes the rapid release of glucagon from pancreatic alpha cells via parasympathetic and

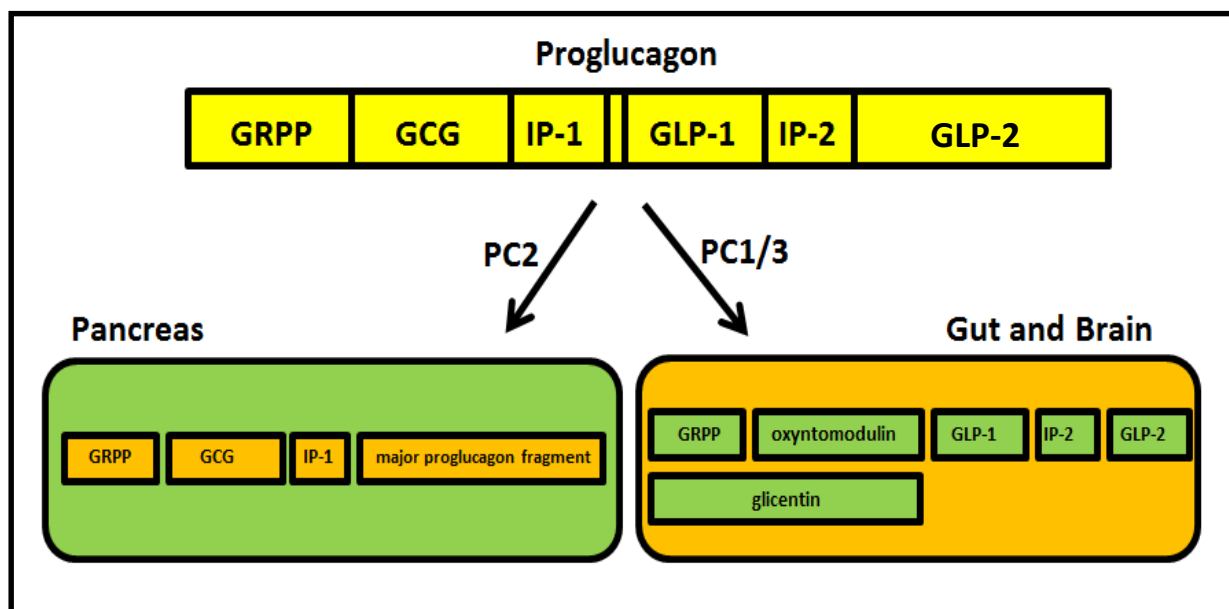


Figure 1.3. Differential processing of proglucagon by site-dependent prohormone convertases (PC) in pancreatic alpha cells and gut L-cells. (GRPP, glicentin-related pancreatic peptide. IP, intervening peptide).

sympathetic nerves that convey signals from the ventromedial nucleus (VMN) to the islets of Langerhans (Ahren, 2000). In addition, the liver contains afferent and efferent connections from the hypothalamus that are implicated in the control of glucose. Interestingly, circulating glucagon activates glucagon receptor signaling in the hypothalamus to inhibit hepatic glucose production and counteract its own direct stimulatory effect in the liver to maintain glucose homeostasis (Mighiu et al., 2013). Circulating glucagon is hydrolysed by dipeptidyl peptidase-4 (DPP-4) resulting in a short half-life of 1-2 minutes (Hinke et al., 2000). Clearance of glucagon also occurs at the liver and kidney (Deacon et al., 2003).

The glucagon receptor (GCGR) was identified in 1971 (Rodbell et al., 1971b). Cloning of the receptor cDNA in 1993 identified it as belonging to the class B GPCR family (Mayo et al., 2003). Although a high degree of homology is seen between GLP-1 and glucagon and their receptors, these peptides bind with a high degree of selectivity to their respective receptors (Hjorth et al., 1994). The glucagon receptor is expressed in a wide variety of tissues, including the liver, kidney, adipose tissue, pancreas, heart, brain, gastrointestinal tract and adrenal glands (Svoboda et al., 1994, Authier and Desbuquois, 2008). Therefore its effects are more far reaching than simply regulating glucose metabolism and it is increasingly recognised that glucagon also plays a key role in energy homeostasis as well as cardiovascular regulation.

In rodents, glucagon levels increase within the first few minutes of ingestion, peak at the end of a meal and gradually return to premeal levels within 10 minutes (Jong et al., 1977). This pattern is also seen during sham feeding (Nilsson and Uvnaswallensten, 1977) suggesting cephalic neural stimuli account for the rise in glucagon, rather than an effect of gut nutrients or mechanical gastric distension. In humans, glucagon is also secreted at the time of meal ingestion (Geary, 1990, Unger et al., 1978). However, obese subjects demonstrate delayed glucagon release following a meal compared to lean controls (Holst et al., 1983). In contrast, patients with type 2 diabetes show blunted glucagon production in response to hypoglycaemia, yet the secretion of glucagon in response to meals is typically exaggerated (Young, 2005). Exogenously administered glucagon, given subcutaneously, intraperitoneally, intravenously or intramuscularly, in animals and humans, decreases food intake (Penick and Hinkle, 1961, Schulman et al.,

1957, Holloway and Stevenson, 1964, Geary et al., 1981, Geary and Smith, 1982, Lesauter et al., 1991, Geary et al., 1992, Geary et al., 1993). Glucagon potently increases satiety and acutely reduces food intake by 12% when infused to mimic plasma levels of the portal vein under physiological conditions (Geary et al., 1992). In contrast, administration of glucagon antibodies increases meal size and duration in rats (Lesauter et al., 1991, Langhans et al., 1982).

Another favourable property, for the purpose of treating obesity, is glucagon's ability to increase energy expenditure by 15% during infusion (Nair, 1987). The mechanism by which glucagon increases energy expenditure is unknown. One possibility is through promoting non-shivering thermogenesis in brown adipose tissue (Billington et al., 1991). Another hypothesis is that glucagon increases futile substrate cycling whereby energy-consuming cyclical metabolic pathways are stimulated but with no net change in product formation in the liver or other tissues (Miyoshi et al., 1988).

In vitro studies suggest that glucagon may increase EE by stimulating oxygen consumption and heat production in BAT (Joel, 1966, Kuroshima and Yahata, 1979). In rats, glucagon administration increases whole body oxygen consumption, body temperature, blood flow, temperature and BAT mass (Yahata et al., 1983, Billington et al., 1991). These effects are attenuated by propranolol, a non-selective adrenergic receptor blocker, as well as denervation of BAT, suggesting a role for the sympathetic nervous system in increased EE following glucagon administration (Dicker et al., 1998, Billington et al., 1987). Longer-term studies also implicate BAT in glucagon-induced thermogenesis. Glucagon infusion via continuous osmotic mini pumps for 5 days increases guanosine diphosphate (GDP) binding to brown fat mitochondria (Billington et al., 1991). It is noteworthy, however, that most studies investigating the role of glucagon and EE administer pharmacological doses of glucagon which could activate the hypothalamic-pituitary-adrenal axis and hence a 'stress response'. These actions suggest that glucagon has a role as a stress hormone as well as in glucose homeostasis (see figure 1.4)(Jones et al., 2012).

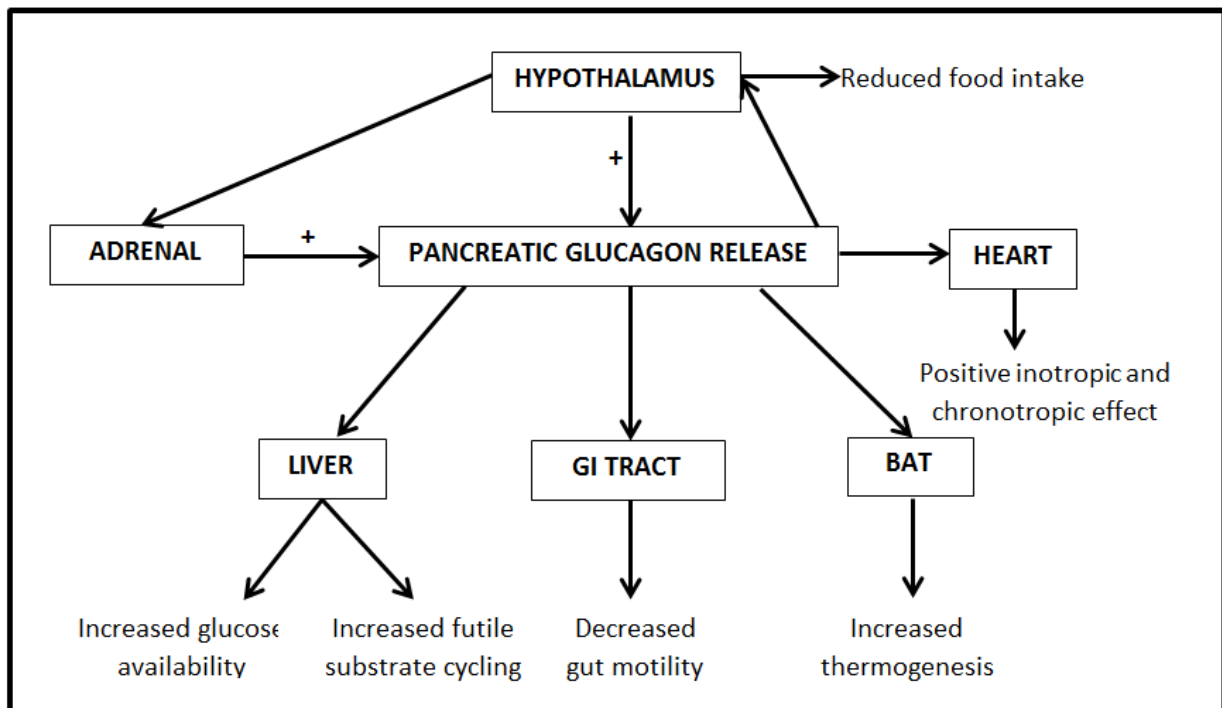


Figure 1.4. Physiological effects of glucagon.

1.2.2.4 Glucagon-like peptide-1 (GLP-1)

GLP-1 is a product of proglucagon, secreted by enteroendocrine L-cells in the intestine. After a meal, it is released in response to direct L-cell stimulation by nutrients within the gut lumen, and indirectly via neuronal pathways within the enteric nervous system. GLP-1 1-37 is processed intracellularly to generate the biologically active peptides, 7-37 and 7-36 amide, although the latter is more prevalent in the circulation. GLP-1 is rapidly inactivated by dipeptidyl peptidase-4 which processes GLP-1 7-37 and 7-36 amide to 9-36 amide, giving the active peptides a half-life of just a few minutes (Deacon et al., 1995).

GLP-1 binds the G-protein coupled GLP-1 receptor found in pancreatic islet cells as well as brain, heart, liver, kidney stomach and lung tissue (Thorens, 1992, Wheeler et al., 1993, Campos et al., 1994). The actions of GLP-1 are best characterized in the beta cell where GLP-1 exerts an incretin effect, the stimulation of glucose-dependent insulin release from pancreatic beta cells (Kreymann et al., 1987). Therefore, until recently, the focus on GLP-1 has been largely as an anti-diabetic agent and several long acting

analogues of GLP-1 (exenatide, liraglutide, lixisenatide, albiglutide and dulaglutide) are licensed for the treatment of diabetes.

GLP-1 also inhibits glucagon release and delays gastric emptying (Nauck et al., 1997). The precise mechanism by which GLP-1 causes inhibition of glucagon is unclear. It is postulated that GLP-1 binds the GLP-1 receptor on pancreatic alpha cells and inhibits glucagon release through a direct mechanism (Heller et al., 1997). Alternatively, glucagon may be inhibited indirectly by GLP-1 induced secretion of insulin, which itself reduces circulating glucagon. Against this, GLP-1 has been shown to suppress glucagon release from pancreatic islets in patients with type 1 diabetes that have no residual cell insulin secretion (Creutzfeldt et al., 1996). Finally, GLP-1 agonists stimulate beta cell proliferation and inhibit apoptosis through the release of transcription factors, thereby increasing beta cell mass (Drucker, 2003).

In contrast to insulin, however, GLP-1 causes a decrease in body weight (Zander et al., 2002). Acute intravenous injection of GLP-1 reduces appetite and calorie intake (Verdich et al., 2001). This effect has been observed in lean, obese and diabetic volunteers (Flint et al., 1998, Todd et al., 1998, Verdich et al., 2001). Additionally, daily subcutaneous injection over 5 days causes an average weight loss of 0.55 kg (Naslund et al., 2004). The actions of GLP-1 on appetite are likely to be related to a direct effect on the CNS via activation of POMC-expressing neurons in the arcuate nucleus (ARC), as well as delayed gastric emptying (Willms et al., 1996). The physiological secretion of GLP-1 after a meal thus mediates key aspects of postprandial metabolism and behaviour. These and other actions of GLP-1 are summarised in figure 1.5. In contrast to glucagon, GLP-1 does not increase energy expenditure. Indeed, a study carried out by Flint et al found that energy expenditure after a standard breakfast was attenuated with GLP-1 infusion compared with saline (Flint et al., 2001).

The indication for GLP-1 analogues is set to expand to aid weight loss in obese individuals who do not have diabetes, and in January 2015, the European Medicines Agency recommended granting a marketing authorisation for Saxenda (liraglutide) for weight management in overweight or obese adults (European Medicines Agency, 2015).

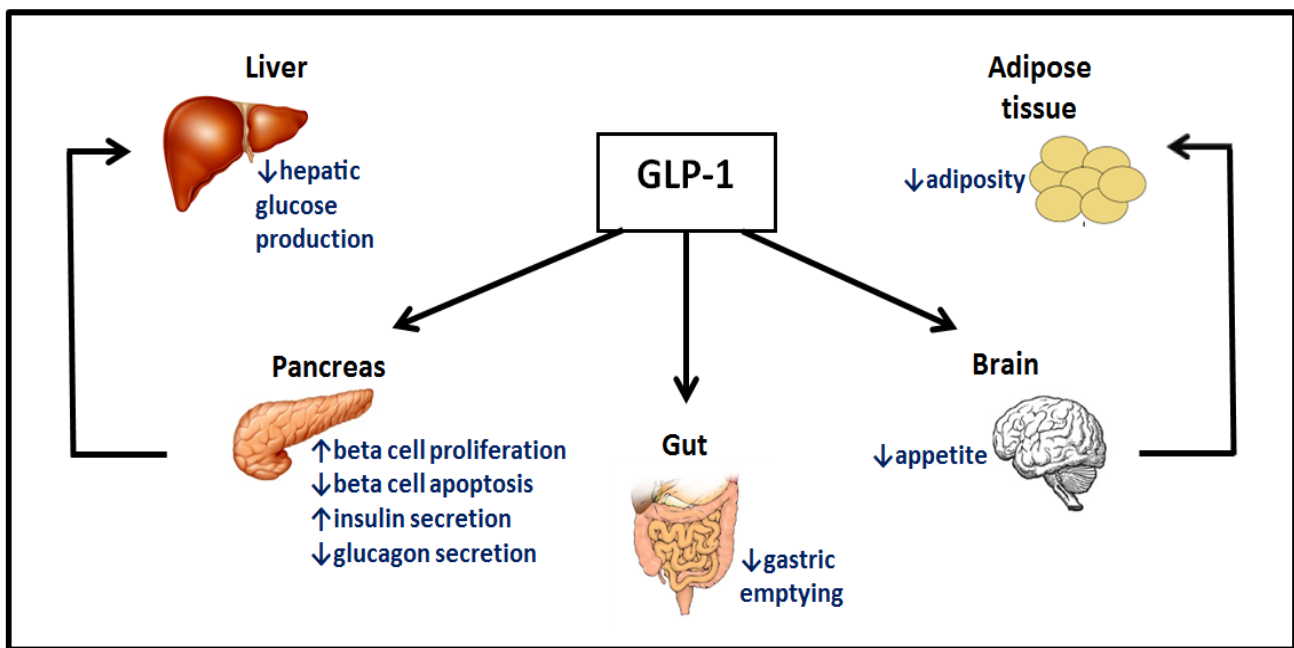


Figure 1.5. Actions of GLP-1.

1.2.2.5 Oxyntomodulin

OXM is a 37 amino acid peptide co-secreted with GLP-1 from intestinal L cells post-prandially. Thus far, no specific receptor has been identified for OXM. The peptide acts as an agonist at both the GLP-1R and GCGR although with lower affinity compared to the individual peptides (Baldissera et al, 1988, Dakin et al., 2001, Baggio et al., 2004). In both rodents and humans, OXM has been shown to reduce food intake and body weight, increase EE and improve glucose control (Dakin et al., 2001, Cohen et al., 2003, Wynne et al., 2006). Following gastric bypass surgery, patients exhibit a two fold rise in OXM, GLP-1 and PYY levels following an oral glucose load compared to controls who had similar body weight loss due to dieting (Laferrere et al., 2010). These findings suggest that these changes in circulating gut hormone levels are due to the surgical procedure rather than weight loss and account for the improved glucose profile seen so rapidly following surgery.

It is postulated that the effects of OXM on food intake occur mainly via the GLP-1R. Food intake and c-fos activation in the ARC following peripheral OXM were blocked by the prior administration of exendin(9-39), a potent GLP-1 antagonist (Dakin et al., 2004). Consistent with this, GLP-1R knockout mice do not show reduced food intake following

centrally administered OXM (Baggio et al., 2004). Peripherally administered OXM also significantly increases c-fos expression in the PVN, AP and NTS. This is abolished in GLP-1R but not GCGR knockout mice (Baggio et al., 2004). This suggests that the presence of a functional GLP-1R is critical for the anorectic effect of OXM to be realised. However, both GLP-1 and OXM increase heart rate in mice. This effect is abolished in GLP-1R knockout mice following GLP-1 administration but not OXM (Gros et al., 2003). Therefore, OXM may have effects that are independent of the GLP-1R.

Chronic treatment with OXM in mice causes greater significant weight loss compared to the GLP-1R agonist, suggesting a beneficial weight lowering effect through GCGR agonism (Kosinski et al., 2012). Studies in GLP-1R and GCGR knockout mice have also demonstrated the important contribution of GCGR agonism on EE and weight loss (Pocai et al., 2009, Day et al., 2009). Repeated administration of a dual GCGR and GLP-1R agonist results in weight loss and reduced food intake in wild-type mice, however this effect is attenuated in GCGR knockout mice (Pocai et al., 2009). Furthermore, administration of a dual glucagon and GLP-1 receptor agonist for one month to DIO mice, significantly decreases food intake, body weight and fat mass and increases EE compared to controls (Day et al., 2009). These effects are still evident following administration in GLP-1R knockout mice (Day et al., 2009). Therefore OXM provides a unique model of dual receptor agonism, causing weight loss through a reduction in food intake via the GLP-1 receptor and increased EE via the GCGR. Dual analogues such as ZP2929 by Zealand Pharma combining these properties are under development (Fosgerau and Hoffmann, 2015).

1.3 Modifying agonist activity at the glucagon receptor

Ideally, the beneficial effects of both glucagon and GLP-1 would be combined into one molecule, which would exert its effects by interacting with both the glucagon and GLP-1 receptors. These native peptides have 50% sequence homology, thus modifying the amino acid structure of glucagon could affect promiscuity at the GLP-1 receptor. Understanding the cellular mechanisms by which glucagon activates the glucagon receptor may therefore enable the development of new therapies for obesity and diabetes based on glucagon.

1.3.1 Glucagon cell signalling

Glucagon binds to the GCGR, a 7 transmembrane G-protein coupled receptor (GPCR), and activates adenylyl cyclase through heterotrimeric G α s G proteins, leading to a rise in intracellular cAMP levels and subsequent activation of protein kinase A (PKA) signalling (Rodbell et al., 1971a, Mayo et al., 2003, Authier and Desbuquois, 2008). In hepatocytes, elevated PKA activity regulates various downstream targets including phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase, fructose-1,6-bisphosphatase and peroxisome proliferator-activated receptor γ coactivator-1, resulting in the suppression of glycolysis and glycogen synthesis, and enhancement of gluconeogenesis and glycogenolysis (Jiang and Zhang, 2003, Cho et al., 2012).

Additionally, glucagon stimulates the phospholipase C-inositol phosphate pathway in hepatocytes via the Gq subunit, inducing intracellular Ca²⁺ signalling and stimulating glycogenolysis and gluconeogenesis (Wakelam et al., 1986, Mayo et al., 2003, Authier and Desbuquois, 2008). Glucagon-induced Ca²⁺ mobilization may be mediated by cAMP (Connelly et al., 1987, Staddon and Hansford, 1989), while others have demonstrated that it may be either potentiated by cAMP (Hansen et al., 1998) or independent of cAMP (Wakelam et al., 1986, Mine et al., 1988a). Thus the exact mechanisms by which inositol phosphate and Ca²⁺ signalling are activated are not fully understood (Authier and Desbuquois, 2008) (see figure 1.6).

Furthermore, glucagon has also been implicated in signalling via 5'-AMP-activated protein kinase (AMPK) (Kimball et al., 2004) mitogen-activated protein kinase (MAPK)

and in a c-Jun n-terminal kinase(JnK)-dependent manner (Chen et al., 1998). Although work to unpick the signalling pathways of glucagon has been underway since the 1970s, it has focussed primarily on ascertaining which pathways are involved in the downstream effects in the liver and the role of glucagon in control of glycaemia. It is yet unknown as to which specific pathways are involved in the extrahepatic roles of glucagon, namely in appetite regulation and control of energy expenditure.

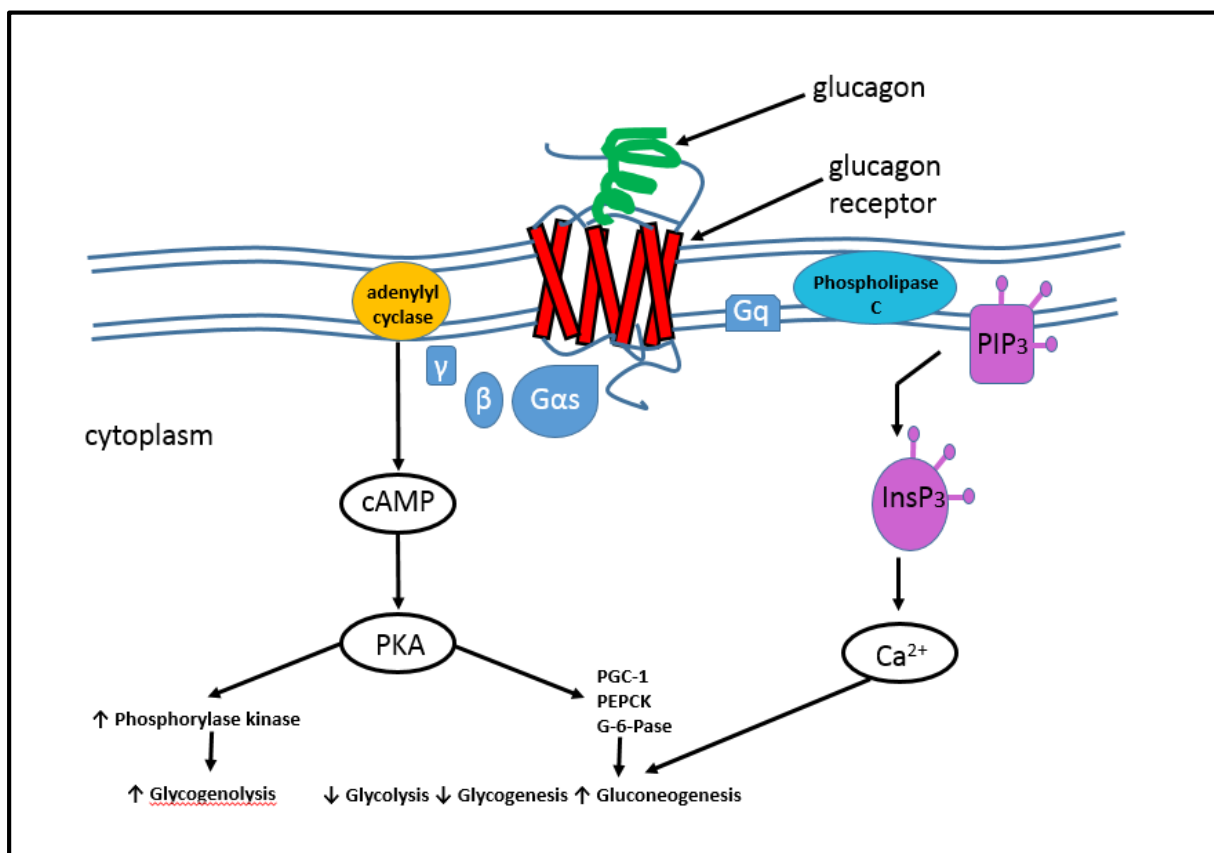


Figure 1.6. Glucagon signalling at the glucagon receptor. Two main pathways are implicated. Firstly, on activation of the receptor, the $G_{\alpha s}$ protein is recruited causing an accumulation of cAMP via adenylyl cyclase accumulation. Secondly, the G_q subunit is recruited and activates phospholipase C to increase the synthesis of inositol triphosphate (InsP3), leading to a subsequent increase in intracellular Ca levels. In the liver these events promote downstream signalling to ultimately cause a rise in glucose.

As a prototypical Class B GPCR, the glucagon receptor is desensitised and sequestered in the cytosol after exposure to glucagon (Authier et al., 1992, Buggy et al., 1997, Merlen et al., 2006). The internalised receptor is then either recycled to the cell surface or targeted for degradation. Therefore recycling of receptors is an important mechanism of resensitization. β -Arrestins modulate postendocytic sorting of some GPCRs, in particular recycling (Oakley et al., 1999, Oakley et al., 2000, Vines et al., 2003). Krilov et al recently undertook two studies to elucidate the mechanisms of GCGR internalisation and the pathways of postendocytic sorting, specifically, receptor recycling and degradation (Krilov et al., 2008, Krilov et al., 2011). They found that the glucagon receptor recycles to the plasma membrane in a β -Arrestin-dependent manner and that downregulation of β -arrestins significantly reduced recycling.

1.3.2 Allosteric modulation of cell signalling pathways

Despite GPCRs such as the glucagon receptor being among the most fruitful targets for marketed drugs, discovery efforts have failed to deliver selective drug candidates. Historically, drug discovery programmes for GPCR ligands were dominated by efforts to develop agonists and antagonists that act at the orthosteric sites for endogenous ligands. However, in recent years, there have been advances in the discovery of novel ligands for GPCRs that act at allosteric sites to regulate receptor function (Conn et al., 2009).

These allosteric modulators are ligands that bind to a site distinct from the orthosteric site. They may modulate ligand binding to the orthosteric site and/or influence the potency of an orthosteric ligand in inducing intracellular signalling. Allosteric modulators may exert their cooperative effects on receptor activation by stabilising particular receptor conformations (Christopoulos and Kenakin, 2002). These influences on the tertiary structure can act to either drive the receptor towards remaining in the inactive state or to advance it to an active state.

Research into allosteric modulation has gained momentum recently for a number of reasons. Firstly there is the saturability effect, which is best described when comparing an allosteric modulator to an orthosteric competitive antagonist (Birdsall et al., 1996). The maximal impact of an orthosteric antagonist is determined by its concentration. If this concentration is too high or too low, the receptor is either over- or under-

stimulated. In contrast, a high concentration of an allosteric modulator can act as a depot for binding to the allosteric site while its maximal activity is instead determined by the co-operativity factor, i.e. the level of influence of the allosteric ligand on receptor activity (Ehlert, 1985). Generally the allosteric ligand cannot have an effect without simultaneously binding the endogenous orthosteric ligand.

Secondly, the effect of allosteric ligands on receptor activation is sensitive to physiological spatial and temporal fluctuations in the local concentrations of the endogenous orthosteric ligand (Birdsall et al., 1996). Allosteric ligands generally only exert their effects when an endogenous agonist is present, despite being constantly present in the receptor compartment.

Finally allosteric ligands, when compared to orthosteric ligands, offer the possibility of much greater receptor subtype selectivity (Lazareno et al., 1998). The rest of the receptor's surface outside the orthosteric site offers greater divergence in structure and therefore also in binding affinity. Furthermore, this structural divergence between receptors means that there will also be differences in the co-operativity factor offered by an allosteric ligand at different receptors.

The potential of allosteric modulation has triggered interest in advancing our knowledge of the endogenous allosteric modulatory systems. These studies have provided evidence that GPCRs are active participants in protein-protein interactions that can occur independently or in conjunction with the receptor coupling to an accessory protein. For example, Dopamine D₁ receptors preferentially signal through G_s proteins to stimulate cAMP accumulation. Recently, a single transmembrane-spanning protein, named calcyon, has been found to physically associate with D₁ receptors in neurons and potentiate their ability to stimulate intracellular calcium release, a typically G_q-coupled response (Lezcano et al., 2000). A further example is that of the chemokine CCR2B receptor which, on stimulation by the monocyte chemotactic protein 1, promotes the rapid association of the receptor with the Janus kinase 2/STAT3 protein pathway. It has been postulated that the association of the CCR2B receptor with its cognate G_i protein requires the allosteric effects induced in the receptor by both monocyte chemotactic protein 1 binding and Janus kinase 2 association (Mellado et al., 1998). A particularly well characterized example of an accessory protein clearly

altering the phenotype of the receptor is a family of single transmembrane proteins required for the transport and ligand specificity known as receptor activity modifying proteins (RAMPs). These are outlined in more detail in the following section.

1.3.3 Receptor Activity Modifying Proteins

In 1998, McLatchie et al reported the discovery of the Receptor Activity Modifying Protein (RAMP) family of accessory proteins (McLatchie et al., 1998). Three RAMPs have been identified thus far, the presence of which can alter the structural confirmation of the receptor and thus ligand binding. The calcitonin receptor like receptor (CRLR) belongs to the class 'B' GPCR family. In the presence of RAMP 1, this receptor binds to and is activated by calcitonin gene related peptide (CGRP). RAMP1 also seems to be important in controlling the translocation of CRLR to the cell surface. However, in the presence of RAMP 2 or RAMP 3, it is activated by adrenomedullin (McLatchie et al., 1998, Buhlmann et al., 1999). Similarly, the calcitonin receptor, in the absence of RAMPs, is activated by calcitonin. In the presence of RAMPs 1, 2 and 3, it changes its phenotype to become an amylin receptor (Udawela et al., 2004, Muff et al., 1999) (see figure 1.7).

There are three RAMPs ubiquitously distributed among tissues and sharing approximately a 31% homology. Although RAMPs are implicated in the trafficking of receptors to the cell membrane and the regulation of the glycosylation (McLatchie et al., 1998, Foord and Marshall, 1999, Fraser et al., 1999), there are effects of RAMPs, particularly RAMP3, that cannot be explained through simple differences in receptor expression. Indeed there is evidence to suggest that RAMPs remain associated with receptors at the cell surface membrane (McLatchie et al., 1998, Leuthauser et al., 2000).

Studies with human calcitonin receptors demonstrate striking effects of RAMPs. Cotransfection of RAMP3 with human calcitonin receptors produces reduced potency of human calcitonin and increased potency of rat amylin (Armour et al., 1999). This effect is consistent with a RAMP3-induced change in calcitonin receptor coupling to G proteins. However, RAMP3 also confers a change in the potency of the peptide calcitonin

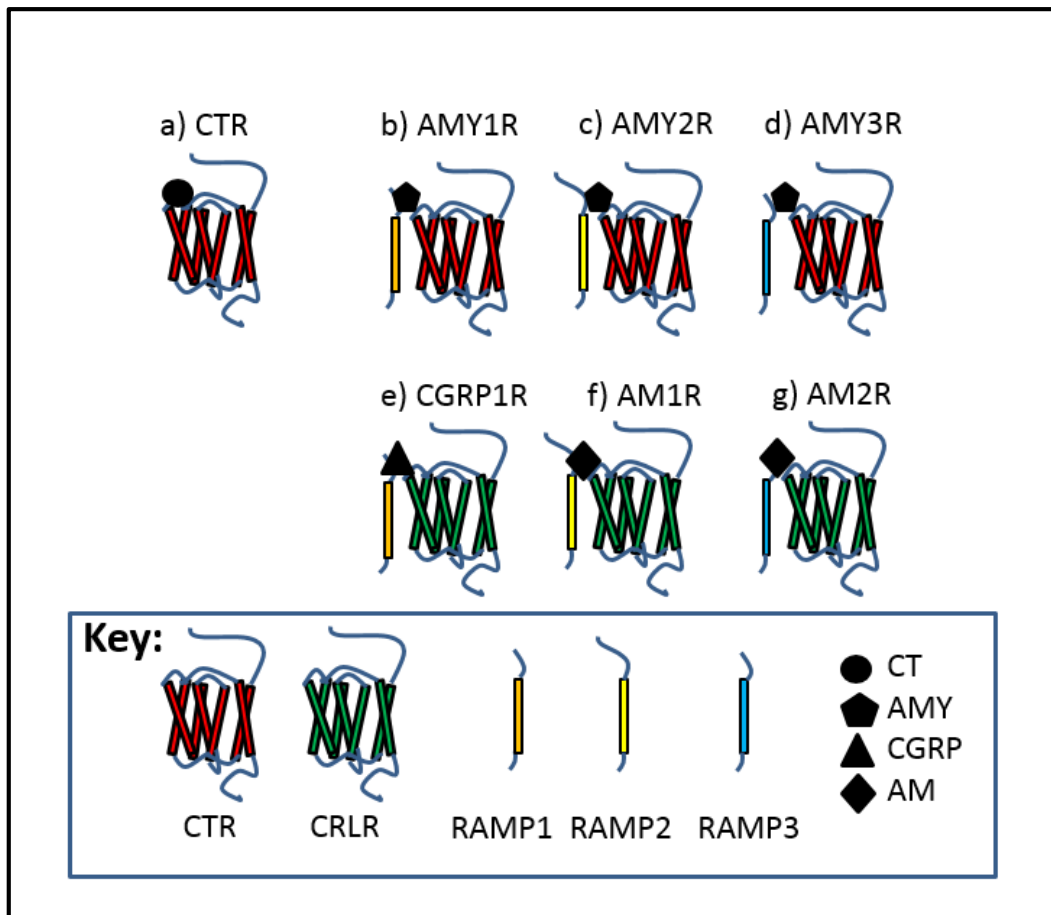


Figure 1.7. The binding of the calcitonin family of peptides to the calcitonin receptor (CTR) and the calcitonin receptor-like receptor (CRLR) is governed by RAMPs. Abbreviations: CT, calcitonin; AMY, amylin; CGRP, calcitonin gene related peptide; AM, adrenomedullin.

antagonist AC66 for antagonism of amylin, but not for human calcitonin response. This work indicates that RAMP3 associates with the receptor to change its behaviour to both agonists and antagonists (Armour et al., 1999). Furthermore, a study by Christopoulos et al (Christopoulos et al., 1999) demonstrates that cotransfection of RAMP1 and RAMP3 produces an improved specific binding of amylin to human calcitonin receptors in COS-7 cells. The binding and functional profiles obtained with these two RAMP subtypes in this study are consistent with the production of two separate amylin-like receptors (Christopoulos et al., 1999).

Thus, RAMPs appear to be able to confer changes in ligand binding and specificity, as well as affecting the ligand's functional down-stream effect. The discovery that RAMPs

are sometimes required to generate receptor phenotypes corresponding to native receptors may be an example of a more generalized phenomenon.

1.3.4 RAMPs and the glucagon receptor

In 2003, Christopoulos and colleagues used immunofluorescence microscopy to show that co-expression of RAMP2 and the GCGR in COS-7 cells translocated RAMP2 to the cell surface, supporting the notion that these two molecules interact (Christopoulos et al., 2003). The same work showed that RAMP1 and RAMP3 do not appear to interact with GCGR. However, to date, no published work has examined whether this interaction has any functional impact on ligand binding and activation. Their presence, however, suggests alternative receptor morphology which may lead to potential biased agonism.

1.4 Summary

Individually, glucagon and GLP-1 analogues do not represent complete solutions for diabetes and obesity. However, dual agonism at both the GLP-1 and glucagon receptors augments appetite reduction and weight loss in rodents (Pocai et al., 2009, Day et al., 2009). This approach combines the appetite suppressive effects of GLP-1 and glucagon with the energy expenditure increasing effects of glucagon. At the same time, the hyperglycaemic effects of glucagon are counterbalanced by GLP-1's insulintropic effects. A dual acting analogue of glucagon and GLP-1 would therefore have additional benefit over current licensed GLP-1 based therapies for diabetes.

These findings are further exemplified by the existence of a third proglucagon derivative, oxyntomodulin (OXM). This native peptide is a weak agonist of both the glucagon and GLP-1 receptors and has been demonstrated to cause weight loss without hyperglycaemia (Wynne et al., 2005).

In addition, it is clear that a long-acting analogue that has promiscuity at both the glucagon and GLP-1 receptors would have promise as a potential therapeutic agent for obesity. Ideally, the anorectic and energy expenditure effects of glucagon would be maximised, while minimising the hyperglycaemic effects. Using allosteric modulation through the RAMP2 system may allow 'biasing' of the signalling pathways to exploit the desirable downstream effects. This work may therefore pave the way for the development of a glucagon/GLP-1 analogue for the treatment of diabetes and obesity.

1.5 Hypotheses

Overall hypothesis: Dual agonism of both the glucagon and GLP-1 receptors is a rational approach to the development of a new therapy for obesity and diabetes.

Specifically, I hypothesise that:

- 1) Co-infusion of Glucagon and GLP-1 will cause a reduction in food intake and increase in energy expenditure in healthy human volunteers. Any hyperglycaemia resulting from agonism at the glucagon receptor will be counterbalanced by the insulinotropic effects of GLP-1 without any deterioration in glucose homeostasis.
- 2) Co-infusion of Glucagon with GLP-1 will cause an improvement in glucose homeostasis after a fixed calorie load, comparable to GLP-1 alone.
- 3) RAMP2 interacts with the GCGR thus mediating or modifying binding and agonist activity of glucagon and its analogues.

1.6 Aims

In this series of experiments, I aim to:

- 1) investigate the acute effects of co-infusion of Glucagon and GLP-1 on food intake, energy expenditure and glucose homeostasis in healthy human volunteers
- 2) investigate the acute effects of co-infusion of Glucagon and GLP-1 on glucose homeostasis after a standardised mixed meal in healthy human volunteers
- 3) investigate the interaction of RAMP2 and the glucagon receptor in vitro

CHAPTER 2: THE ACUTE EFFECTS OF CO-ADMINISTRATION OF GLUCAGON AND GLP-1 ON ENERGY BALANCE IN HUMANS

2.1 Introduction

GLP-1R agonists are licensed for the treatment of patients with type 2 diabetes and obesity, improving glycaemic control through their insulinotropic effect and resulting in weight loss through a reduction in food intake (Flint et al., 1998). However, the magnitude of weight loss is modest and restricted by dose-limiting nausea (Astrup et al., 2009). Glucagon reduces food intake in rodents (Geary and Smith, 1982, Geary and Smith, 1983, Lesauter et al., 1991) and humans (Geary et al., 1992, Penick and Hinkle, 1961) and increases energy expenditure (Davidson et al., 1960, Nair, 1987). Similarly, its use is limited by nausea and vomiting (Yuen et al., 2013). In addition, glucagon's hyperglycaemic effect, predominantly through an acute stimulation of glycogenolysis is undesirable.

Individually, therefore, glucagon and GLP-1 analogues do not represent complete solutions for the treatment of diabetes and obesity. However, dual agonism at both the GLP-1 and glucagon receptors has been shown to augment appetite reduction and weight loss in rodents (Pocai et al., 2009, Day et al., 2009). This approach combines the appetite suppressive effects of GLP-1 and glucagon with the energy expenditure increasing effects of glucagon. Furthermore, the hyperglycaemic effects of glucagon are counterbalanced by GLP-1's insulinotropic effects. Dual infusion of glucagon and GLP-1 would therefore have additional benefit over current licensed GLP-1 based therapies for diabetes and obesity. These findings are further exemplified by the existence of a third proglucagon derivative, oxyntomodulin (OXM) which has been demonstrated to cause weight loss without hyperglycaemia (Wynne et al., 2005, Wynne et al., 2006). Of note, the anorectic effects of oxyntomodulin are not borne out in GLP-1 receptor knockout mice, indicating that the effect of oxyntomodulin on energy intake is mediated through the GLP-1 receptor (Baggio et al., 2004). However, oxyntomodulin is a relatively weak agonist at the GLP-1 receptor, at least two-fold less potent than GLP-1 itself (Schepp et al., 1996). This raises questions regarding the mechanism of oxyntomodulin's anorectic effect, as GLP-1 and oxyntomodulin are secreted at similar concentrations post-prandially (Ghatei et al., 1983b). Furthermore, chronic treatment with OXM in mice causes greater significant weight loss compared to a GLP-1R agonist, suggesting a beneficial weight lowering effect through GCGR agonism (Kosinski et al., 2012). Thus

dual agonism of both the GCGR and GLP-1R may provide an enhanced anorectic effect, while also increasing energy expenditure.

Previous studies have examined the effect of dual combinations of gut hormones on energy intake in humans. It has been demonstrated that GLP-1/PYY co-infusion (Neary et al., 2005) and OXM/PYY co-infusion (Field et al., 2010) reduce food intake additively. However, co-administration of anorectic doses of OXM/PYY resulted in one-third of participants experiencing nausea, despite the low doses used. In contrast, no nausea was reported following GLP-1/PYY co-infusion (Neary et al., 2005). Additionally, the duration of hormone infusion appears to have an effect on the incidence of nausea. Nausea was reported following 110 minutes intravenous OXM infusion in healthy volunteers (Field et al., 2010). In contrast, OXM infusion at the same dose for 90 minutes demonstrated no side effects (Cohen et al., 2003). Similarly, following intravenous PYY(3-36) infusion in healthy human participants, reported nausea increased with the duration of infusion (Witte et al., 2009).

There is currently no data investigating the duration of GLP-1 or glucagon infusion and the incidence of nausea. In a previous study, intravenous glucagon was infused at 0.8 pmol/kg/min and was demonstrated to reduce food intake with no reported nausea compared to saline controls (Geary et al., 1992). Although plasma glucagon levels were not measured in that study, previous studies have found that the same dose of glucagon administered intravenously increases plasma glucagon levels from 30 to 75-150 pmol/l (Sherwin et al., 1976, Bomboy et al., 1977, Rizza and Gerich, 1979). Intravenous administration of GLP-1 at 0.83 pmol/kg/min reduced food intake by 12% when infused over 2 hours with peak levels reaching 60-90 pmol/l (Flint et al., 1998). Although GLP-1 infusion reduced satiety and feelings of hunger, the effect on nausea was not specifically reported on. In a further study, 0.4 pmol/kg/min GLP-1 similarly reduced food intake compared to placebo (although this did not reach statistical significance) and circulating GLP-1 levels rose to 61.5 pmol/l following 90 minutes infusion (Neary et al., 2005). Throughout that study, there were no reported symptoms of nausea.

Professor Bloom's group have recently shown that a combination of high-dose glucagon (14 pmol/kg/min) and GLP-1 (0.4 pmol/kg/min) does indeed increase energy expenditure in humans (Tan TM et al., 2012). Thus far, the effect of combination on energy intake has not been examined. This chapter investigates the effect of co-administration of low-dose glucagon and GLP-1 on energy balance for the first time in humans.

2.2 Hypothesis and aims

2.2.1 Hypothesis

I hypothesise that co-infusion of glucagon and GLP-1 will cause reduction in food intake in healthy human volunteers over that observed when the respective peptides are given singly. The combination of glucagon and GLP-1 will cause an increase in resting energy expenditure. Additionally, any hyperglycaemia resulting from agonism at the glucagon receptor will be counterbalanced by the insulinotropic effects of GLP-1 without any deterioration in glucose homeostasis.

2.2.2 Aim

To examine the acute effects of intravenous infusion of GLP-1 alone, glucagon alone, and the combination of glucagon with GLP-1 on three outcomes:

1. Food intake during *ad libitum* meal
2. Energy expenditure
3. Plasma glucose homeostasis

2.3 Materials and Methods

2.3.1 Participants

Sixteen non-diabetic subjects with a mean BMI of 27 kg/m² (range 24-32.9) were recruited through an advertisement placed in the London Evening Standard and Metro newspapers. A volunteer information leaflet was sent to those who responded to the advertisement. Potential participants were invited to attend a screening visit at the NIHR/Wellcome Clinical Research Facility, Hammersmith Hospital, which comprised a medical history, routine physical examination and basic investigations (full blood count, urea and electrolytes, liver function tests, thyroid function tests, plasma glucose, lipid profile, HbA1C and electrocardiogram), and assessment of eating behaviour using the Dutch Eating Behaviour Questionnaire (DEB-Q) (Wardle, 1987) and the SCOFF questionnaire (Morgan et al., 1999). Suitability was determined following the screening visit according to the following criteria:

Inclusion criteria

- Aged 18 – 65 years
- Male or female
- BMI between 20 and 35 kg/m²
- Stable body weight for at least 3 months

Exclusion criteria

- History of a medical, psychological or other condition, or use of any medications, including over-the-counter products, which, in the opinion of the investigators, would either interfere with the study or potentially cause harm to the volunteer.
- Pregnancy or breastfeeding
- Unable to maintain adequate contraception for the duration of the study and for one month afterwards
- History of hypersensitivity to any of the components of the infusions
- Donated blood during the preceding 3 months or intention to do so before the end of the study
- High level of restrained pattern of eating, as determined by DEB-Q and SCOFF questionnaires

All participants gave their written consent to take part (see Appendix A), and were free to withdraw from the study at any time. Ethical approval was granted for the study by the West London Research Ethics Committee (reference number 10/H0707/80) and carried out in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki.

Of the initial 16 participants recruited, 3 were excluded from the final analysis, prior to unblinding, as they were found to display abnormal eating behaviour which had not been identified by the standard questionnaires. One subject consumed less than the minimum required 300 kcal at the acclimatisation visit, one subject did not finish eating within the allotted 20 minute time-frame and the third demonstrated progressive aversion to his chosen meal throughout the course of the study as demonstrated by visual analogue scores (VAS). Participants' demographics are described below in Table 2.1.

Volunteer	BMI (kg/m²)	Age (Years)	Sex
1	24.0	25	M
2	24.2	41	M
3	24.8	32	M
4	26.3	40	F
5	26.6	33	M
6	26.8	21	F
7	26.8	40	F
8	26.3	35	M
9	32.9	28	F
10	29.4	23	M
11	27.6	39	M
12	28.5	32	M
13	27.4	22	M
Mean	27.0	31.6	9M, 4F

Table 2.1. Demographics of the study participants. M, Male; F, Female.

2.3.2 Peptides

Natural sequence peptides, including standard pharmaceutical grade glucagon (GlucaGen, Novo Nordisk, UK) and GLP-1₍₇₋₃₆₎ amide (Clinalfa basic, Bachem, Switzerland), were used. Both peptides were manufactured to EU Good Manufacturing Practice quality standards.

Peptide was made up in Gelofusine (B. Braun, Crawley, UK) in 50 ml syringes pre-coated with Gelofusine 30 minutes before administration. Previous testing within the department has demonstrated that this approach minimises peptide loss through adsorption onto the syringe wall (Kraegen et al., 1975).

2.3.3 Study protocol

A double-blind, randomised, crossover design was used for the study. Each subject was scheduled to attend 5 study visits at least two days apart. The first visit was a 'sham' visit in order to acclimatise the subject to the study protocol during which participants were infused with placebo alone (Gelofusine). For each subsequent visit, subjects received intravenous infusions of either:

- 1) placebo (Gelofusine)
- 2) GLP-1₇₋₃₆ amide (0.4 pmol/kg/min)
- 3) glucagon (2.8 pmol/kg/min)
- 4) combined GLP-1 and glucagon at the above doses.

The rate of infusion of glucagon was based on a preliminary dose-finding study performed in the department to establish a sub-anorectic dose of glucagon. A known sub-anorectic dose of GLP-1 previously reported in the literature was used (Verdich et al., 2001, Neary et al., 2005).

Subjects were instructed not to consume alcohol or perform strenuous exercise for 24 hours prior to the study day to control for factors that may affect appetite. They consumed a standardised meal of their choosing the evening before the study. Subjects were asked to fast overnight from 10 pm onwards.

On arrival at 9 am, an intravenous cannula was inserted in each antecubital fossa and baseline blood samples taken for measurement of plasma glucose, insulin, glucagon and

GLP-1 ($t = -60$ min). Indirect calorimetry was performed from $t = -30$ to $t = 0$ to assess baseline resting energy expenditure. The intravenous infusion was started at 10 am ($t = 0$), and lasted for 120 minutes. In order to rapidly achieve steady state concentration, a ramping infusion protocol was used. For $t = 0$ until +5 mins the infusion rate was 4x the steady state rate, for $t = +5$ until +10 min it was 2x the steady state rate, and thereafter it remained constant at the steady state rate. Indirect calorimetry was performed again from $t = +40$ to $t = +70$ min to assess resting energy expenditure during the infusion. Blood samples were taken at a number of time points ($-30, 0, +40, +70, +90, +120, +150, +180$ min) for plasma glucose, serum insulin, plasma glucagon and active GLP-1. Pulse and blood pressure were taken at each time point, and volunteers were asked to fill in visual analogue scales (VAS – see appendix B) to record appetite and nausea levels. An *ad libitum* meal, provided to excess, was served at 11.30 am ($t = +90$ mins) and volunteers were allowed 20 minutes to eat in private until $t = +110$ mins. On each occasion, they were instructed to eat until comfortably full. Each subject received the same meal at each study visit with a standardised preparation and serving ritual. The infusion was stopped at 12.00 pm ($t = +120$ mins). Blood sampling and VAS measurement then continued at 30 minute intervals, until 1.00 pm ($t = +180$ min). At 180 min the cannulae were removed, and the participant was asked to empty their bladder. Urinary volume was measured in order to calculate urinary nitrogen excretion for estimation of protein oxidation. The participant was then discharged home. This protocol is summarised in figure 2.1.

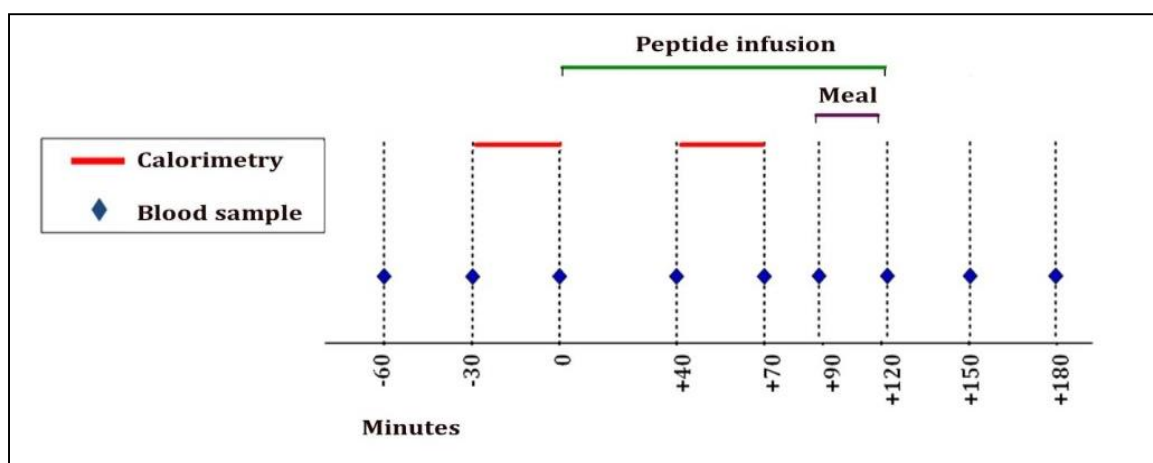


Figure 2.1 Study day protocol.

2.3.4 Assessment of energy intake

Pre-prepared, standardised ready meals (Sainsbury's Supermarkets Ltd, London, U.K.) of known macronutrient and calorific content were used (spaghetti bolognese (188 kcal/100g), chicken tikka masala (178 kcal/100g) or macaroni cheese (194 kcal/100 g)). Meals were weighed immediately before and after eating. Thus, calorie intake was calculated according to weight of food consumed. Participants had the opportunity to try their meal choice during the acclimatisation visit and all had deemed it to be palatable.

2.3.5 Assessment of energy expenditure

At 0930h ($t = -30$ minutes) the volunteer was placed under the canopy of the indirect calorimeter (GEM Nutrition, Daresbury, Cheshire, UK). Prior to measurement of energy expenditure, the calorimeter was calibrated with 'zero' (0.000% O₂, 0.000% CO₂) and 'span' (20% O₂, 1.125% CO₂) gases (BOC Gases, Surrey, UK). Energy expenditure was determined by alternatively measuring O₂ and CO₂ concentrations of inspired and expired air. Flow rate was continually measured to determine the dilution factor. Gas collection was via a ventilated hood placed over the subject's head. Air flow through the hood was set between 30 and 40 litres/min to give optimum conditions for subject comfort. Lightweight flexible tubing connected the hood to the calorimeter, allowing the unit to be some distance away from the hood. Resting energy expenditure (REE), respiratory quotient (RQ), carbohydrate and fat oxidation rates were calculated from the VO₂ and VCO₂ measured at one minute intervals, using the Weir equation adjusted for urinary nitrogen excretion (Weir, 1949, Frayn, 1983).

Calorimetry was carried out for 30 minutes as described previously (Tan TM et al., 2012), with measurements from the final 10 minutes of this phase being used to calculate the baseline resting energy expenditure. Volunteers lay supine on beds at a 45° inclination and were allowed to watch television or to listen to music. At $t = 0$ minutes, a hormone infusion was started and calorimetry was performed again between $t = +40$ and $t = +70$ minutes. As with the baseline, measurements from the last 10 minutes of this phase were used to calculate the infusion resting energy expenditure.

2.3.6 Assessment of glucose homeostasis

Blood samples for measurement of plasma glucose and serum insulin were drawn carefully through the intravenous cannula to avoid haemolysis. Samples for glucose were collected into fluoride oxalate tubes, centrifuged and separated immediately. Plasma glucose was assayed using an automated hexokinase/glucose-6-phosphate dehydrogenase method (Abbott Architect analyzer, Abbott reagents, within run C.V. 2.0% at 4.3 mmol/L). Samples for insulin were collected into serum separator tubes, allowed to clot at room temperature and then immediately centrifuged and separated. Serum was transported on ice to the laboratory and insulin was assayed using an automated microparticle enzyme immunoassay (Abbott AxSYM, Abbott reagents, within run C.V. 2.7% at 106.5 mU/L). Thus, glucose and insulin excursions in response to peptide infusion and then to food ingestion were monitored.

2.3.7 Glucagon radioimmunoassay (RIA)

Samples for glucagon were collected in lithium heparin tubes containing 1000 kallikrein inhibitor units of aprotinin (Bayer, Newbury, UK) and were measured using established in-house radioimmunoassay (RIAs) (Ghatei et al., 1983a). All samples were assayed in duplicate. Glucagon was purchased from Bachem Ltd (Switzerland). All other reagents and materials were supplied by Sigma (Poole, Dorset, UK). The glucagon label was prepared by Professor M. Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) who iodinated the peptide using the iodogen method (Conn, 1989) and was purified by reverse-phase HPLC.

Assays were performed in veronal buffer (1l distilled water containing 10.3g sodium barbitone, 0.3g sodium azide), at pH 8 with 0.3% BSA (VWR, UK). A standard curve was prepared in assay buffer at 0.5 pmol/ml, added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 μ l. The glucagon antibody (RCS5) was raised in rabbits against the C-terminal of glucagon and is therefore specific for pancreatic glucagon. In this assay the antibody was used at a dilution of 1:50000. Experimental samples of 100 μ l, 100 μ l antibody solution and 100 μ l of glucagon label solution were used and all tubes were buffered to a total volume of 700 μ l with assay buffer. The assays were incubated for 96 hours at 4°C. Free peptide was separated from bound using charcoal adsorption. To

each tube, 4 mg of charcoal, suspended in 0.06 M phosphate buffer with gelatine, was added immediately prior to centrifugation. The samples were then centrifuged at 1500 rpm, 4°C, for 20 minutes. Bound and free label were separated and both the pellet and supernatant counted for 180 seconds in a γ -counter (model NE1600, Thermo Electron Corporation). Plasma glucagon concentrations in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard. C.V.s were <10% across the working range.

2.3.8 Active GLP-1 ELISA

Samples for active GLP-1 were collected in lithium heparin tubes containing 1000 kallikrein inhibitor units of aprotinin (Bayer, Newbury, UK). Active GLP-1 was measured using a commercially available ELISA kit specific for the physiologically active forms of GLP-1 (GLP-1₇₋₃₆ amide and GLP-1₇₋₃₇) (Millipore, Livingston, UK) according to the manufacturer's instructions. C.V.s were <7% across the working range.

2.3.9 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). Two-way repeated measures ANOVA with Bonferroni post hoc test was used to compare differences in glucose, insulin and VAS. One-way repeated measures ANOVA with Newman-Keuls and Bonferroni post hoc tests was used to compare food intake, change in REE and substrate oxidation rates between groups. Area under the curve (AUC) was calculated using the trapezoidal rule and differences between treatment arms were compared using one-way repeated measures ANOVA with Tukey post hoc test. Data are reported $\pm$ SEM unless otherwise stated.

2.4 Results

2.4.1 Plasma hormone levels

At baseline (-30 min), plasma active GLP-1 levels were 4-5 pmol/l. In the treatment groups receiving GLP-1 alone or GLP-1 in combination with glucagon, plasma levels increased to 11-16 pmol/l at 70 min post-infusion (Figure 2.2A). Mean plasma glucagon levels at baseline (-30 min) were 14-19 pmol/l, rising to 147-173 pmol/l at 70 min in those who received glucagon infusion (Figure 2.2B).

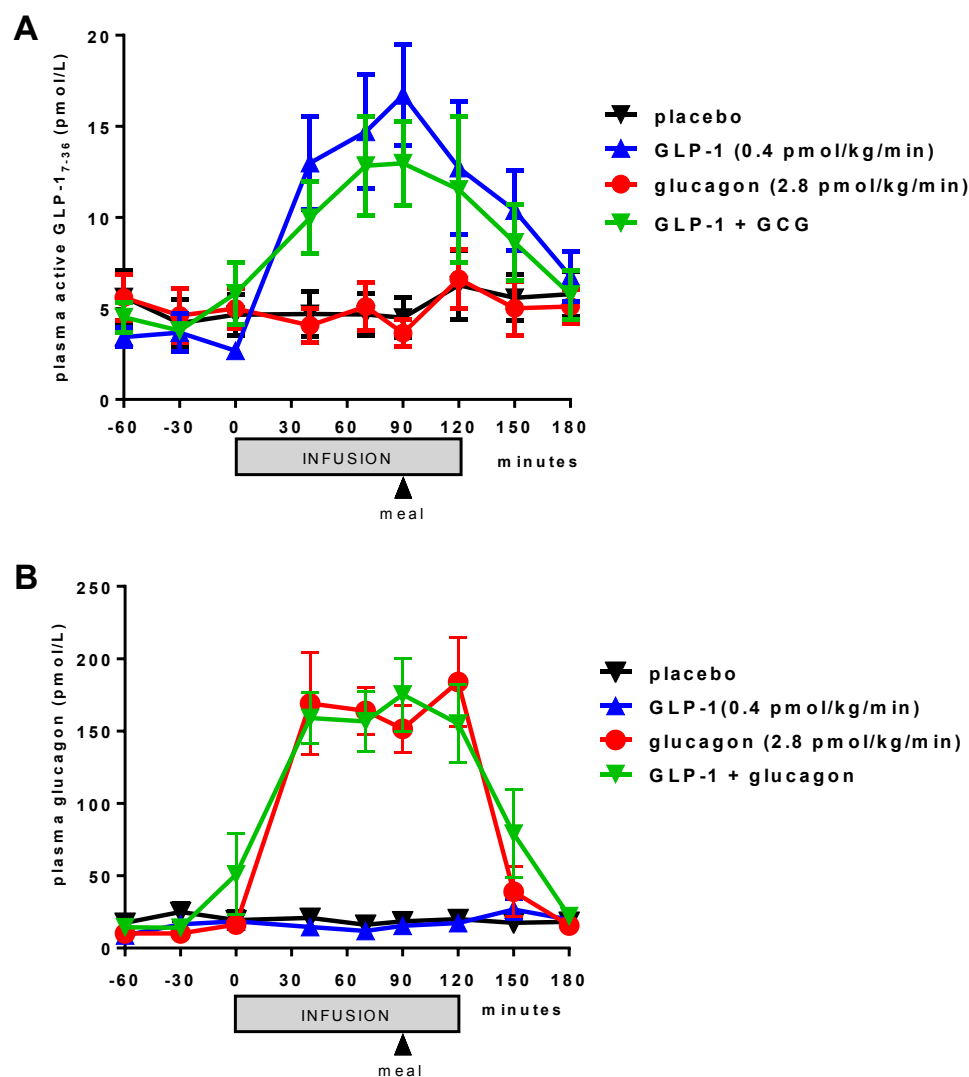


Figure 2.2. Plasma active GLP-1 (A) and glucagon (B) levels before, during and after peptide infusion. Mean $\pm$ SEM plasma levels plotted. Infusion denoted by grey bar and meal served at the arrow.

2.4.2 Effect on energy intake

Glucagon alone and GLP-1 alone, at the selected sub-anorectic doses administered, did not significantly reduce food intake. However, co-infusion of glucagon and GLP-1 significantly decreased food intake by 13% compared to placebo ($p<0.05$). This was also a significantly lower than food intake during individual glucagon ($p<0.05$) or GLP-1 infusions ($p<0.05$) (mean energy intake: 1086 ± 110.1 kcal [placebo], 1086 ± 96.9 kcal [glucagon], 1052 ± 81.3 kcal [GLP-1] and 879 ± 94.2 kcal [combined glucagon + GLP-1] – Fig 2.3).

Perceived hunger, satiety and palatability of the meal were not altered significantly by any of the infusions (Fig 2.4A, C and D). The perceived nausea rating increased significantly post-meal (120 min) during the combination infusion of glucagon and GLP-1 (Fig 2.4B). Mild nausea was reported by three volunteers after the combined infusion and two volunteers vomited following glucagon infusion. In all cases, this occurred after the meal between 120 and 160 min.

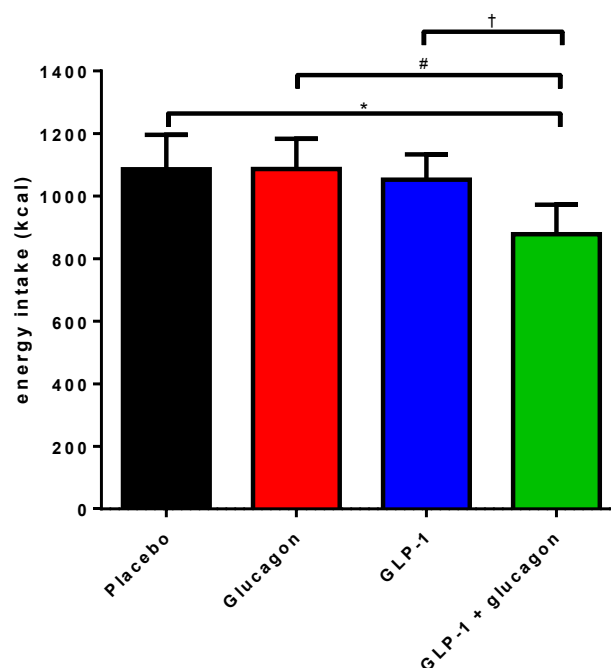


Figure 2.3. Energy intake at the *ad libitum* meal at 90 min. Mean $\pm$ SEM absolute energy intake; * $p<0.05$ compared with placebo, # $p<0.05$ compared with glucagon, † $p<0.05$ compared with GLP-1.

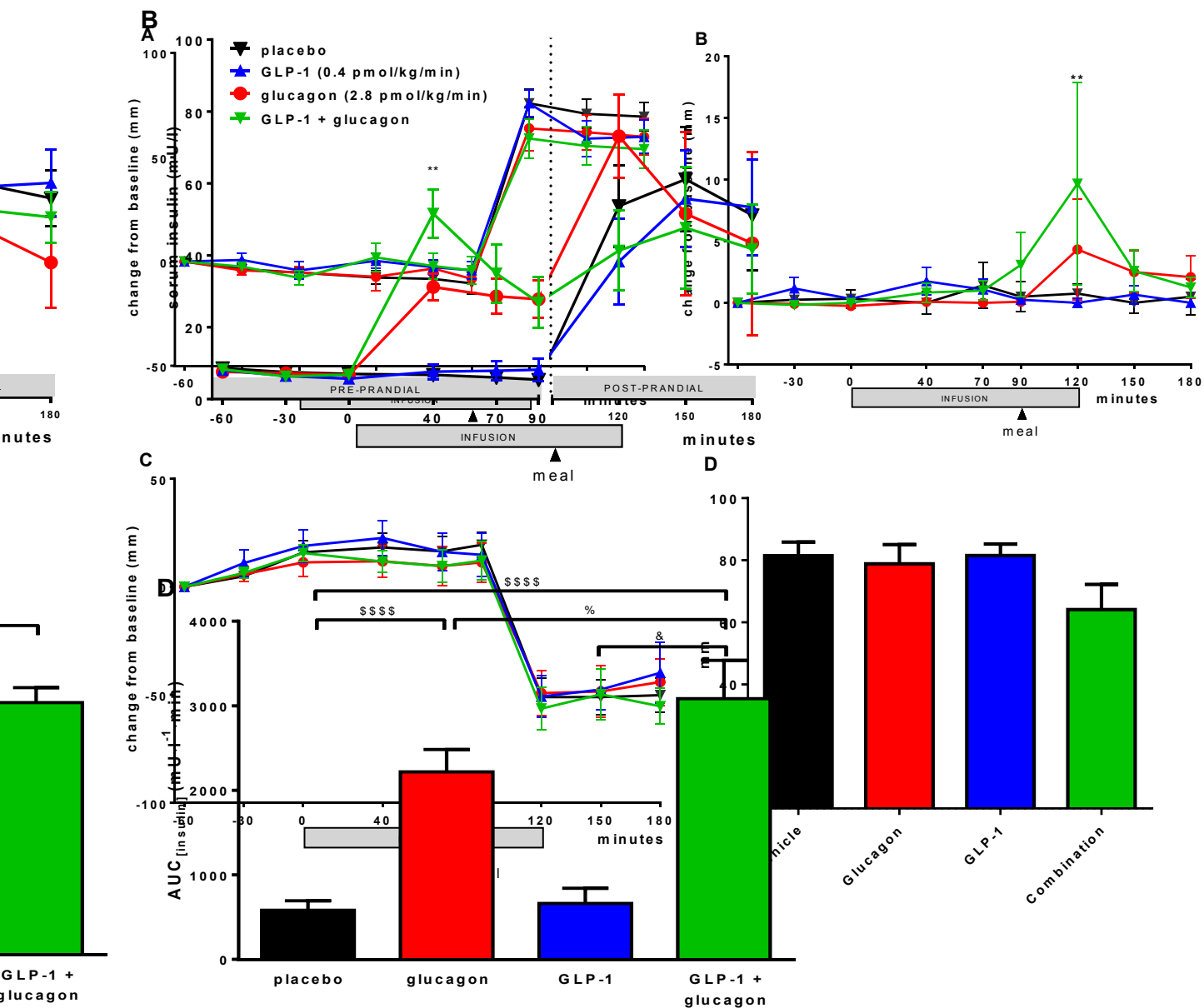


Figure 2.4. Visual Analogue Score (VAS) response for subjective rating of satiety (A), nausea (B), hunger (C) and palatability (D). For A-C, scores are presented as change from baseline value (millimetres). ** $p < 0.01$ compared with placebo. For D, an absolute value (millimetres) is shown.

2.4.3 Effect on energy expenditure

Baseline REE values were not significantly different between groups: 1336 $\pm$ 65.8 kcal/day [placebo], 1314 $\pm$ 53.0 kcal/day [glucagon], 1330 $\pm$ 71.9 kcal/day [GLP-1] and 1341 $\pm$ 56.6 kcal/day [combined glucagon + GLP-1] $p=0.7275$. Following infusion, REE was increased in response to glucagon alone and glucagon/GLP-1 co-administration by a mean of 66.8 and 52.5 kcal/day respectively but these increases were not statistically significant (Fig 2.5A). RQ values and carbohydrate oxidation rates were similar in all treatment arms at baseline. GLP-1 infusion did not affect RQ, however, a significant rise in RQ and carbohydrate oxidation was observed with both the glucagon and combination infusions (Fig 2.5B and 2.5C). Glucagon alone and in combination with GLP-1 caused a significant reduction in fat oxidation rates (Fig 2.5D). Protein oxidation rate, calculated over the entire study period for each treatment arm, was not significantly different from placebo.

2.4.4 Effect on glucose homeostasis

Plasma glucose and serum insulin responses to placebo, glucagon, GLP-1 or combination infusions are depicted in Figure 2.6. Glucose and insulin were constant during placebo infusion and as expected, increased in response to the meal at 90 min. Glucagon infusion caused glucose to rise from 4.8 $\pm$ 0.08 mmol/l to a peak of 6.5 $\pm$ 0.3 mmol/l at 40 min with a corresponding increase in insulin to 31.2 $\pm$ 3.8 mU/l. GLP-1 infusion caused a reduction in plasma glucose during infusion from 4.9 $\pm$ 0.1 mmol/l to 4.1 $\pm$ 0.3 mmol/l at 40 min, with serum insulin levels comparable to the placebo arm. Following co-administration of glucagon and GLP-1, AUC_{glucose} was similar to the placebo arm and significantly lower than observed with glucagon alone (Fig 2.6C). The significant increase in insulin during the glucagon/GLP-1 co-infusion was of greater magnitude than seen with GLP-1 or glucagon alone (Fig 2.6D). Post-meal, where calorie intake differed between treatment arms, glucose and insulin levels were not significantly different between groups.

2.4.5 Effect on safety parameters

No significant changes in pulse, systolic or diastolic blood pressure (Fig 2.7) were observed with any of the peptide infusion, alone or in combination.

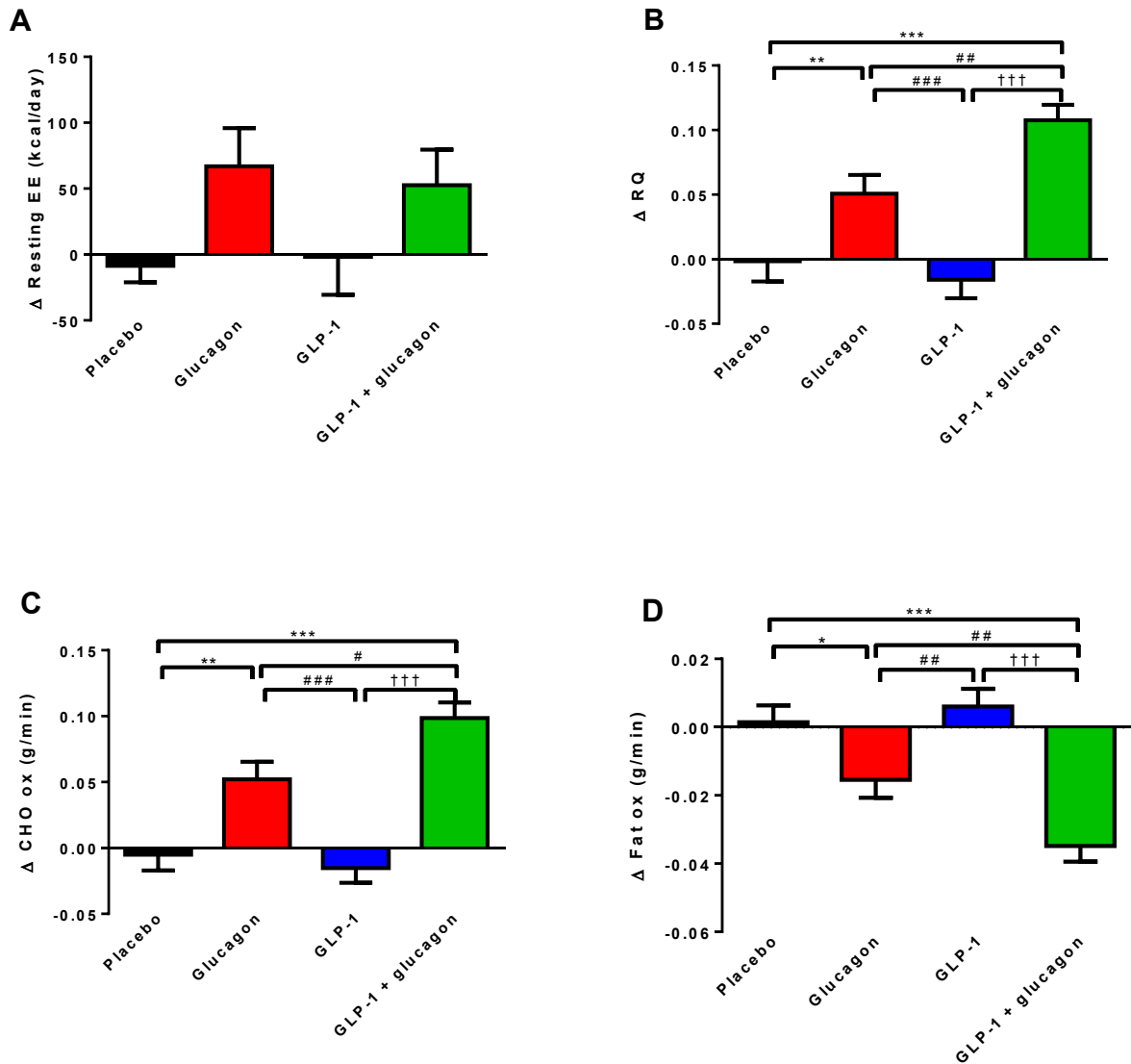


Figure 2.5. The effects of glucagon and GLP-1, alone and in combination, on resting energy expenditure (A), Respiratory Quotient (B), Carbohydrate oxidation (C), and Fat oxidation (D). Mean absolute change for each parameter ($\pm$ SEM) between baseline and infusion phases presented. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with placebo; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with glucagon; ††† $p < 0.001$ compared with GLP-1.

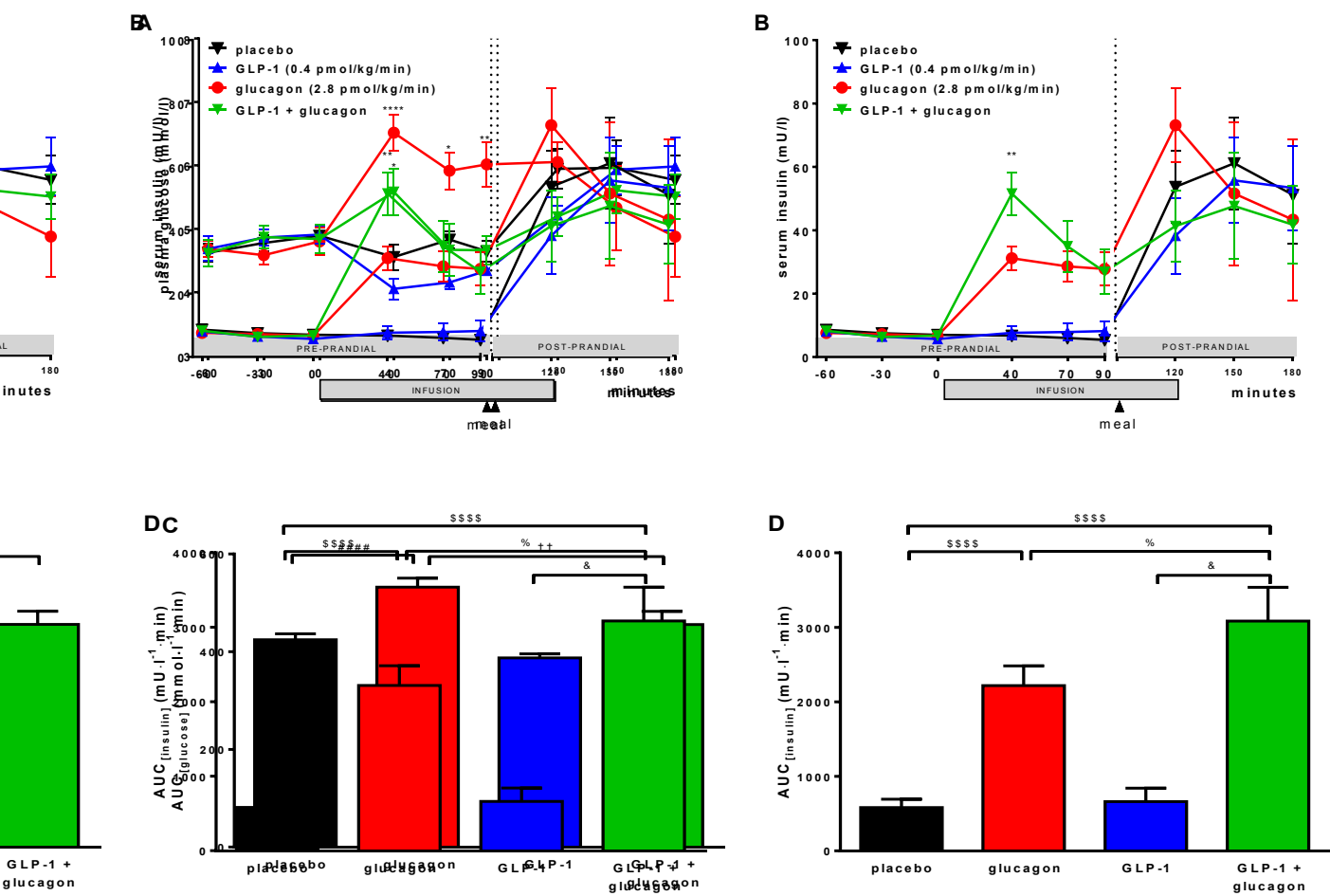


Figure 2.6. The effects of glucagon and GLP-1, alone and in combination, on plasma glucose (A and C) and serum insulin (B and D). A and B: Mean $\pm$ SEM plasma glucose and serum insulin levels. Infusion represented by the grey bar and meal served at the arrow. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared with placebo. C: Area under the curve for glucose levels from 0 to 90 min (during infusion, pre-meal). #### $p < 0.0001$ compared with placebo; †† $p < 0.01$ compared with glucagon. D: Area under the curve for insulin levels from 0 to 90 min. \$\$\$\$ $p < 0.0001$ compared with placebo; % $p < 0.01$ compared with glucagon; & $p < 0.0001$ compared with GLP-1.

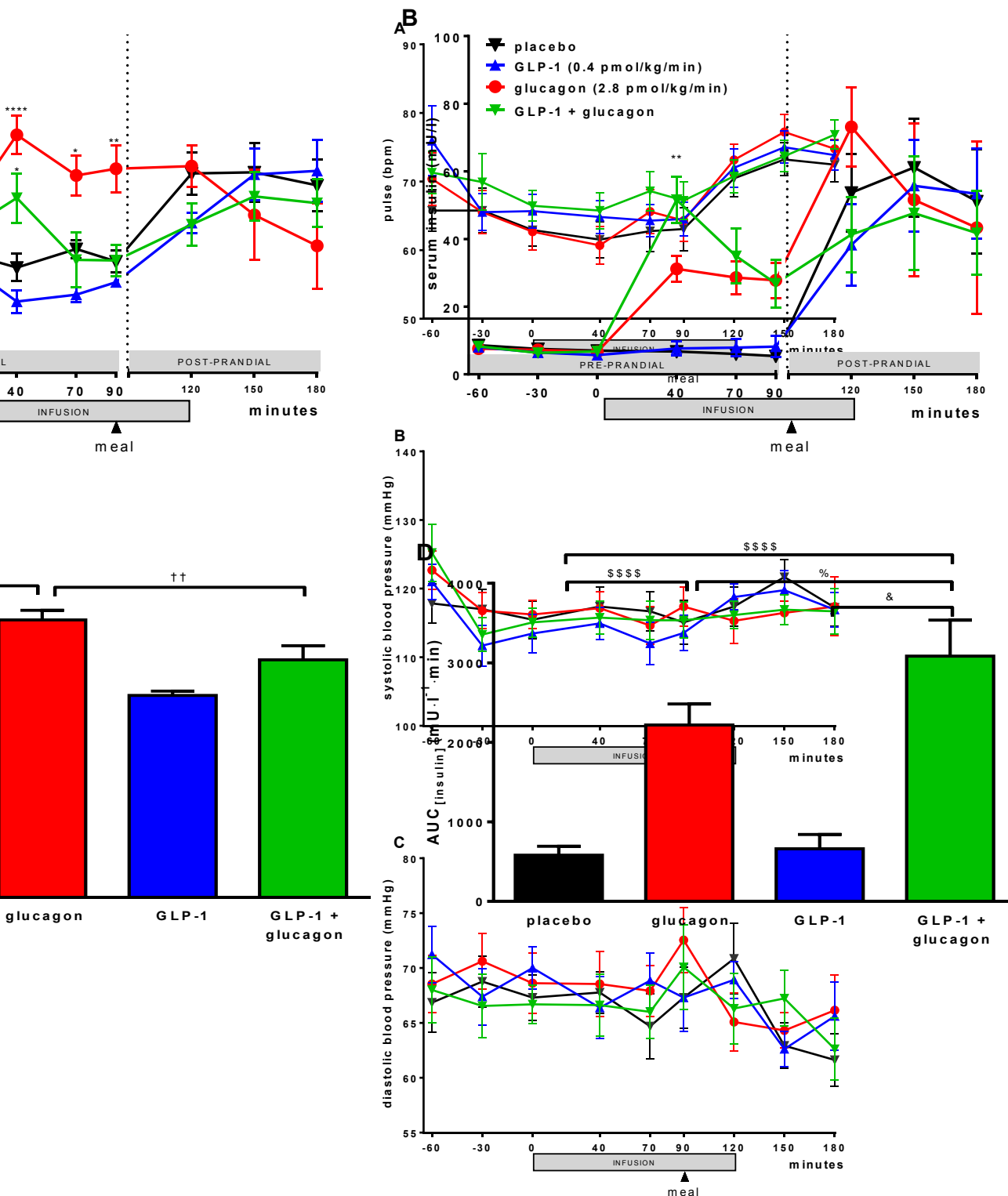


Figure 2.7. The effect of glucagon and GLP-1 on heart rate (A), systolic (B) and diastolic (C) blood pressure. Two way repeated measures ANOVA showed no significant differences between treatment groups.

2.5 Discussion

This chapter examines the effect of co-infusion at low doses of two endogenous peptide hormones, glucagon and GLP-1, on food intake, energy expenditure and glucose homeostasis.

2.5.1 Food intake

This study demonstrates, for the first time in humans, that dual infusion of GLP-1 and glucagon reduces food intake significantly whereas the same low doses of glucagon and GLP-1, when administered separately, do not exert a similar anorectic effect. The mechanism of food intake reduction for both hormones is thought primarily to be at the level of the hypothalamus, which expresses both glucagon and GLP-1 receptors (Hunt et al., 2012, Hoosein and Gurd, 1984). These effects are postulated to be mediated by both central and peripheral pathways as intracerebroventricular injections of both glucagon and GLP-1 cause a reduction in food intake (Meeran et al., 1999, Honda et al., 2007), and as vagotomy attenuates the anorectic effect of glucagon and GLP-1 following peripheral administration (Abbott et al., 2005, Geary and Smith, 1983). Within our laboratory, we have also shown that co-administration of GLP-1 and glucagon causes a reduction in circulating levels of the orexigenic hormone ghrelin (Tan TM et al., 2012). In that study, infusion of GLP-1 alone had no effect on ghrelin levels, whilst glucagon infusion resulted in a non-significant reduction in circulating ghrelin. However, the co-infusion of both hormones achieved a significant reduction in ghrelin levels. Other studies have also shown an inhibition of ghrelin secretion by glucagon (Arafat et al., 2005). This suppression of ghrelin may therefore enhance the anorectic effect seen during administration of glucagon and GLP-1 (Tan TM et al., 2012). It is noteworthy that this study has only examined the acute effects of GLP-1/glucagon co-agonism and further chronic food intake studies need to be performed in humans to establish a therapeutically useful effect.

Three subjects receiving the combination of glucagon and GLP-1 experienced nausea. It is well established that patients taking GLP-1 based therapies for diabetes do experience nausea (Vilsboll et al., 2012) and it is for this reason that the dose of such

therapies is only increased incrementally. With careful dose titration, the nausea caused by GLP-1 resolves whilst the anorectic effect persists. Glucagon is also known to cause nausea, for example when given for the treatment of hypoglycaemia or for the investigation of ACTH deficiency (Leong et al., 2001). However, the doses used in this study were far smaller than those administered in these clinical situations. The mechanism of nausea for both hormones is thought to be via the same signalling pathways of satiety in the hypothalamus mentioned above. The post-prandial nausea seen in this study may be accounted for by a rise in endogenous satiety factors (including the gut hormones PYY and PP) with concomitant infusion of glucagon and GLP-1 resulting in mildly supraphysiological levels, when compared to normal postprandial glucagon and GLP-1 plasma concentrations (Flint et al., 1998). Additionally, both glucagon and GLP-1 are known to cause a delay in gastric emptying (Chernish et al., 1978, Flint et al., 1998), which may exacerbate any pre-existing mild nausea.

2.5.2 Energy expenditure

Consistent with the results of a previous study performed in the department (Tan TM et al., 2012), a trend towards an increase in REE of ~50 kcal/day is demonstrated in both the glucagon alone and combination infusion groups. It is not surprising that the rise in REE did not reach statistical significance in this current study as the dose of glucagon used was a fifth that of the previous study (Tan TM et al., 2012). Moreover, the chronic sustained effects of a small increase in REE of such a magnitude may have an important impact on body weight long term when combined with the energy intake reduction induced by these peptides. The mechanism by which glucagon increases REE is uncertain. This phenomenon could be due to increased thermogenesis in brown adipose tissue (Billington et al., 1991) and/or by futile substrate cycling (Miyoshi et al., 1988). These effects may be mediated directly, via glucagon receptors on brown adipose tissue or indirectly, via increased catecholamines (Jones et al., 2012).

2.5.3 Glucose homeostasis

The hyperglycaemic effect of glucagon is undesirable in patients with diabetes and impaired glucose tolerance, despite the greatest element resulting from a one-off stimulation of glycogenolysis. Co-administration with GLP-1 appears to ameliorate this

hyperglycaemia, perhaps due to enhanced insulin release and glucose disposal. Two independent groups have shown this to be the case in rodents on administration of analogues exerting dual agonism at the GLP-1 and glucagon receptors (Day et al., 2009, Pocai et al., 2009). This group has previously used native peptides to show that GLP-1 infusion blunted glucagon-induced hyperglycaemia when infused in combination (Tan TM et al., 2012). This is likely to be mediated via the insulinotropic properties of both hormones. Both GLP-1 and glucagon act directly on the beta cell to activate release of insulin. Also the glucagon-induced hyperglycaemia itself may be a stimulus for insulin release (Christophe, 1996). Furthermore, as shown in this study, the reduction in food intake observed with the dual infusion is very likely to contribute to the attenuated postprandial glycaemic response. A further study examining the effect of dual glucagon and GLP-1 administration following a standardized calorie load is presented in Chapter 3.

2.5.4 Cardiovascular safety

It is essential that any novel therapeutic undergoes rigorous cardiovascular safety checks. This is particularly pertinent in the fields of diabetes and obesity where recently, both rosiglitazone and sibutramine have been withdrawn from the market due to concern over increased cardiovascular deaths. Both glucagon and GLP-1 have receptors in cardiac tissue and this raises that question of off-target effects. Glucagon is known to be positively ino- and chronotropic and is used therapeutically in the treatment of beta blocker overdose (Regan et al., 1964). Here, there was no discernible change seen in either pulse or blood pressure with glucagon infusion.

Recently, various cardiovascular benefits of GLP-1 have been reported in the literature including improvement of systolic function and atherosclerosis (Ussher and Drucker, 2012). Again, no clear changes in pulse or blood pressure were seen with GLP-1 infusion.

It must be noted that the number of participants presented here is very small and many more patients will need to be studied before drawing further conclusions regarding safety. Furthermore, this study only looks at the acute effects, and the chronic effects of infusion have not been studied.

2.5.5 Future work

A complementary study examining the effect of glucagon and GLP-1 infusion on carbohydrate tolerance using a mixed meal tolerance test is presented in Chapter 3. The aim of this study is to assess whether insulin sensitivity is affected by co- infusion of glucagon and GLP-1 following a fixed calorie load. This is important as a significant proportion of obese patients have concomitant type 2 diabetes and the combination infusion may have direct, as well as indirect, consequences on diabetes.

Ideally, the beneficial effects of both glucagon and GLP-1 would be combined into one molecule, which would exert its effects by interacting with both the glucagon and GLP-1 receptors. Additionally, its half-life would be prolonged in order to facilitate once-weekly administration. The development of a co-agonist of glucagon and GLP-1 is currently in progress in the department.

2.5.6 Conclusion

In conclusion, this study demonstrates that co-administration of glucagon and GLP-1, at doses that are individually sub-anorectic, significantly reduces food intake in humans. In addition, the co-administration of GLP-1 attenuates the hyperglycaemia of glucagon in the fasting state. This work is consistent with the findings seen with acute infusion of OXM (Wynne et al., 2006). These observations provide further support for the continued development of GLP1/glucagon receptor co-agonists as a therapeutic approach for obesity. Establishing the effects of long-term co-agonism at the glucagon and GLP-1 receptors; in particular, harnessing the potential anorectic effect without exerting nausea as well as maintaining euglycaemia, is the key to therapeutic exploitation.

CHAPTER 3: THE ACUTE EFFECTS OF CO-ADMINISTRATION OF GLUCAGON AND GLP-1 ON GLUCOSE HOMEOSTASIS IN HUMANS

3.1 Introduction

The worldwide prevalence of Type 2 Diabetes Mellitus (T2DM) continues to rise with a projected 600 million people affected by 2035 (International Diabetes Federation, 2014). The most potent stimulus for this is the parallel rise in obesity, which is estimated to account for 80-85% of the risk of developing T2DM therefore any future obesity therapy would ideally also have a beneficial effect on glucose homeostasis. Currently, the most effective treatment for obesity is Roux-en-Y Gastric Bypass (RYGB) surgery which results in a mean sustained 27% reduction in presurgical weight (Sjostrom et al., 2007). Remarkably, there is a rapid improvement in T2DM that occurs within hours of the operation suggesting a mechanism independent of weight loss (Wickremesekera et al., 2005, Dhabuwala et al., 2000). This remission in T2DM occurs in 40-80% of patients, depending on the definition of remission (Buchwald et al., 2009, Pournaras et al., 2012, Mingrone et al., 2012).

The mechanism by which RYGB results in both weight loss and T2DM remission is currently unknown, however, it has been proposed that the observed rise in gut hormones post-surgery may have a causative role (le Roux et al., 2007). The chief gut hormone implicated is glucagon-like-1 peptide (GLP-1). The post prandial release of GLP-1 is exaggerated after RYGB and as described in chapters 1 and 2, has multiple effects that could contribute to weight loss and improvements in glucose tolerance (Kreymann et al., 1987, Willms et al., 1996, Verdich et al., 2001, Zander et al., 2002).

Several studies have also shown that post RYGB, there is a post prandial elevation in glucagon levels, with peak levels doubling the fasting state (Falken et al., 2011, Jacobsen et al., 2012, Nannipieri et al., 2013). In normal glucose tolerant individuals, the highest levels of glucagon occur in the fasting phase and decrease in the context of post prandial hyperglycaemia (Unger and Orci, 1976). However, in patients with diabetes, glucagon levels are no longer suppressed in the post prandial phase, and paradoxically rise by 35%, which may contribute to their glucose intolerance (Henkel et al., 2005, Shah et al., 1999). Further evidence to support the role of elevated glucagon in the pathology of diabetes are rodent studies that successfully used GCGR antagonists to improve glucose tolerance (Wang et al., 2015). As discussed in chapter 2, despite promoting hyperglycaemia, glucagon has many other actions that make it an attractive anti-obesity

therapeutic agent (Geary et al., 1992, Nair, 1987). Furthermore, as RYGB patients have improved carbohydrate tolerance it would suggest that glucagon does not cause an elevation in post prandial glycaemia, or that the observed raised levels of GLP-1 balance out any glucagon induced hyperglycaemia.

Additionally, post RYGB, there is also an elevated postprandial level of oxyntomodulin (LaFerrere et al., 2010). Acute infusions of OXM have demonstrated reduction in weight and increase in energy expenditure without any deterioration in glucose tolerance (Wynne et al., 2006, Cohen et al., 2003). This is further evidence that dual agonism of GLP-1 and GCG prevents any worsening of postprandial hyperglycaemia.

In chapter 2, it is demonstrated that co infusion of GLP-1 and glucagon (each at sub-anorectic doses) resulted in a significant reduction in food intake in man (Cegla et al., 2014). It has previously been shown that acute intravenous administration of GLP-1 and glucagon also results in an increase in resting energy expenditure (Tan TM et al., 2012). These observations together support the premise that the associated elevation in both GLP-1 and GCG may contribute to the beneficial reduction in food intake and increased energy expenditure after RYGB. Furthermore they support the rationale of developing anti-obesity treatments based on dual GLP-1 and GCG receptor agonism. However, there remains a concern regarding the potential of hyperglycaemia with glucagon. The effect of dual infusion on glucose homeostasis has thus far only been tested in the fasting state where an amelioration of glucagon-provoked hyperglycaemia is observed. The investigations in this chapter set out to determine the effect on glucose homeostasis of single and combined infusions of GLP-1 and GCG after a fixed calorie load. Homeostatic model assessment (HOMA-IR), a method for assessing beta cell function and insulin resistance from glucose and insulin, was used to assess the effect of GLP-1 and GCG and glucose homeostasis.

3.2 Hypothesis and aims

3.2.1 Hypothesis

I hypothesise that co-infusion of glucagon and GLP-1 will improve glucose homeostasis following a fixed-calorie load, compared to glucagon alone.

3.2.2 Aim

To examine the acute effects of intravenous infusion of GLP-1 alone, glucagon alone, and the combination of glucagon with GLP-1 on glucose homeostasis following a fixed-calorie load.

3.3 Materials and Methods

3.3.1 Participants

Ten non-diabetic, overweight volunteers with a mean BMI of 27.2 kg/m² (range 25.0 to 30.0) were recruited by advertisement. All participants underwent health screening including medical history, physical examination, biochemical and haematological testing and 12-lead electrocardiogram as described section 2.3.1. Any abnormal eating behaviour was assessed using the Dutch Eating Behaviour Questionnaire (DEB-Q) (Wardle, 1987) and the SCOFF questionnaire (Morgan et al., 1999).

Suitability was determined following the screening visit according to the following criteria:

Inclusion criteria

- Aged 18 – 65 years
- Male or female
- BMI between 20 and 35 kg/m²
- Stable body weight for at least 3 months

Exclusion criteria

- History of any medical, psychological or other condition, or use of any medications, including over-the-counter products, which, in the opinion of the investigators, would either interfere with the study or potentially cause harm to the volunteer
- Pregnancy or breastfeeding
- Unable to maintain adequate contraception for the duration of the study and for one month afterwards
- History of hypersensitivity to any of the components of the infusions
- Donated blood during the preceding 3 months or intention to do so before the end of the study
- High level of restrained pattern of eating, as determined by the DEB-Q

All participants gave their written consent to take part (see Appendix A), and were free to withdraw from the study at any time. Ethical approval was granted for the study by

the West London Research Ethics Committee (reference number 10/H0707/80) and carried out according to the principles of Good Clinical Practice and the Declaration of Helsinki.

Participants' demographics are described below in Table 3.1.

Table 3.1. Demographics of the study participants. M, Male; F, Female.

Volunteer	BMI (kg/m ²)	Age (Years)	Sex
1	25.1	32	M
2	26.3	21	M
3	29.1	49	F
4	27.8	35	M
5	28.1	33	M
6	25.3	36	M
7	25.0	25	F
8	28.4	22	F
9	26.5	20	M
10	30.0	32	F
Mean	27.2	30.5	6M, 4F

3.3.2 Peptides

Natural sequence peptides, including standard pharmaceutical grade glucagon (Glucagen, Novo Nordisk, UK) and GLP-1₍₇₋₃₆₎ amide (Clinalfa basic, Bachem, Switzerland), were used. Both peptides were manufactured to EU Good Manufacturing Practice quality standards.

Peptide was made up in Gelofusine (B. Braun, Crawley, UK) in 50 ml syringes pre-coated with Gelofusine 30 minutes before administration. Previous testing within the department has demonstrated that this approach minimises peptide loss through adsorption onto the syringe wall (Kraegen et al., 1975).

3.3.3 Study protocol

A double-blind, randomised, crossover design was used for the study. Participants attended for 5 study visits. The first visit was an unblinded acclimatisation visit, during which participants become familiar with the study protocol. For each subsequent visit, subjects received intravenous infusions of either:

- 1) placebo (Gelofusine)
- 2) GLP-1₇₋₃₆ amide (0.4 pmol/kg/min)
- 3) glucagon (1.4 pmol/kg/min)
- 4) combined GLP-1 and glucagon at the above doses.

A known sub-anorectic dose of GLP-1 previously reported in the literature was used as per chapter 2 (Verdich et al., 2001, Neary et al., 2005). This dose has been previously shown to result in plasma active GLP-1 levels of 15-20 pmol/l, similar to the levels observed in the post prandial RYGB patient. With regards to glucagon, in the study described in Chapter 2, nausea was experienced by 3 of the 13 volunteers during infusion with 2.8 pmol/kg/min of glucagon. Given that the nausea uniformly occurred in the post-prandial state, we elected to use a lower dose of glucagon for this investigation. Previous studies have used infusions of glucagon at 0.86 pmol/kg/min to reproduce the non-suppressed levels observed in diabetics. We used a higher dose of 1.4 pmol/kg/min to simulate the elevated post prandial levels seen in RYGB (Sherwin et al., 1976).

The study protocol is shown in Figure 3.1. Volunteers attended the research centre at 0900h, having fasted from 2200h the night before, and refrained from alcohol and strenuous exercise for the preceding 24 hours. Female participants underwent a urinary β -HCG assessment at the start of each study visit to exclude pregnancy. The study room was kept at a consistent temperature of 21°C, which was centrally controlled.

The study commenced at -10 minutes with the placement of two venous cannulae, one for blood sampling and one for intravenous hormone infusion. Following cannulation, volunteers were seated in reclining chairs. The hormone infusion was ramped from initiation (0 minutes) in order to rapidly achieve a steady state plasma concentration of

hormone. Ramping was carried out at four times the nominal infusion rate for 5 minutes, then two times the nominal infusion rate for a further 5 minutes, and was then reduced to the nominal rate for the remainder of the 220 minute infusion. At 30 minutes a meal was served, consisting of liquid (Ensure Plus, 13.8 g of protein, 10.8 g of fat, 44.4g of carbohydrates, 330kcal 220ml, Abbott laboratories, Abbott Park, IL) and solid components (Nutri-grain bar, 1.5g of protein, 3g of fat, 26g of carbohydrates, 133kcal Kellogg company, UK). Participants were allowed 10 minutes to consume the meal. The hormone infusion continued for 220 minutes in total. Participants remained in the study room for 60 minutes following termination of the infusion, at which point the cannulae were removed. The participant was then discharged home.

During the study, pulse and blood pressure were measured at 0, 15, 30, 60, 100, 130, 160, 190 and 220 minutes. Blood samples were taken for the measurement of glucose and insulin at the following time points: 0, 30, 50, 60, 70, 80, 90, 100, 115, 130, 145, 160, 190 and 220 minutes. Blood samples were taken for the measurement of glucagon and active GLP-1: 0, 30, 60, 100, 160 and 200 minutes. To assess nausea, the participants were given visual analogue scale (VAS) sheets at the following time points; 0, 30, 60, 100, 160 and 220 minutes.

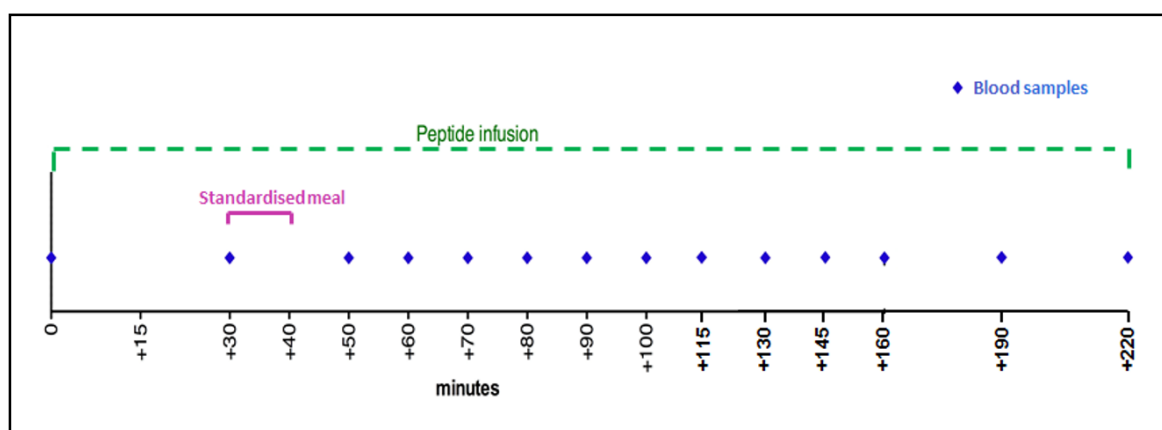


Figure 3.1: Study protocol

3.3.4 Assessment of glucose homeostasis

Blood samples for measurement of plasma glucose and serum insulin were drawn carefully through the intravenous cannula to avoid haemolysis. Samples for glucose were collected into fluoride oxalate tubes, centrifuged and separated immediately. Plasma glucose was assayed using an automated hexokinase/glucose-6-phosphate dehydrogenase method (Abbott Architect analyzer, Abbott reagents, within run C.V. 2.0% at 4.3 mmol/L). Samples for insulin were collected into serum separator tubes, allowed to clot at room temperature and then immediately centrifuged and separated. Serum was transported on ice to the laboratory and insulin was assayed using an automated microparticle enzyme immunoassay (Abbott AxSYM, Abbott reagents, within run C.V. 2.7% at 106.5 mU/L). Thus, glucose and insulin excursions in response to peptide infusion were monitored.

3.3.5 Glucagon radioimmunoassay (RIA)

Samples for glucagon were collected in lithium heparin tubes containing 1000 kallikrein inhibitor units of aprotinin (Bayer, Newbury, UK) and were measured using established in-house radioimmunoassays (RIAs) (Ghatei et al., 1983a), (Kreymann et al., 1987) as described in section 2.3.7.

3.3.6 Active GLP-1 ELISA

Samples for active GLP-1 were collected in lithium heparin tubes containing 1000 kallikrein inhibitor units of aprotinin (Bayer, Newbury, UK). Active GLP-1 was measured using a commercially available ELISA kit specific for the physiologically active forms of GLP-1 (GLP-1₇₋₃₆ amide and GLP-1₇₋₃₇) (Millipore, Livingston, UK) according to the manufacturer's instructions. C.V.s were <7% across the working range.

3.3.7 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). Two-way repeated measures ANOVA with Bonferroni post hoc test was used to compare differences in glucose, insulin and VAS. The insulinogenic index was calculated as the ratio of meal-induced increment of insulin to glucose; (Insulin_{t60-t30})/(Glucose_{t60-t30}). Insulin resistance (IR) was assessed by the homeostasis model

assessment (HOMA-IR) was calculated using the HOMA-IR 2 calculator (Wallace et al., 2004). Area under the curve (AUC) was calculated using the trapezoidal rule and differences between treatment groups were compared using one-way repeated measures ANOVA with Tukey post hoc test. Data are reported $\pm$ SEM unless otherwise stated.

3.4 Results

3.4.1 Plasma hormone levels

Mean plasma levels of active GLP-1 30 minutes before the infusion were 6-7 pmol/l. In the experimental groups receiving GLP-1 alone or GLP-1 with glucagon, mean levels rose to 10-12 pmol/l. There was no significant difference in the levels of active GLP-1 between those that received GLP-1 alone or in combination with glucagon (figure 3.2A). Mean plasma levels of GCG 30 minutes prior to infusion were between 17 and 21 pmol/l, rising to 64 - 76 pmol/l at 30 minutes in those receiving glucagon (figure 3.2B).

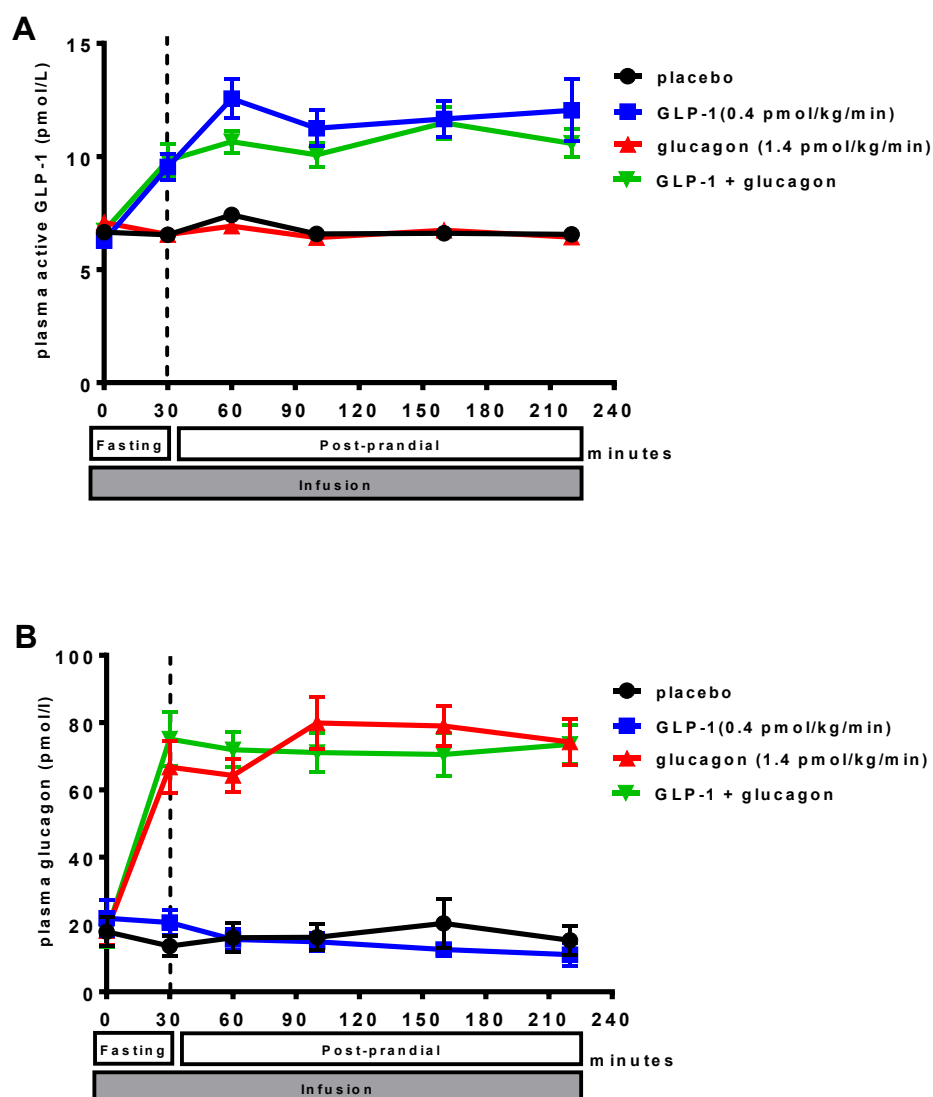


Figure 3.2. Plasma active GLP-1 (A) and glucagon (B) levels during peptide infusion. Mean $\pm$ SEM plasma levels plotted. Infusion denoted by grey bar.

3.4.2 Effect on glucose homeostasis

Plasma glucose and serum insulin responses to vehicle, glucagon, GLP-1 or combination are shown in figures 3.3. Prior to any infusion being initiated, the mean plasma glucose was between 4.9 and 5.1 mmol/l with no significant difference between the groups. In the vehicle group, mean plasma glucose stayed constant for the first 30 minutes, but then rose in response to consuming the mixed meal to 6.1 mmol/l at T=70 minutes. Mean plasma glucose levels then returned to a baseline level at 160 minutes. All infusion groups returned to their baseline at this time point.

In those receiving glucagon infusion, mean preprandial plasma glucose rose to 6.1 mmol/l after 30 minutes infusion, which was significantly different to the vehicle group ($p<0.001$) (figure 3.3A). Levels peaked at 6.6 mmol/l, 30 minutes after meal commencement (T=60), before dropping to baseline at T=160 minutes. AUC glucose₃₀₋₁₆₀ was unchanged relative to vehicle (figure 3.4A). After 30 minutes of GLP-1 infusion, compared to vehicle there was a significant fall in mean plasma glucose levels from baseline (drop from 5.1 to 4.4 mmol/l; $P<0.001$) (figure 3.3A). Glucose levels fluctuated during the GLP-1 infusion but never rose above the preinfusion fasting level. AUC glucose₃₀₋₁₆₀ in the GLP-1 group was significantly reduced compared to vehicle ($P<0.01$) (figure 3.4A). Coinfusion of GLP-1 and glucagon did not result in any significant changes in pre-prandial glucose compared to vehicle (figure 3.3A). Post-prandially, however, glucose levels were significantly lower than those observed during placebo ($P<0.05$) and glucagon infusion ($P<0.01$) (figure 3.4A).

Following consumption of the mixed meal, serum insulin level rose in all infusion groups peaking between 40-50 minutes post-meal. GLP-1 alone and in combination with glucagon exerted a biphasic insulin response. Relative to vehicle, the AUC insulin₃₀₋₁₆₀ was significantly lower in those receiving GLP-1 alone ($P<0.01$) but was unchanged with glucagon alone or GLP-1 and glucagon in combination. However, the AUC insulin₃₀₋₁₆₀ was significantly lowered by the addition of GLP-1 to glucagon compared to the single infusion of glucagon ($P<0.05$) (figure 3.4B)

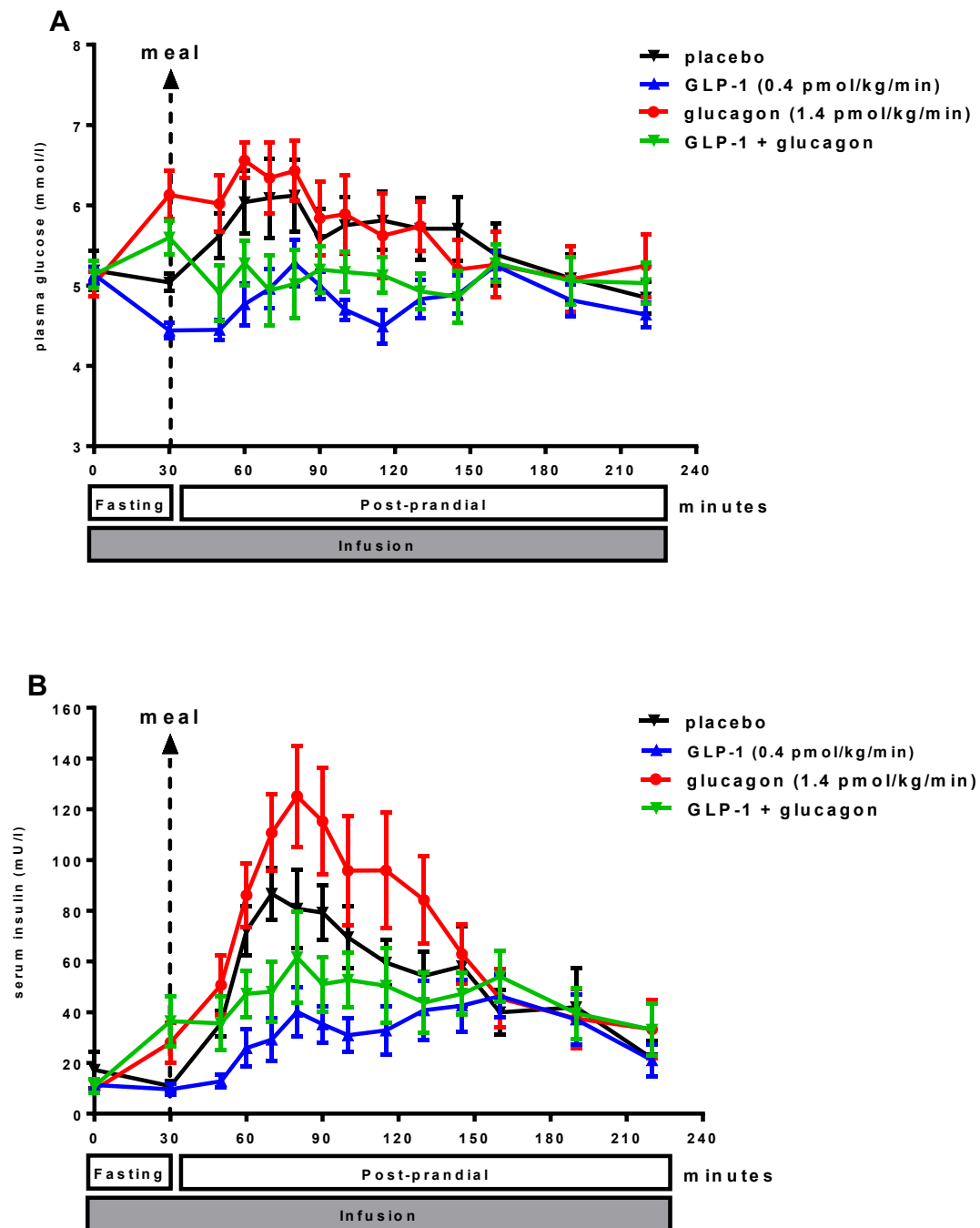


Figure 3.3. The effects of glucagon and GLP-1, alone and in combination, on plasma glucose (A) and serum insulin (B). Mean $\pm$ SEM plasma levels plotted. Infusion denoted by grey bar and meal served at the arrow.

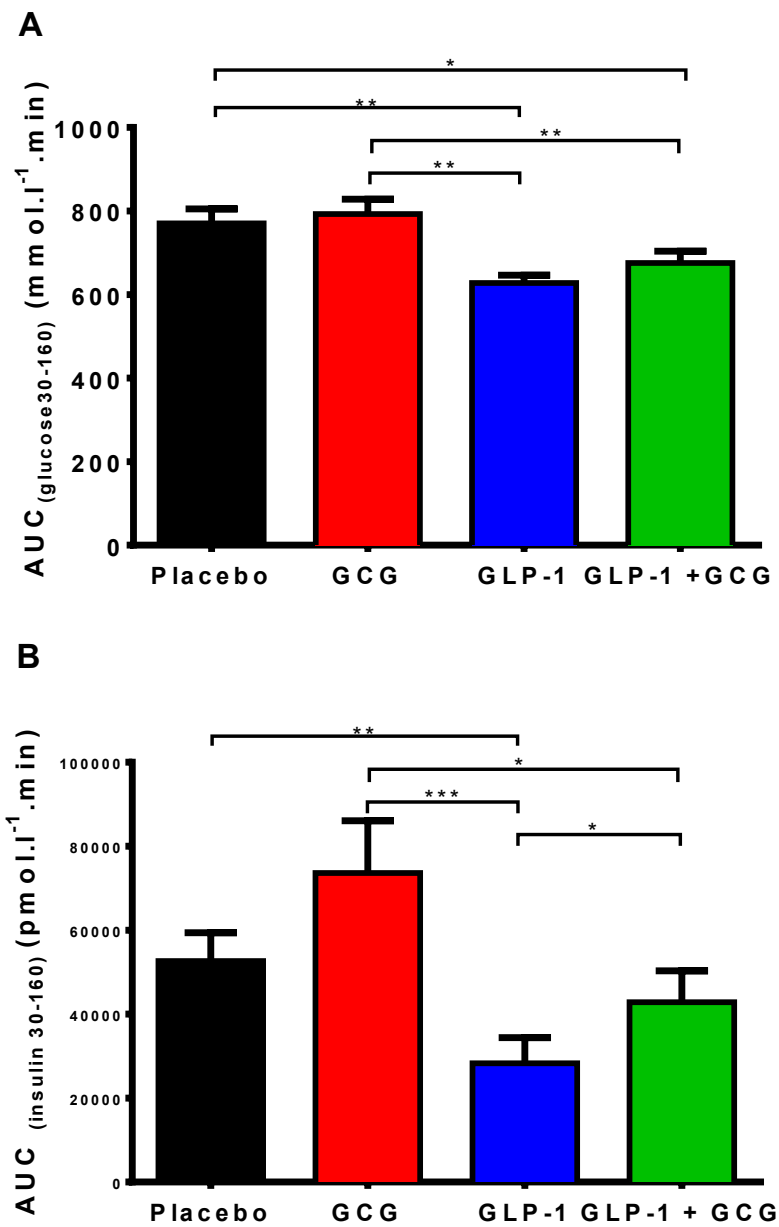


Figure 3.4 Area under the curve for A) glucose and B) insulin levels from 30 to 160 min (during infusion, pre-meal). Mean $\pm$ SEM plasma levels plotted. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

The effects of glucagon and GLP-1 infusion on insulin resistance and secretion are shown in table 3.2.

	Vehicle	GCG	GLP-1	GLP-1 & GCG
Fasting glucose (mmol/l)	5.2±0.2	5.1±0.2	5.0±0.1	5.1±0.2
Fasting glucose after 30 minutes infusion (mmol/l)	5.0±0.1	6.1±0.3 ^{ab}	4.4±0.1 ^a	5.6±0.2
Peak insulin (mU/l)	86.7±10.2	125.2±19.9	46.4±8.5 ^{cd}	61.62±17.9
Time for mean insulin peak (mins post-meal)	40	50	130	50
HOMA-IR (t=0)	1.95±0.66	1.22±0.24	1.47±0.30	1.41±0.34
HOMA-IR on infusion (t=30)	1.42±0.27	3.24±0.54 ^e	1.21±0.26 ^g	3.88±0.61 ^{cj}
AUC glucose 30-160 (mmol/l/min)	744±41	765±42	623±18 ^{cf}	662±29 ^{ef}
AUC insulin 30-160 (pmol/l/min)	52646±6742	73606±12484	28277±6118 ^{cd}	42841±7457 ^{gh}
AUC insulin ₃₀₋₁₆₀ /glucose 30-160	70.4±8.1	93.4±8.9 ^b	44.4±12.9 ^c	62.4±9.3 ^{gi}
Insulinogenic index	87.1±39.6	130.6±62.8	33.9±9.6	50.4±30.4

Table 3.2 Measures of glucose homeostasis. Values are calculated as mean ± SEM.

^aP<0.001 vs vehicle, ^bP<0.0001 vs GLP-1, ^cP<0.01 vs vehicle, ^dP<0.001 vs GCG, ^eP<0.05 vs vehicle, ^fP<0.01 vs GCG, ^gP<0.05 vs GCG, ^hP<0.05 vs GLP-1, ⁱP<0.01 vs GLP-1, ^jP<0.001 vs GLP-1. Fasting glucoses, baseline HOMA-IR results and insulinogenic indices were not significantly different between groups.

2.4.3 Effect on safety parameters

Neither the single nor combination infusions of GLP-1 and glucagon had an effect on pulse and blood pressure (figure 3.5). None of the participants reported nausea and the visual analogue score for nausea remained unchanged throughout all infusions (figure 3.6).

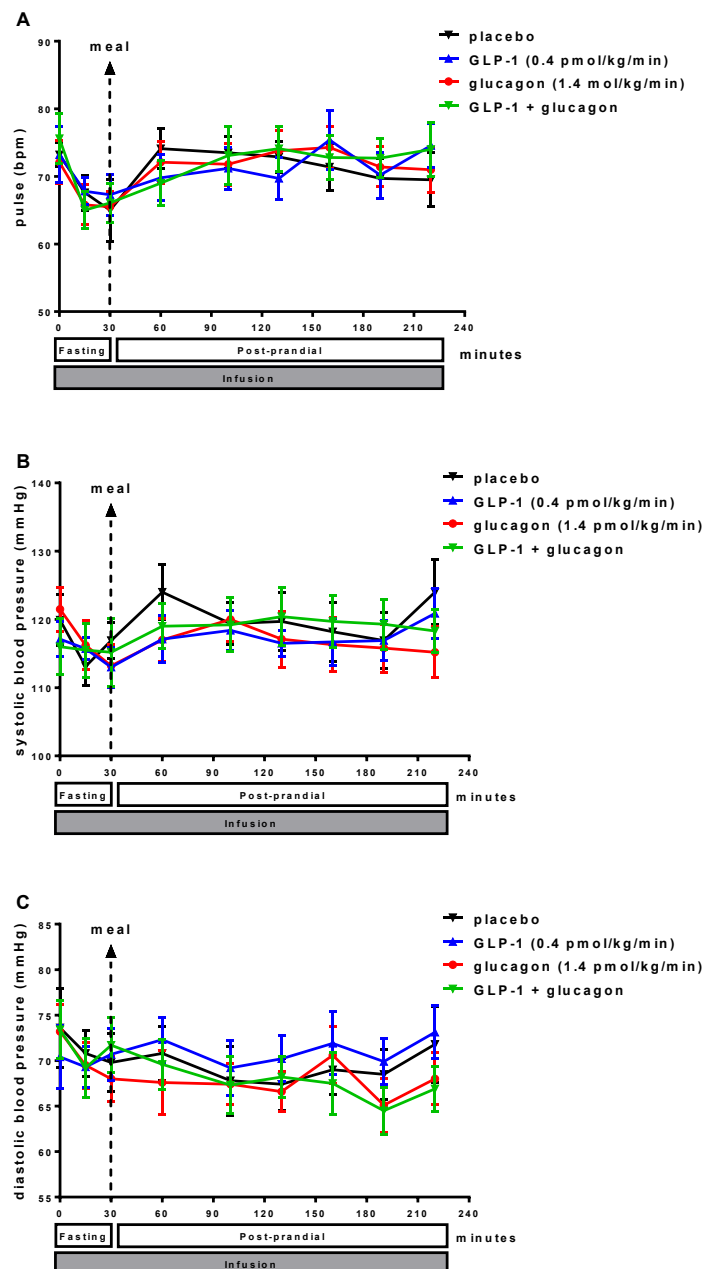


Figure 3.5. The effect of low dose glucagon and GLP-1 on heart rate (A), systolic (B) and diastolic (C) blood pressure. Mean $\pm$ SEM values plotted. Two way repeated measures ANOVA showed no significant differences between treatment groups.

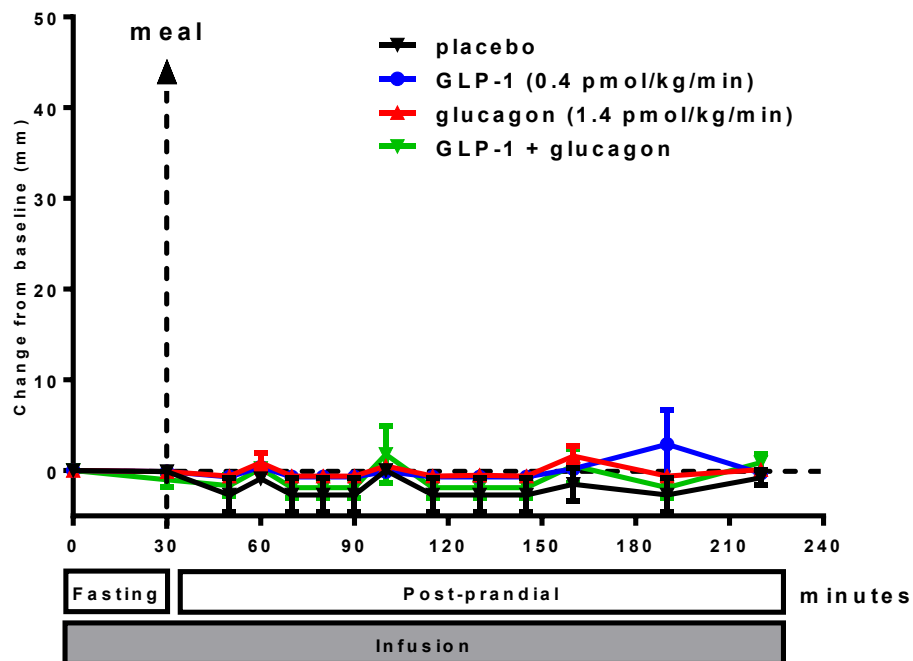


Figure 3.6. Subjective rating of nausea as measured by Visual Analogue Score (VAS) response. Scores are shown as change from baseline value(millimetres). Mean $\pm$ SEM values plotted.

3.5 Discussion

This study examines the effect of co-infusion of two endogenous peptide hormones, glucagon and GLP-1, on glucose homeostasis. The doses chosen reflect post-prandial levels of glucagon and GLP-1 in the RYGB patient, thus mimicking the hormonal milieu observed post-bypass. Here it is demonstrated that co infusion of GLP-1 and glucagon significantly attenuates the fasting hyperglycaemia induced by glucagon alone. In addition, it is observed for the first time that the combined infusion of GLP-1 and glucagon causes a significant reduction in post prandial glycaemia relative to both placebo and single glucagon infusion.

The glucagon-mediated elevation of glucose is not unexpected, rather, it is an established action and recognised treatment for conditions characterised by hypoglycaemia. We have previously demonstrated that acute intravenous glucagon increases preprandial glucose levels (Tan TM et al., 2012, Cegla et al., 2014). A fasting plasma glucose of 6.1 mmol/l observed here with glucagon infusion is clinically relevant as this is the threshold at which impaired fasting glucose (IFG) is defined. IFG is a strong risk factor for developing diabetes and/or cardiovascular disease (Alberti et al., 1998). This illustrates the potential danger of using glucagon alone in the treatment of obesity. The significantly lower preprandial glucose levels observed with the combined infusion highlight the advantage of dual hormone treatment.

Although, glucagon alone did not cause a significant rise in post prandial glucose relative to placebo, there was a significant difference in the AUC glucose₃₀₋₁₆₀ between vehicle and co-infusion of GLP-1 and GCG, demonstrating that the combination of peptides can actually improve glycaemia, rather than just 'ameliorate' hyperglycaemia. This provides further support for the utility of this dual infusion.

These findings are supported by previous studies examining the effects of single glucagon infusions. Sherwin et al administered a 3ng/kg/min (0.9 pmol/kg/min) infusion to obese subjects for duration of 6 hours during which an oral glucose tolerance test (100mg of glucose) was performed (Sherwin et al., 1976). They demonstrated a transient rise in preprandial glucose with no significant difference in

postprandial glycaemia relative to saline. Franks et al also used a 3ng/kg/min intravenous infusion of glucagon but tested its effect on glucose tolerance in overweight subjects using a mixed meal test as the carbohydrates stimulus (Frank et al., 1998). They again showed no significant elevation in post prandial glucose levels with glucagon infusion relative to saline. However, they did not demonstrate an elevated fasting glucose whilst on the glucagon infusion. This could be due to a lower glucagon concentration (compared to the current study) or the lower BMI (compared to the study by Frank et al (Frank et al., 1998)). A potential mechanism for the lack of post prandial hyperglycaemia during infusion with glucagon is a compensatory rise in insulin. The basis for this is the observation that in Type 1 diabetic patients, an infusion of 3ng/kg/min results in a significantly higher post prandial glucose relative to a saline infusion (Dinneen et al., 1995). However, against this is the observation that no significant difference in AUC insulin was found between our single GCG and saline group, despite a trend being noted. This finding has previously been replicated in the study by Franks et al (Frank et al., 1998).

The single infusion of GLP-1 resulted in a reduction in post prandial glucose and insulin levels relative to the saline infusion. Ratios of AUC insulin₃₀₋₁₆₀ to AUC glucose₃₀₋₁₆₀ and insulinogenic indices suggest that the GLP-1 infusion was the least insulinotropic infusion. As first glance this seems counter-intuitive as GLP-1 is thought of as an insulinotropic hormone, however, this relative reduction in post prandial insulin is well established. Nauck et al have previously reported that post-prandial insulin response was diminished by the administration of GLP-1 (Nauck et al., 1993). This is most likely explained by the inhibition of gastric emptying, which slows the transit of nutrients into the duodenum and jejunum, where the absorption of glucose, fructose, and amino acids takes place, as is illustrated by plasma glucose concentrations that remain below basal levels in all experiments involving exogenous GLP-1 (Willms et al., 1996). Because the mechanism of GLP-1's insulinotropic effect is to potentiate substrate-induced secretion, it is understandable that the insulin response is decreased despite increased GLP-1 concentrations. In a further study, Nauck et al have shown that GLP-1 infusion displays tachyphylaxis of gastric emptying and this may explain the disparity in insulin levels post meal with the GLP-1 infusion in Chapters 2 and 3. In Chapter 2, where there was a marked rise in insulin post-prandially, the meal was given at 90 mins compared to

30 mins in this current investigation where we observe an attenuated rise in insulin. Holst et al demonstrate rapid tachyphylaxis of gastric emptying in response to GLP-1. They serve two meals during GLP-1 infusion and show the two different insulin profiles that we have also observed (Nauck et al., 2011).

The addition of glucagon to GLP-1 did not result in any deterioration in carbohydrate tolerance as measured by AUC glucose₃₀₋₁₆₀ relative to the single GLP-1 infusion but there was a significant reduction compared the single glucagon infusion. The dual infusion caused a significantly lower AUC insulin₃₀₋₁₆₀ than glucagon by itself but significantly higher than GLP-1. This observation offers a valuable insight into the action of these hormones in the post-prandial phase. Both GLP-1 and glucagon are known to be insulinotropic, but in our study, both the single glucagon and the combination infusion stimulated a higher insulin response relative to GLP-1.

3.5.1 Future work

The work presented in chapters 2 and 3 reflects acute infusion data and further work must be done to investigate the chronic effects of dual infusion in both euglycaemic and diabetic individuals.

As mentioned previously, the beneficial effects of both glucagon and GLP-1 would ideally be combined into one molecule, stimulating both the glucagon and GLP-1 receptors. The pharmacokinetic profile would need to be prolonged in order to facilitate once-weekly administration. Work to develop a long-acting analogue of glucagon and GLP-1 is currently in progress in the department.

3.5.2 Conclusion

In conclusion, this study has demonstrated that the co-infusion of GLP-1 and glucagon results in improved carbohydrate tolerance relative to saline. In RYGB, there is a paradoxical post prandial increase in glucagon plasma levels with a significant improvement in glucose tolerance. Acute infusions of oxyntomodulin yield a significant improvement in glucose tolerance and our data confirms that simultaneous agonism at the glucagon and GLP-1 receptors does not result in postprandial hyperglycaemia.

These data provide further support for the safety of dual GLP-1 and GCG agonists in the treatment of obesity.

CHAPTER 4: INTERACTION OF THE GLUCAGON RECEPTOR AND RAMP2

4.1 Introduction

The discovery of Receptor Activity Modifying Proteins (RAMPs), as accessory proteins for G-protein coupled receptor (GPCR) localization and function constituted a fundamental change in our understanding of GPCR physiology. There are three members of the RAMP family; they are crucial in determining the pharmacology of the calcitonin receptor like receptor (CRLR), including translocation from the endoplasmic reticulum (ER) to the Golgi, internalisation and recycling of the receptor. Since then, RAMPs have been shown to heterodimerize with a number of Class B and C GPCRs and influence their binding, function and subcellular distribution.

4.1.1 RAMPs and ligand specificity

As described above, the discovery of RAMPs was made with the finding that interaction of three different RAMPs with the CRLR and CTR gave rise to receptors for different ligands (McLatchie et al., 1998, Takei et al., 2004, Roh et al., 2004). These four ligands (calcitonin, amylin, calcitonin gene related peptide and adrenomedullin) bind to two receptors to give rise to seven different receptors with distinct pharmacology. Thus far, the ability of RAMPs to determine which ligand binds the receptor, depending on which RAMP is present, has only been demonstrated for the CRLR and CTR.

4.1.2 RAMPs and cell trafficking

On ligand binding, GPCRs undergo a number of events including G-protein coupled signalling, receptor endocytosis, degradation or recycling (Rajagopal et al., 2010, Reiter and Lefkowitz, 2006, Lefkowitz et al., 2006). A variety of studies using biochemical and confocal microscopy approaches have shown that RAMPs and the CRLR interact in the ER and remain partners throughout the life cycle of the receptor (Kuwasako et al., 2000, Hilairet et al., 2001, Hilaire et al., 2001, Cottrell et al., 2007, Kuwasako et al., 2006, Bomberger et al., 2005a, Bomberger et al., 2005b, Kuwasako et al., 2009). In particular, RAMP3 has been demonstrated to regulate internalisation of the CRLR, as well as post-endocytic receptor recycling in response to adrenomedullin (Bomberger et al., 2005a, Bomberger et al., 2005b). Although RAMP2-CRLR is also an adrenomedullin receptor, only RAMP3 is involved in the receptor's life cycle regulation.

In addition to the CRLR and CTR, other class B GPCRs have also been found to be capable of associating with at least one of the RAMPs (Christopoulos et al., 2003). On co-transfection with the GPCR, the RAMP partner translocates to the cell surface. The vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide (VPAC)-1 receptor interacts with all 3 RAMPs; the parathyroid hormone-1 receptor (PTH1R) and glucagon receptor interact solely with RAMP2 while PTH2R only interacts with RAMP3 (Christopoulos et al., 2003). However the effect of RAMPs on intracellular cell trafficking has not yet been studied for these receptors.

More recently, Harikumar et al used a combination of morphological fluorescence techniques, bioluminescence resonance energy transfer, and bimolecular fluorescence complementation to demonstrate that the secretin receptor associates with RAMP3, but not with RAMP1 or RAMP2 (Harikumar et al., 2009). There were no observed changes in receptor internalization in COS or CHO-K1 cells expressing the secretin receptor in the presence or absence of RAMP. The secretin receptor trafficked normally to the cell surface in these cells in a RAMP-independent manner, resulting in both free and RAMP-associated receptor on the cell surface. However, RAMP3 association with this receptor was shown to be capable of rescuing a receptor mutant (G241C) that is normally trapped intracellularly in the biosynthetic machinery (ER and golgi).

The calcium-sensing receptor (CaSR), a class C GPCR, has also been shown to be regulated by RAMPs. Recombinant CaSR expressed in COS7 cells only translocates to the cell surface in the presence of RAMPs 1 or 3. RAMPs also facilitate trafficking of the CaSR from the ER to the Golgi as well as mediating glycosylation of the receptor (Desai et al., 2014). Furthermore, siRNA mediated knockdown of RAMP1 expression was found to impair the trafficking of CaSR in HEK293 cells (Bouschet et al., 2005).

4.1.3 RAMPs and cell signalling bias

The ability of RAMPs to influence downstream signalling pathways is an exciting concept as it may enable the creation of biased agonists which fully exploit the therapeutic potential of clinically important receptors. Several examples of biased agonism exist for RAMPs. For the VPAC1R, RAMP2 enhances receptor-mediated phosphoinositide hydrolysis (a surrogate marker of the Gq pathway) without changing the cAMP response or agonist binding. RAMP2 co-expression increases the E_{max}

(maximal action of the agonist) while causing no effect on the EC₅₀ (molar concentration of agonist required to cause 50% of the maximal possible effect of that agonist) (Christopoulos et al., 2003).

For the calcitonin receptor, RAMP3 changed the potency of calcitonin and amylin at the CTR. When co-expressed with RAMP3, the relative potency was reversed by a 3.5-fold loss in sensitivity to calcitonin and a 19-fold increase in sensitivity to amylin. AC66, an inverse agonist, produced apparent simple competitive antagonism of calcitonin and amylin. However, the potency of AC66 was changed in the blockade of amylin but not calcitonin responses with RAMP3 coexpression (Armour et al., 1999).

4.1.4 RAMP2 and the glucagon receptor

As mentioned above, it has recently been found that the glucagon receptor may interact with RAMP2 (Christopoulos et al., 2003). The effect of this interaction on ligand binding and receptor activation has not yet been studied. This chapter is concerned with understanding the effect of RAMP2 on the pharmacology of the glucagon receptor.

4.2 Hypothesis and aims

4.2.1 Hypothesis

I hypothesise that:

1. RAMP2 functionally interacts with the GCGR.
2. This interaction is important in mediating or modifying the binding and agonist activity of GCG, GCG-related peptides and a GCG analogue we have devised in the laboratory.
3. This interaction is important in enabling co-localisation of RAMP2 with GCGR on the cell surface.

4.2.2 Aims

1. To investigate the effect of RAMP2 on glucagon binding at the GCGR
2. To investigate the effect of RAMP2 on glucagon signalling via:
 - a. The $G_{\alpha s}$ pathway through measurement of cAMP accumulation.
 - b. The G_q pathway through measurement of intracellular calcium flux.
3. To investigate the effect of RAMP2 on glucagon induced β -Arrestin recruitment at the GCGR.
4. To investigate the effect of RAMP2 on other ligands including
 - a. oxyntomodulin (a native glucagon/GLP-1 agonist)
 - b. GLP-1
 - c. A Glucagon/GLP-1 analogue
5. To investigate the effect of knock-down of RAMP2 using siRNA
 - a. on the $G_{\alpha s}$ pathway
 - b. on the G_q pathway
 - c. β -Arrestin recruitment
6. To visualise using confocal microscopy:
 - a. Whether RAMP2 and GCGR colocalise
 - b. Whether the presence of RAMP2 affects the subcellular distribution of GCGR

4.3 Materials and Methods

4.3.1 Peptides

Human GCG, GLP-1 and OXM were purchased from Bachem, Ltd. (Merseyside, UK). GLP-1(7-36)NH₂ was the form used in all experiments, and will now be referred to simply as GLP-1. The peptide analogue G(X) was custom synthesised by Bachem, Ltd. (Merseyside).

4.3.2 Custom synthesis of peptides

A dual glucagon/GLP-1 analogue, G(X), was designed by Professor Stephen Bloom, Department of Investigative Medicine, Imperial College London. G(X) was custom synthesised using solid phase peptide synthesis (SPPS) methodology (Bachem Ltd). Peptide purity was measured by reverse-phase high performance liquid chromatography (HPLC) and by Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS).

A chimeric peptide consisting of the N-terminal of glucagon and the C-terminal of GLP-1 has been shown to confer affinity and activity at both the glucagon and GLP-1 receptors (Hjorth et al., 1994, Runge et al., 2003). G(X) contains identical amino acid sequences to glucagon from positions 1 to 15 as the N-terminal of glucagon has been shown to be critical for glucagon receptor binding and activation (Hjorth et al., 1994).

In order to create a dual agonist that is also effective at the GLP-1 receptor, G(X) has been modified to resemble exendin-4. This peptide, first isolated from the venom of the lizard *Heloderma* species, has been found to be a potent agonist at the human GLP-1 receptor (Eng et al., 1990, Eng et al., 1992). Also favourable is its prolonged pharmacokinetic profile compared to native GLP-1. Therefore, from positions 16-34, amino acid substitutions have been made to resemble exendin-4. An example of this is removal of the Gly at position 16. Underwood (2010) examined the crystal structure of GLP-1 bound to the extra-cellular domain of its receptor and demonstrated that GLP-1 actually formed a continuous helix from residues 13 to 33 but with a kink around the glycine residue at position 16 (Underwood et al., 2010). Exendin-4 has a greater helical

propensity in this region than GLP-1, which has been suggested to be due to the absence of the helix disrupting Gly at position 16 (Neidigh et al., 2001, Andersen et al., 2002).

Residues 22 and 23 have also been identified as critical for GLP-1 receptor activation (Adelhorst et al., 1994, Gallwitz et al., 1994). Phenylalanine at position 22 is a conserved residue in GLP-1, glucagon and exendin-4 whereas valine replaces isoleucine at position 23 in glucagon. Rather than direct interaction with the receptor, it has been postulated that these two residues may enable a specific conformation of the C-terminal which contributes to the selective recognition of GLP-1 by the receptor (Adelhorst et al., 1994). The peptide sequence of analogue G(X), along with glucagon and GLP-1, can be found in Appendix C.

4.3.3 Establishing a cellular co-expression system for RAMP2 and GCGR.

4.3.3.1 Production of competent bacteria

Gram negative bacteria such as *E. coli* have a cell wall which has evolved to prevent entry of exogenous DNA. To enable entry of plasmids into bacteria, they have to be rendered 'competent' to take up DNA. Several methods can be used, including electroporation and treatment with cations. The method of choice is treatment with cations, which is reliable and relatively easy. There have been several adaptations to the original method including the ions used, their concentration and incubation times (Hanahan, 1983).

Materials

Liquid Broth (LB) (Appendix E)

Tetracycline (Sigma, Poole, Dorset, UK) 10 mg/ml in absolute ethanol

Transformation buffer I (TFB I) (Appendix E)

Transformation buffer II (TFB II) (Appendix E)

XL1-Blue *E. coli* (XL1B) (Stratagene Ltd)

Method

One hundred millilitres of LB, supplemented with 5µg/ml tetracycline (LBtet), was inoculated with a colony of XL1-B and incubated overnight in a shaking incubator at 37°C. One millilitre of this culture was inoculated into 100 ml of fresh LBtet (prewarmed to 37°C) and incubated at 37°C, with vigorous shaking, until the bacteria were in log phase growth (OD550 = 0.4 - 0.5). The bacteria were recovered by centrifugation at 800 x g for 15 minutes. The bacteria were resuspended in 40 ml ice cold TFB I and incubated on ice for 10 minutes. The bacteria were recovered as above and resuspended in 4 ml TFB II and incubated on ice for 15 minutes. They were divided into 50µl aliquots, frozen in a dry ice/ethanol bath and stored at -70°C.

4.3.3.2 Transformation of competent bacteria

Although treating bacteria with cations renders them competent to take up DNA, to obtain efficient transformation requires several additional steps. The initial step, incubation on ice, allows attachment of the plasmid to the bacterial cell membrane. The heat shock step enables efficient transport of the plasmid into the bacteria. The subsequent incubation allows expression of the resistance genes before exposure to the antibiotic.

Materials

LB

LB(amp) plates

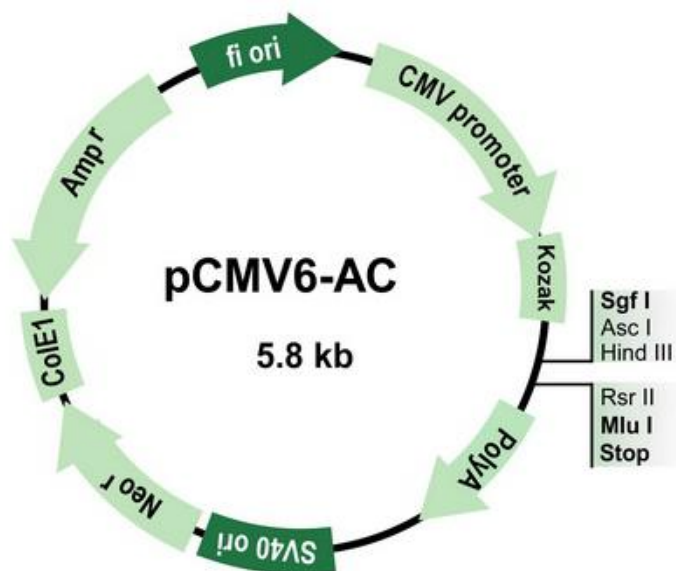
Competent bacteria (section 4.3.3.1)

pCMV6-AC-hRAMP2 (Origene, Rockville, MD, USA) (See Figure 4.1)

Method

Competent XL1B bacteria stored at -80°C were allowed to thaw in a waterbath at 42°C. Plasmid DNA containing human RAMP2 (pCMV-AC-hRAMP2) (up to 1 µg) in 1 µl sterile dH₂O was added to the bacteria and left on ice for 5 minutes. This was then heatshocked at 42°C for exactly 40 seconds and then transferred back on ice for 2 minutes. 200 µl of LB containing ampicillin 50 µg/ml (LB(amp)) was added to the plasmid/ bacteria solution and incubated at 37°C for 30 minutes. 30 µl of this solution was transferred to a

dried L-agar plate (as for LB(amp) with 15 g Agar per litre of LB) which was incubated upside down at 37°C overnight. A single colony was identified and placed into a large conical flask containing LB (+ ampicillin 50 µg/ml) at 37°C.



Schematic of the multiple cloning sites:

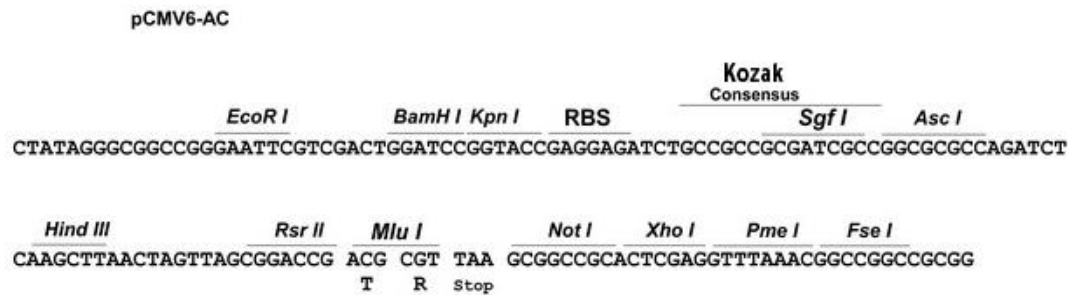


Figure 4.1: Plasmid map of pCMV6-AC-hRAMP2 as supplied by Origene. cDNA of the gene of interest (human RAMP2) was purchased from Origene (Origene, Rockville, MD, USA), and was supplied in the pCMV6-AC plasmid. Over expressing cell lines were produced using the protocols outlined in section 4.3.3.8.

4.3.3.3 Large scale plasmid purification from bacterial cells by maxiprep

To isolate plasmids from bacteria it is necessary to disrupt the cell wall to release the plasmid and simultaneously remove contaminating proteins, genomic DNA and RNA (Sambrook and Russell, 2001). The method used involves disruption of the cell wall by treatment with alkaline SDS followed by precipitation of bacterial debris with sodium acetate. This step also removes most of the genomic DNA, since in bacteria this is anchored to the cell wall. The RNA is removed at a later stage by either treatment with RNase A or alkali.

Materials

LB (see Appendix E)

GTE solution (glucose /Tris EDTA) (see Appendix E)

Lysozyme (Sigma)

Alkaline SDS (see Appendix E)

5M potassium acetate pH 4.6 (Sigma)

1x TE (see Appendix E)

DNase free RNaseA (Sigma)

2M sodium acetate (Sigma)

Propan-2-ol (Sigma)

Phenol/chloroform

Method

Following overnight growth of plasmid-containing bacteria in a conical flask, the bacteria were recovered by centrifugation for 8 minutes at 3000 x g (HS-4 rota in RC-5B super speed centrifuge, Du Pont) at 4°C. The pellet was resuspended in 25 ml GTE supplemented with 2mg/ml of lysozyme and the samples incubated at room temperature for 5 minutes. Fifty millilitres of alkaline SDS were added, the sample mixed very gently until clear and incubated on ice for 5 minutes. Then 37.5ml of 5M potassium acetate were added, the samples mixed gently and left on ice for 10 minutes. The bacterial debris was then removed by centrifugation for 15 minutes 9000 x g (HS-4 rota in RC-5B super speed centrifuge) at 4°C. The supernatant was filtered through

nylon gauze into a clean tube. 34ml of propan-2-ol was added and then incubated on ice for 15 minutes. The DNA was then recovered by centrifugation (9000 x g, 4°C). The pellet was dissolved in 10ml 1x TE, RNase A added to a concentration of 0.1mg/ml and the reaction incubated at 37°C for 30 minutes. Addition of an equal volume of phenol/chloroform terminated the reaction, the mixture mixed and phases separated by centrifugation for 20 minutes at 10000 x g and 4°C. The aqueous phase was collected and DNA was precipitated using 1ml 2M sodium acetate, pH 5.2 and 10ml of cold propan-2-ol added, and the reaction incubated at -20°C for at least one hour.

4.3.3.4 Caesium chloride gradient purification

The technique was used to purify large quantities of plasmid (Sambrook and Russell, 2001). The purification method depends on the decrease in density of nucleic acids when they bind ethidium bromide. Ethidium bromide binds by intercalation of DNA, causing the DNA helix to partially unwind. Binding occurs to a higher degree in linear genomic DNA and nicked plasmid DNA than the closed circular supercoiled plasmid DNA, so the different states of DNA form separate bands in the caesium gradient.

Materials

TES buffer (Appendix E)

Caesium chloride

Ethidium bromide (10mg/ml)

Propan-2-ol, caesium chloride saturated (Appendix E)

1x TE (Appendix E)

Mineral oil

Ethanol

2M sodium acetate

Method

The DNA obtained from section 4.3.3.3 was recovered by centrifugation for 20 minutes at 24000 x g (HB4 rotor in RC5B super speed centrifuge, Du Pont) and 4°C. The supernatant was poured off and the pellet dissolved in 8.2ml of TES. Eight grams of caesium chloride were dissolved in the DNA solution and the solution mixed by hand. 200µl of ethidium bromide was added. The sample was loaded into a polyallomer tube

(Ultracrimp, Du Pont), overlaid with mineral oil, the tube sealed and centrifuged for 16 hours at 20°C and 185500 x g (A1256 rotor, OTD-55B centrifuge, Du Pont). After centrifugation, the DNA bands were visualised by UV illumination and the band containing the closed circular DNA removed using a 20G needle and a 2ml syringe. The ethidium bromide was removed from the plasmid by repeated extraction with an equal volume of caesium chloride saturated propan-2-ol, until both phases were colourless. The DNA was precipitated by addition of two volumes of glass-distilled water (GDW) and six volumes of room temperature absolute ethanol. The DNA was recovered by centrifugation for 15 minutes at room temperature and 24000 x g, the supernatant removed and the pellet dissolved in 0.4ml GDW. The solution was then transferred into a 1.5ml eppendorf and ethanol precipitated for at least 1 hour at -20°C by adding 40ul 2M NaAc, then 1ml cold ethanol and shaking. The solution was then centrifuged for 7min at 12300 x g. The supernatant was removed and the pellet allowed to air dry for about 10 min, resuspended in 1ml GDW and quantified spectrophotometrically (section 4.3.3.5).

4.3.3.5 Quantification of DNA by a spectrophotometer

DNA was diluted 1:100 in GDW and 1ml placed into a quartz cuvette. The absorbance was read at 260 and 280 nm (UV0160 spectrophotometer, Kyoto, Japan). The concentration of DNA was calculated using the following formula based on Beer-Lambert's Law:

$$\text{concentration } (\mu\text{g/ml}) = (A_{260} \times \text{dilution factor}) \times 50.$$

4.3.3.6 Restriction endonuclease digestion of plasmid DNA

In order to ensure that the purified DNA contained the correct plasmid, DNA was digested with restriction endonuclease enzymes and analysed by gel electrophoresis.

Materials

Plasmid DNA

Restriction endonucleases: EcoR I and Xho I (New England Biolabs, Hitchin, Hertfordshire, UK)

10x restriction buffer (as supplied)

Method

In two separate reaction tubes, the product of the large scale preparation of plasmid was diluted in autoclaved GDW and restriction buffer added to give a final concentration of 1x restriction buffer. Restriction endonucleases were added to five times excess; the volume of enzyme added was kept below 10% of the final volume as their solution contains glycerol which inhibits enzyme activity and increases non-specific cutting. The reaction was incubated for a minimum of one hour at 37C. Reaction volumes are outlined below:

TV = 10µl	Uncut (control) Plasmid	Cut Plasmid
GDW	8 µl	7.2 µl
10x ECOR1 buffer	1 µl	1 µl
Plasmid (2mg/ml)	1 µl	1 µl
Xho I (20U/µL)	0 µl	0.4 µl
Eco RI (20U/µl)	0 µl	0.4 µl

4.3.3.7 Electrophoresis of DNA fragments

After restriction digestion, the DNA was size fractionated by electrophoresis on an agarose gel to confirm if an insert was present.

Materials

Agarose, type II-A medium EEO

50x TAE (Appendix E)

2M Tris-Acetate pH 8.5

0.5M EDTA pH 8.0

Ethidium bromide (10mg/ml)

DNA marker (1KB plus ladder, Invitrogen)

Gel loading buffer (Appendix E)

Method

A 0.7% (w/v) agarose gel was prepared by dissolving the agarose in 1x TAE using a microwave oven. The gel was cooled to 45°C and ethidium bromide added to a final concentration of 0.5 µg/ml. Three microliters of loading buffer were added to 10µl of restriction enzyme digest, 1µl of DNA marker was added to 9µl GDW and treated the same way. The samples were loaded onto the gel and electrophoresed at 7.5V/cm. The DNA was visualised by illumination with UV light (300 nm).

4.3.3.8 Maintenance of cells

Materials

CHO-K1 cells (GeneBLAzer® GCGR-CRE-*bla* CHO-K1 cells (K1855A), Invitrogen)

Dulbecco's modified medium (DMEM) without sodium pyruvate containing 4.5g/l glucose (Invitrogen)

Foetal bovine serum (FBS) (Invitrogen)

Blastocidin (Sigma)

Non-essential amino acids (Sigma)

HEPES (pH7.3) (Sigma)

100IU/ml penicillin 100µg/ml streptomycin

EDTA (Sigma)

Phosphate buffer solution (PBS) (Sigma)

Method

Chinese hamster ovarian (CHO-K1) cells expressing the glucagon receptor were cultured in DMEM supplemented with 10% FBS, 0.1 mM NEAA, 25 mM HEPES (pH7.3), 100IU/ml penicillin, 100µg/ml streptomycin and 5 µg/ml blastocidin which was the selection marker for this cell line. Medium was changed every 2-3 days and the cells passaged when 70% confluent using EDTA. Briefly, the medium was aspirated from the flask, the cells were rinsed with PBS and 2ml/50cm² flask surface area of room temperature EDTA was added. Cells were incubated at room temperature until they detached from the flask. Five millilitres of fresh medium was added to the flask and cells

recovered by centrifugation for five minutes at 200 x g. The cells were resuspended in fresh medium and transferred to a new flask at a dilution of 1:10.

4.3.3.9 Polyethylenimine (PEI) mediated in vitro gene transfer

In order to create a co-expression system, the plasmid containing the DNA construct of RAMP2 was transfected into Chinese hamster ovarian (CHO-K1) cells already expressing the glucagon receptor (Kunapuli et al., 2003). This was achieved using Polyethylenimine (PEI), a cationic polymer containing a repeating unit composed of an amine group and two carbon aliphatic CH_2CH_2 spacers. PEI and DNA interact, in the presence of 0.15M NaCl, forming stable complexes which are taken up by pinocytosis across the cell membrane (Abdallah et al., 1996). Once inside the cell protonation of the amines results in an influx of counter-ions and a lowering of the osmotic potential. Osmotic swelling results and bursts the vesicle releasing the polymer-DNA complex (polyplex) into the cytoplasm. If the polyplex unpacks then the DNA is free to diffuse to the nucleus.

Materials

0.1 M 25kD PEI (Sigma) (Appendix E)

0.3M NaCl

Plasmid DNA (pCMV6-AC-RAMP2)

CHO-K1-GCGR cells

Method

Twenty four hours prior to transfection, cells were sub-cultured and plated at a density of 40000 cells/well (Invitrogen) in standard media in a six well plate. Transfections were carried out when the cells were 50% confluent. PEI/DNA complexes were made with PEI (average Mw ~ 25000) according to the method described by Abdallah et al (Abdallah et al., 1996). A 0.1 M stock solution of PEI pH 7.0 was prepared and filtered through a 0.2µm filter prior to use. The plasmid DNA was prepared such that each plate was transfected with 6µg DNA in 0.15M NaCl (the 0.3M NaCl stock solution was also sterilised by passage through a 0.2µm filter). The cells were transfected with pCMV6-AC-RAMP2 (containing a neomycin resistance gene) and 9 nitrogen equivalents of PEI. The amount of PEI required was calculated according to the ratio of PEI amine nitrogen

equivalents to DNA phosphate where 1µl of 0.1 M PEI = 100nmol amine nitrogen and 10µg DNA = 30nmol DNA phosphate. Thus for 9 nitrogen equivalents 45µg DNA (135nmol DNA phosphate) requires 810nmol amine nitrogen i.e. 8.1µl of 0.1 M PEI. PEI solution was slowly added to the DNA solution, vortexed for 30 seconds and allowed to stand at room temperature for 10 minutes before use. Four wells were transfected with the PEI/DNA/NaCl mix and two wells with PEI/NaCl mix only (controls). The mix was added slowly to the cells in a circular motion and incubated for three hours at 37°C, 5% CO₂, after which the media was removed and replaced with standard media (as described in 4.3.3.8). Forty eight hours later, media was supplemented with 800µg/ml geneticin, and media replaced every 48 hours with fresh geneticin until all control cells were dead, usually 10 days after the start of treatment. Remaining cells from each plate were then transferred to T-10 flask (1 plate to 1 flask), and then maintained as described in 4.3.3.8.

4.3.3.10 Confirmation of gene expression

In order to confirm gene expression of RAMP2, RNA was extracted from the cells, reverse transcribed and cDNA was then amplified by quantitative polymerase chain reaction (qPCR) as outlined below.

4.3.3.10.1 RNA extraction

Materials

Tri-reagent

bromo-chloropropane

Purelink RNA Mini Kit (Invitrogen, Paisley, UK)

Purelink DNase set (Invitrogen, Paisley, UK)

60% ethanol

Method

Cells were detached from their flasks and pelleted via centrifugation as described in section 4.3.3.8. Total RNA was extracted using the Tri-reagent method according to the manufacturer's protocol. The pelleted cells were homogenised in 1 ml Tri-reagent and the suspension transferred to 1.5 ml microcentrifuge tubes to incubate at room temperature for 10 minutes. 100 µls of bromo-chloropropane was then added, mixed

and incubated for 10 minutes at room temperature. The solution was centrifuged for 10 minutes at 14,000 x g at 4°C and the upper aqueous phase transferred to autoclaved microcentrifuge tubes.

To ensure sample purity and absence of DNA, RNA samples were purified using a Purelink RNA Mini Kit (Invitrogen, Paisley, UK) and Purelink DNase set (Invitrogen, Paisley, UK) and instructions followed according to the manufacturer's protocol. Four hundred microliters of 70% ethanol was added to each sample. Each sample was mixed and added to a spin cartridge, prior to centrifugation at 14000 x g for 15 seconds at room temperature. The flow-through was discarded leaving RNA bound to the cartridge membrane. The spin cartridge then underwent a series of buffer washes and centrifugation, followed by the addition of Purelink DNase. For each sample the following constituents were added to create the Purelink DNase mixture: 8 µl 10x DNase I reaction buffer, 10 µl resuspended DNase and 62 µl RNase free water. Samples contained within the spin cartridges were allowed to incubate at room temperature for 15 minutes followed by a further buffer wash and centrifugation at 14000g for 15 seconds. A final wash using 500 µl buffer containing 60% ethanol, was added to the cartridge and centrifuged at 14000 x g for 15 seconds. The flowthrough was discarded, leaving the dry membrane with bound RNA which was placed into a recovery tube. RNase-free water (30 µl) was added to the membrane. Samples were incubated at room temperature for 1 minute and then centrifuged for 1 minute at 14000 x g to elute the RNA from the membrane into the recovery tube. Purified RNA in the recovery tube was then stored at -20°C.

Finally, RNA in each sample was quantified using a UV 1101 spectrophotometer (Biochrom, Cambridge, UK). A 1:50 dilution of the RNA purified sample was made (2µls RNA sample added to 98 µls GDW) and the resultant 100 µls solution placed in a disposable cuvette (Brand manufacturers, Germany). The absorbance was read at 260 nm and the concentration of RNA calculated using the following formula:

$$\text{Concentration } (\mu\text{g/ml}) = (\text{absorbance}_{260} \times \text{dilution factor}) \times 40$$

An appropriate dilution was made to achieve a concentration of 100 µg/ml of total RNA, as required for appropriate amplification using qPCR.

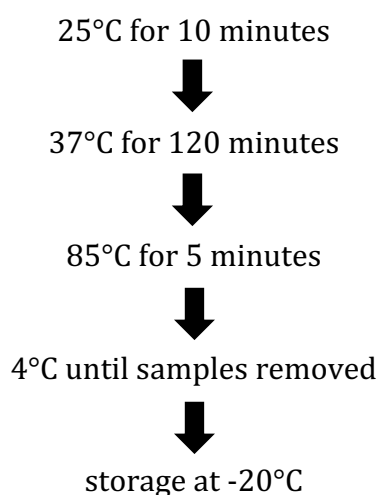
4.3.3.10.2 Reverse transcription

Materials

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK)

Methods

To perform qPCR, mRNA needs to be transcribed into its complementary DNA sequence (cDNA) by reverse transcription. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) was used and the protocol followed according to the manufacturer's instructions. 2X RT Master Mix was prepared using the following components: 2 µl 10XRT buffer, 0.8 µl 25X 100mM dNTP mix, 2 µl 10X RT random primers, 1 µl Multiscribe Reverse Transcriptase and 4.2 µls RNase free water. 1µg (10 µl of 100µg/ml sample) of each RNA sample was added to 0.5ml eppendorfs containing 10µl 2X RT master mix and pipetted several times to mix. Each eppendorf was transferred to a thermal cycler (PTC-100 7307 Programmable Thermal Controller, MJ Research Inc., US) under the following conditions:



4.3.3.10.3 Quantitative polymerase chain reaction (qPCR)

Materials

TaqMan Universal PCR Master Mix

human RAMP2 probe Hs00359352_m1 (Life Technologies, UK)

GDW

Methods

QPCR involves specific gene quantification through the use of a reporter-tagged probe, which is complementary to the cDNA of interest (Figure 4.2). The TaqMan probe anneals specifically to the complementary sequence which has been amplified using a set of primers. When the probe is intact, the proximity of the reporter dye to the quencher suppresses the fluorescence of the reporter. However, addition of DNA polymerase extends the primer and as it synthesises a second strand of DNA, the 5' to 3' activity of the polymerase degrades the probe. This causing release of the reporter dye from the quencher and thus fluorescence of the reporter. This is shown below in Figure 4.3. In order to quantify the expression of human RAMP2, the probe Hs00359352_m1 was obtained (Life Technologies, UK).

For each rtPCR reaction, the following constituents were added to each well within a 384 well plate: 10 μ l TaqMan Universal PCR Master Mix, 1 μ l 20x TaqMan Gene Expression Probe, 1 μ l cDNA and 8 μ l GDW. A gene expression assay targeting the 18S ribosomal subunit was used as a housekeeping gene (control), using 1:10 dilution of sample. Each reaction was mixed and centrifuged for 1 minute at 1000 x g at room temperature to remove bubbles from within the reaction mixture. The plate was then loaded into a qPCR 108 machine (7900HT Fast Real-Time PCR System, Applied Biosystems, Warrington, UK) and PCR performed under the following conditions:

50°C for 2 minutes



95°C for 10 minutes



Thermal cycling: 95°C for 15s followed by 60°C for 1 minute

Repeated for 40 cycles

Each probe was validated prior to experimental procedures, using a randomly selected sample and compared to the 18S control. The amplification data for each target gene was normalised to the expression of the 18S endogenous control and expressed relative to an internal calibrator using the $2^{\Delta\Delta CT}$ method (SDS v2.3 software, Applied Biosystems, UK). This method allows data to be expressed as the fold change in gene expression normalised to a reference gene (18S rRNA) and relative to an internal control. 18S was chosen as the internal standard (rather than GAPDH or β -actin) as it has previously been shown that assessment of gut hormone mRNA expression may be greatly influenced by the choice of housekeeping gene and that 18S rRNA is the most stable (Yamada et al., 1997).

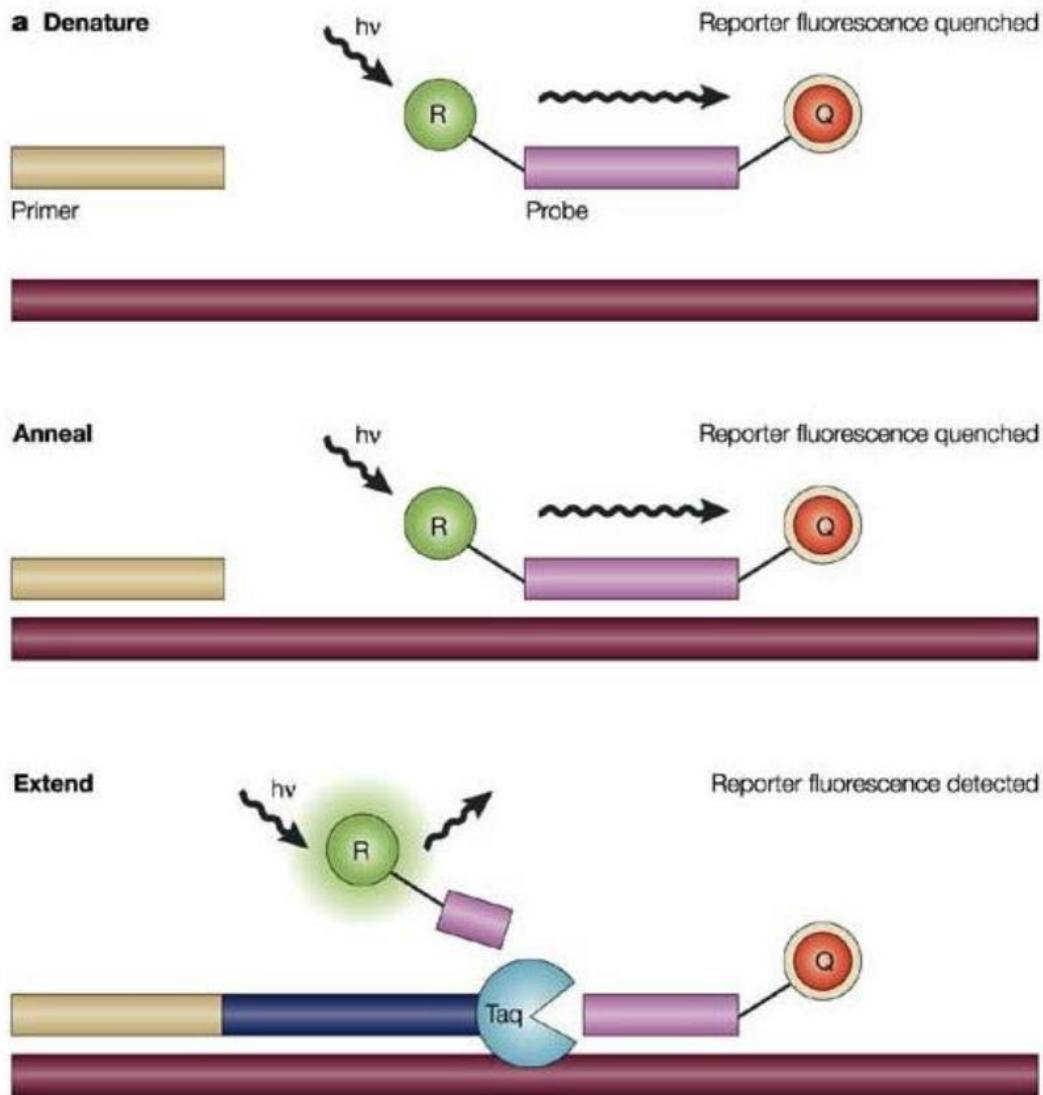


Figure 4.2 Schematic diagram quantitative polymerase chain reaction (qPCR). TaqMan probes consist of a fluorophore (6-carboxyfluorescein, FAM) attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end (dihydrocyclopyrroloindole tripeptide minor groove binder, MGB). The PCR cyclers' light source under normal conditions excites the fluorophore using Förster-type energy transfer. However, close proximity of the quencher to the fluorophore prevents this excitement. The probe anneals to a region of interest within DNA which has been amplified using a set of primers. As the Taq polymerase extends the primer and synthesises a second strand of DNA the 5' to 3' activity of the polymerase degrades the probe. This releases the fluorophore from the probe, and hence proximity to the quencher, resulting in fluorescence. Therefore, fluorescence detected in qPCR is directly proportional to the amount of DNA present in the sample. Figure reproduced from Koch WH (2004) (Koch, 2004)

4.3.4 Whole cell binding assays

Materials

Assay buffer (described below)

I¹²⁵-glucagon

Methods

Competition between binding of unlabelled and labelled ligand at the glucagon receptor was assessed in live whole cells. CHO-K1 cells expressing the human glucagon receptor with/without RAMP2 were compared. Ligands tested were: 1) glucagon 2) GLP-1 3) oxyntomodulin and 4) analogue G(X). Cells were grown in T-75 flasks to 70% confluence. Cells were detached from their flasks and pelleted via centrifugation as described in section 4.3.3.8. The pellet was resuspended in 1.5 ml assay buffer.

The assays were performed in 1.5ml siliconised microtubes, with a total volume of 500µl. The assay buffer was prepared (25mM HEPES (pH 7.4), 2mM MgCl₂, 1% BSA, 0.05% (w/v) Tween 20, 0.1mM diprotin A and 0.2mM PMSF). 50 µl of I¹²⁵-glucagon dissolved in assay buffer at 1000 counts per second was added to each tube. Increasing concentrations of unlabelled peptide made up in 400 µl of assay buffer were added to each tube. Each peptide concentration was assayed in duplicate or triplicate. Once competing unlabelled ligand and radio-labelled glucagon were added, 50 µl of the cell suspension was added to each tube. The microtubes were then vortexed and incubated at room temperature for 90 minutes. Microtubes were then centrifuged at 15781 x g at 4°C for 3 minutes, the supernatant removed and discarded, another 500µl of assay buffer added to wash the pellet of unbound radio-ligand, and then centrifuged as before. The supernatant was again discarded and the pellets measured for γ radiation for 240 seconds (Gamma counter, NE 1600, NE Technology Ltd, Reading, UK).

The half-maximal inhibition concentrations (IC₅₀), a measure of binding affinity, were then calculated and compared for CHO-K1-GCGR and CHO-K1-GCGR-RAMP2 cells. IC₅₀ values were calculated using the Prism 5.01 program (GraphPad Software Inc., San Diego, USA) using the following regression fit line:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})$$

Where Y=% specific binding and X=concentration of the agonist

4.3.4.1 Bradford assay

Materials

Bradford reagent (Sigma)

bovine serum albumin (Sigma)

Method

0.1 ml cell suspension (CHO-K1-GCGR or CHO-K1-GCGR-RAMP2 cells) was added to 3ml of Bradford reagent (Sigma). A standard curve, constructed using known concentrations of bovine serum albumin (Sigma) (0.1ml 0, 0.25, 0.5, 1.0, 1.5 mg/ml solution), was added to 3ml of Bradford reagent. All solutions were incubated at room temperature for 45 minutes. A sample of each solution was added to a cuvette and absorbance was measured at 595nm using a spectrophotometer (WPA UV 1101). The standard curve was used to determine the unknown concentration of protein in the cell samples.

4.3.5 cAMP accumulation assay for activation of adenylyl cyclase

Materials

3-isobutyl-1-methylxanthine (Sigma-Aldrich, UK)

Forskolin (Sigma-Aldrich, UK)

direct cyclic AMP ELISA kit (ADI-900-066, EnzoLifeSciences, UK)

Methods

This assay measured cAMP accumulation in cells in response to incubation with GCGR agonists as a measure of activation of the G α s pathway. CHO-K1 cells overexpressing the human glucagon receptor (with and without RAMP2) were plated onto 48 well plates one day prior to assay at 100,000 cells/ml in 250 μ l of standard media (DMEM 6429, with L-glutamine, 4.5 g glucose, sodium pyruvate, Sigma-Aldrich, UK) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic (w/v) (5000 Units/ml Penicillin; 5000 μ g/ml Streptomycin, Sigma). On the following day, the media was removed and replaced with FCS-free DMEM media and cells incubated for 1 hour. An appropriate range of concentrations of peptide to be tested (glucagon, GLP-1,

oxyntomodulin and analogue G(X)) were made in serum free DMEM containing 100µM of IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich, UK), a phosphodiesterase inhibitor. The serum free media was aspirated carefully from each well and replaced with incubation media containing the test peptide. All concentrations were performed in duplicate. Forskolin (100µM), a direct adenylyl cyclase activator, was used as a positive control in 2 wells per cell line per assay (Seamon et al., 1981). The cells were incubated for exactly 30 minutes from this point. After the incubation, media was removed and replaced with 110ul lysis buffer (0.1M HCl with 0.5% Triton-X) and incubated for 10 minutes to allow sufficient cell lysis. The lysate, now containing accumulated cAMP, was stored at -20°C before assay by ELISA.

The lysate was diluted at a ratio of 1:10 and assayed using a direct cyclic AMP Enzyme-linked Immunosorbent assay (ELISA) kit (ADI-900-066, EnzoLifeSciences, UK) as described in the assay manual. Briefly, 50 µL of 'Neutralising Reagent' was added to each well except the total and blank wells. Provided standards (100µl) and samples (100µl) were added to appropriate wells before adding 50 µl of blue conjugate and 50 µl of the yellow antibody into each well. The plate was then sealed and left to incubate at room temperature for 2 hours on a plate shaker (~500 rpm). Contents were emptied from wells and washed three times with 400µl of wash buffer. 'Substrate solution' (200µl) was then added to each well and left to incubate on the bench top for 1 hour. After this incubation, 50µl of 'stop solution' was added into each well, and optical density was read at 405 nm on a plate reader (Multiskan RC 381, Labsystems, MA, USA). Concentrations of cAMP were calculated from a standard curve and plotted using Prism 5.01 program (GraphPad Software Inc. San Diego, USA). cAMP response was then corrected for well protein levels (a surrogate marker of cell number) as described in 4.3.4.1 and expressed as a percentage of forskolin response. The maximal response (Emax) and the half-maximal effective concentrations (EC50) were then calculated and compared for each peptide tested between CHO-K1-GCGR and CHO-K1-GCGR-RAMP2 cells. EC50 values were calculated using the following regression fit line:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})$$

where Y= cAMP response and X=agonist concentration.

4.3.6 Intracellular calcium flux assay

Materials

ATP (Sigma-Aldrich, UK)

DiscoverX Ca NW^{PLUS} Assay Kit (90-0091, DiscoverX Corporation Ltd, Birmingham, UK)

Method

Intracellular calcium flux was assayed as a measure of activation of the Gq pathway. The DiscoverX Ca NW^{PLUS} Assay Kit (90-0091, DiscoverX Corporation Ltd, Birmingham, UK) was used as per the manufacturer's protocol to detect changes in intracellular calcium in CHO-K1 cells overexpressing the human glucagon receptor (with and without RAMP2) in response to glucagon, GLP-1, oxyntomodulin and analogue G(X).

CHO-K1 cells overexpressing the human glucagon receptor (with and without RAMP2) were plated onto 96 well plates one day prior to assay at 50,000 cells/well in 200 µl of standard media (DMEM 6429, with L-glutamine, 4.5 g glucose, sodium pyruvate, Sigma-Aldrich, UK) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic (w/v) (5000 Units/ml Penicillin; 5000 µg/ml Streptomycin, Sigma). On the following day, the media was removed and replaced with 75 µl Ca NW^{PLUS} working reagent. The cells were then incubated for 1 hour at 37°C. Agonists of increasing concentration were prepared in a designated reagent 96 well plate. All concentrations were performed in duplicate. Adenosine triphosphate (ATP) was used as a positive control in 2 wells per cell line per assay. The reagent plate and cell plate were both placed into the fluorescent microplate reader (NOVOstar, BMG Labtech Ltd, Aylesbury, UK) and 25 µl of glucagon (or related peptide) was applied from the reagent plate to the cell plate via an integrated transfer pipettor. Upon stimulation, the receptor signaled release of intracellular calcium, resulting in an increase of dye fluorescence. Fluorescence signal was measured from 5 seconds prior to 30 seconds post injection of agonist.

Calcium response was expressed as a percentage of ATP response. The maximal response (Emax) and the half-maximal effective concentrations (EC50) were then calculated and compared for each peptide tested between CHO-K1-GCGR and CHO-K1-GCGR-RAMP2 cells. EC50 values were calculated using the following regression fit line:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})$$

where Y= calcium response and X=agonist concentration.

4.3.7 β -Arrestin recruitment assay

Materials

PathHunter™ eXpress CHO-K1 GCGR β -Arrestin GPCR assay (DiscoverX Corporation Ltd, Birmingham, UK)

Method

PathHunter™ eXpress CHO-K1 GCGR β -Arrestin GPCR assay (DiscoverX Corporation Ltd, Birmingham, UK) was used to determine the effect of RAMP2 on the potency of GCGR ligands for recruitment of β -Arrestin-1 to the GCGR. The CHO-K1-D-GCGR cells are engineered to detect the interaction of β -Arrestin-1 with the activated GCGR using β -galactosidase (β -gal) enzyme fragment complementation. In this system, the GCGR is fused in frame with the small, 42 amino acid fragment of β -gal called ProLink™ and co-expressed in cells stably expressing a fusion protein of β -Arrestin and the larger, N-terminal deletion mutant of β -gal (called enzyme acceptor or EA). Activation of the GCGR stimulates binding of β -Arrestin to the ProLink-tagged GPCR and forces complementation of the two enzyme fragments, resulting in the formation of an active β -gal enzyme. This action leads to an increase in enzyme activity that can be measured using PathHunter® Detection Reagents which produce a chemiluminescent signal in the presence of the active β -gal enzyme. This chemiluminescent signal was detected using the LumiLITE™ microplate reader.

CHO-K1-D-GCGR cells were stably transfected with and without RAMP2 using antibiotic selection as described in Section 4.3.3. The β -Arrestin assay was carried out for both cell lines in parallel. Briefly, cells were re-suspended in the provided culture media and plated at 100 μ l per well into a 96-well tissue culture plate. The plates were then incubated at 37°C and 5% CO₂ for 24 hours. Increasing concentrations of glucagon (or GLP-1, oxyntomodulin, G(X)) were prepared in culture media at 11x the final screening concentrations for a 12 point dose response. Each point was performed in duplicate. 10 μ l of each peptide concentration were then added to the cell culture plate and incubated

for 90 minutes at 37°C and 5% CO₂. After 90 minutes incubation, 55µl of the provided PathHunter™ detection reagents was added to each well and the microplate was incubated at room temperature (23°C) for a further 60 minutes. Microplates were then read using the LumiLITE™ microplate reader (DiscoverX Corporation Ltd, Birmingham, UK). Dose-responses were plotted using Prism 5.01 program (GraphPad Software Inc. San Diego, USA) and EC₅₀ and Emax calculated using the following regression fit line:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope})})$$

where Y= β-Arrestin recruitment and X=agonist concentration.

4.3.8 SiRNA knockdown

Materials

SiRNA (fully deprotected and desalted, Sigma, UK)

siPORT™ NeoFX™ (Ambion)

Method

SiRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 and CHO-K1-D-GCGR-RAMP2 cells was performed using pooled siRNA to RAMP2 previously validated by Albertin et al to knock down the human AM1 receptor in human umbilical vein endothelial cells (Albertin et al., 2006).

Cells were maintained as described previously in section 4.3.3.9. Prior to transfection with siRNA, cells were pelleted, resuspended in normal growth medium and maintained at 37°C while the transfection complexes were prepared. SiRNA complexes (fully deprotected and desalted, Sigma, UK), added in a single pool (containing 4 duplexes) at a final concentration of 10 nM and 50 nM, were used for the transfection with a lipid-based agent siPORT™ NeoFX™ (Ambion). SiPORT NeoFX was diluted (1:20) into serum-free medium. The diluted siPORT NeoFX and RNAs were combined (1:1) and incubated for 10 min at room temperature to allow the formation of transfection complexes. The complexes (200 µl per well) were then dispensed into empty wells of a 6-well culture plate. The cells were pipetted into the culture plate wells containing the transfection complexes (2.3 ml of cell suspension containing 150,000 cells /well). The effects of the transfected RNA were assessed 24 hours later.

To assess effective knockdown, gene expression was quantified as described in section 4.3.3.10 involving cell detachment, RNA extraction, reverse transcription and qPCR. For these experiments, four control wells were included: two wells containing CHO-K1-GCGR-RAMP2 without siRNA and two wells containing scrambled siRNA. The same format was used for siRNA knockdown in CHO-K1-D-GCGR-RAMP2 cells. SiRNA sequences can be found in table 4.1.

To assess whether knockdown of RAMP2 affected GCGR cell signalling, the siRNA knockdown protocol was followed as above except that it was carried out in a 96-well plate with the volumes of siRNA, SiPORT NeoFX and cells adjusted as follows:

	Volume per well
siRNA	10 µl
SiPORT NeoFX	10 µl
Cell suspension	80 µl (6000 cells)

The effect of RAMP2 on cAMP response, calcium flux and β -Arrestin recruitment was assayed 24 hours later.

Sequence range		siRNA sequence
RAMP2-siRNA(1) 545-563	Sense	5'-CCUCAUCACUCUUGUAGUA-3'
	Antisense	5'-UACUACAAGAGUGAUGAGG-3'
RAMP2-siRNA(2) 290-308	Sense	5'-UCAAAUGGAUCCUAUCGAA-3'
	Antisense	5'-UUCGAUAGGAUCCAUUUGA-3'
RAMP2-siRNA(3) 358-376	Sense	5'-GAGAUUGCCUGGAGCACUU-3'
	Antisense	5'-AAGUGCUC CAGGCAAUCUC-3'
RAMP2-siRNA(4) 417-435	Sense	5'-GAGAGGAUCAUCUUUGAGA-3'
	Antisense	5'-UCUCAAGAUGAUCCUCUC-3'
RAMP2-siRNA(1) SCRAMBLE	Sense	5'-ACCAUGCCUUUAUUCGCUA-3'
	Antisense	5'-UAGCGAAUAAAGGCAUGGU-3'
RAMP2-siRNA(2) SCRAMBLE	Sense	5'-UAAAGUACGUUCUCGUGUA-3'
	Antisense	5'-UACACGAGAACGUACUUUA-3'

Table 4.1 SiRNA sequences (fully deprotected and desalted, Sigma, UK)

4.3.9 Confocal microscopy

Materials

Lipofectamine 2000 (Life Technologies Ltd, Paisley, UK)

GFP-tagged GCGR (Origene, Rockville, MD, USA)

CFP-tagged RAMP2 (Tebu-bio Ltd, Peterborough, UK)

paraformaldehyde (PFA) (Sigma, UK)

Vectashield (Vector Laboratories Ltd, Peterborough, UK)

Method

In order to assess the sub-cellular distribution of RAMP2 and GCGR, HEK293 cells were stably transfected with C-terminal green fluorescent protein (GFP)-tagged GCGR with/without cyan fluorescent protein (CFP)-tagged RAMP2 and visualized using confocal microscopy.

HEK293 cells were stably transfected with C-terminal GFP-tagged GCGR (Origene, Rockville, MD, USA) using Lipofectamine 2000 (Life Technologies Ltd, Paisley, UK) as per the manufacturer's protocol. Briefly, cells were seeded in 2ml of media per well in a 6-well plate so that they were 70-90% confluent on the day of transfection. 7 µl of Lipofectamine reagent was diluted in 193 µl of Opti-MEM medium (Gibco, UK) in tube 1 and 2 µg of plasmid DNA containing GFP-tagged GCGR were diluted in Opti-MEM medium (final volume 200 µl) in tube 2. Tubes 1 and 2 were combined and incubated at room temperature for 20 minutes. Meanwhile, media was aspirated from the cells and 1.6mL of Opti-MEM medium was added to each well. 400 µl of Lipofectamine-DNA mixture was then added to each well and swirled gently. Cells were incubated at 37°C overnight and the cell media was replaced with the cells' usual media. After selection with G418 (800 ug/ml) cells were then FACS-sorted (BD FACSAria IIu) to produce single cell colonies with known GFP signal, and a clonal population with above average GFP signal was used for subsequent experiments.

GFP-GCGR expressing HEK293 cells were seeded on sterile coverslips coated with poly-L-lysine (MW 70,000-120,000: 0.01%, Sigma) on a 6 well plate. Using the same Lipofectamine protocol, the cells were transiently transfected with/without C-

terminally CFP-tagged RAMP2 (Tebu-bio Ltd, Peterborough, UK). The following day, they were fixed with 2% paraformaldehyde (PFA) (Sigma, UK) and mounted with Vectashield (Vector Laboratories Ltd, Peterborough, UK) Slides were visualised with confocal microscopy using a 405 nm laser to capture CFP excitation and 488 nm for GFP. Images were captured using a Crest X-Light spinning disk module coupled to a Nikon Eclipse Ti microscope, 63x 1.4 NA oil immersion objective and Orca-Flash4.0 CMOS.

4.3.10 Statistical analysis

Comparison of RAMP2 expression between two groups was performed by an unpaired t-test whereas a one-way ANOVA was used to compare more than two groups. Emax and EC50 values were compared by paired t-test, where values were compared for individual experiments in which RAMP +ve and -ve cells were examined in parallel. Prism Version 5.01 (GraphPad Software Inc. San Diego, USA) was used for statistical analysis. In all cases, values of $p < 0.05$ were considered statistically significant.

4.4 Results

4.4.1 Confirmation of plasmid propagation

In producing large scale replication of the pCMV6-AC-RAMP2 plasmid, it was important to confirm that the purified DNA did indeed contain the plasmid. This was performed by restriction digest and gel electrophoresis. This is shown in figure 4.3. In the uncut control, only one band was seen at 5.8 kb which indicated the intact plasmid. In the cut sample, two bands were visualised: one of 5.1kb which represented the cleaved plasmid and the other of approximately 750 bp which represented the RAMP2 insert.

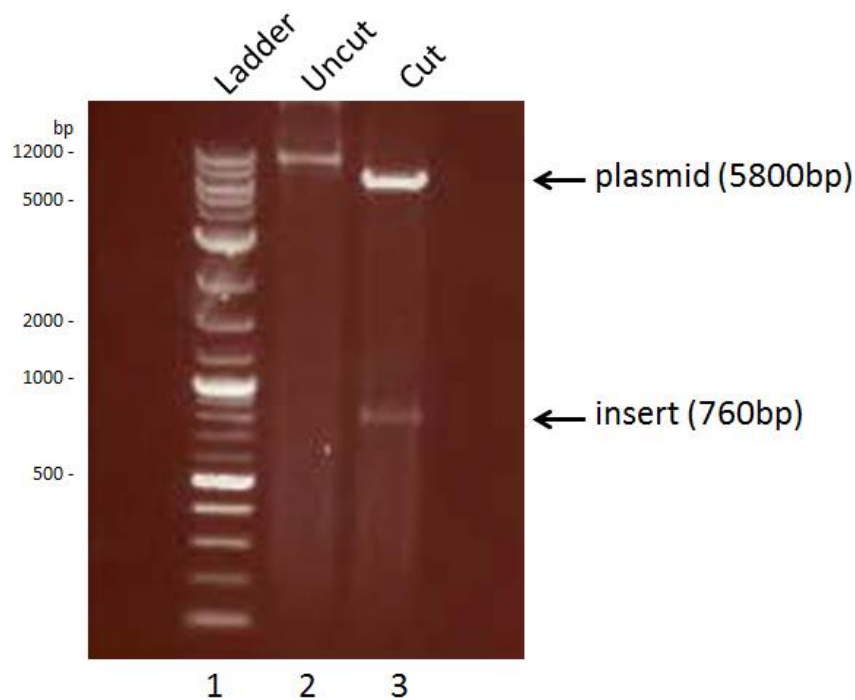


Figure 4.3: Electrophoresis of DNA fragments. After restriction digestion, the DNA was size fractionated by electrophoresis on an agarose gel to confirm if an insert was present. Lane 1: 1kb plus ladder; lane 2: uncut plasmid; lane 3: cut plasmid.

4.4.2 Confirmation of transfection of CHO-K1-GCGR and CHO-K1- D - GCGR cells with RAMP2

Successful transfection into CHO-K1-GCGR cells of the pCMV6-AC-RAMP2 plasmid was confirmed by qPCR. Subsequently, a second CHO-K1 cell line expressing the GCGR containing the β -arrestin recruitment reporter signal (CHO-K1-D-GCGR) was also transfected with RAMP2. Figure 4.4 demonstrates that transfection was successful for both cell lines and that cells transfected with the pCMV6-AC-RAMP2 plasmid did indeed express RAMP2 while control cells did not.

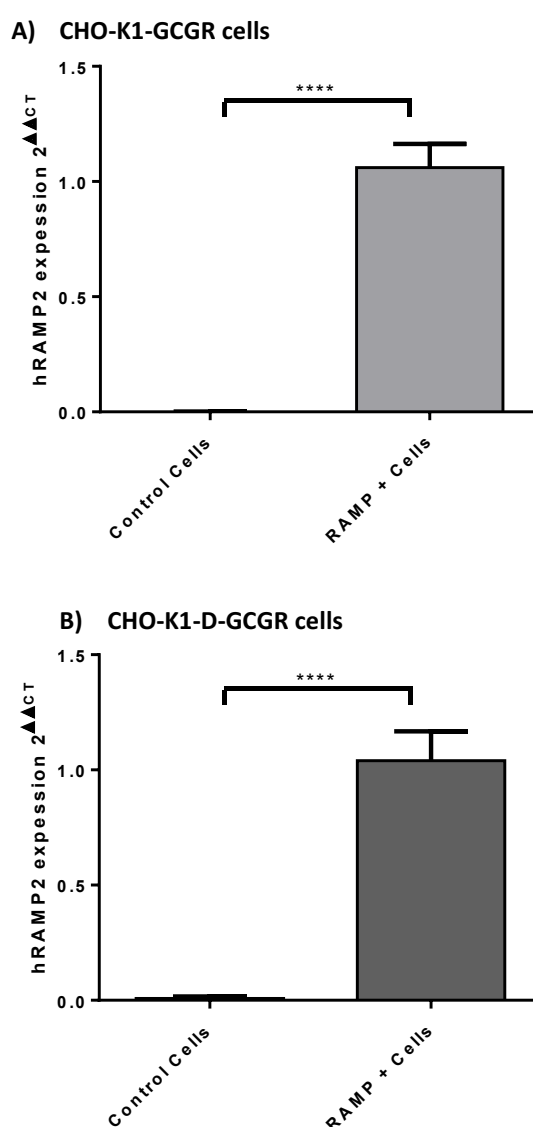


Figure 4.4 Expression of RAMP2 in control cells and cells transfected with the pCMV6-AC-RAMP2 plasmid in two different cell lines A) CHO-K1-GCGR cells and B) CHO-K1-D-GCGR cells. **p<0.0001. Errors shown are ±SEM.**

4.4.3 Whole cell binding assays

Glucagon bound to the GCGR with an IC₅₀ of 1.43 nM. This was not significantly different when the GCGR was co-expressed with RAMP2 (figure 4.5A). As expected, GLP-1 had poor affinity for the GCGR (figure 4.5B). Oxyntomodulin and analogue G(X) had a 7-fold and 2.5 fold lower affinity to the GCGR than the native peptide respectively (figure 4.5C and D). Similar to glucagon, the presence of RAMP2 had no effect on the binding affinity at the GCGR for GLP-1, oxyntomodulin or analogue G(X). IC₅₀ values for all ligands tested are displayed in table 4.2. However, when total glucagon binding to the GCGR, detected using an excess of I-125-glucagon, was compared in RAMP2 positive and negative cells, it was found that the total binding was 10-fold lower in the presence of RAMP2 (see figure 4.6A). This was despite the protein content, i.e. the number of cells, being similar in both groups (see figure 4.6B).

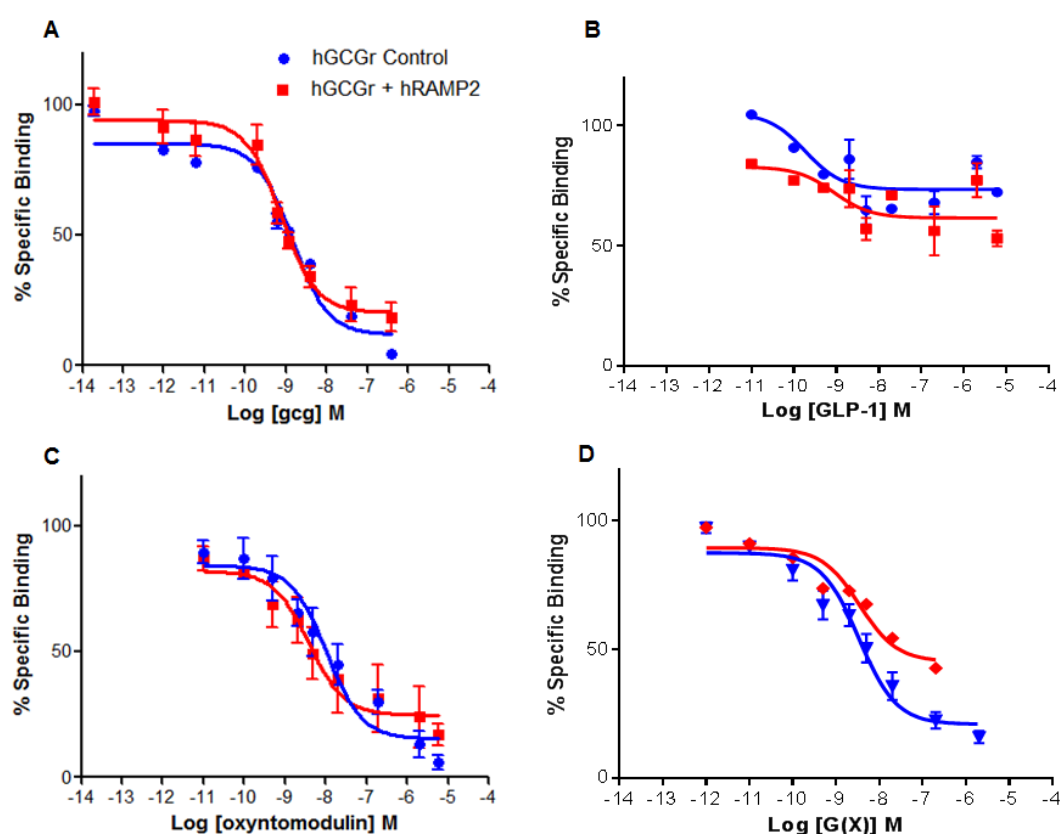


Figure 4.5: Binding affinities of A) glucagon, B) GLP-1, C) oxyntomodulin and D) analogue G(X) to the human glucagon receptor. Whole CHO-K1-GCGR cells $\pm$ RAMP2 were used. I¹²⁵-glucagon was used as the competing peptide in all assays and IC₅₀ values were calculated as a mean of four separate experiments (except for GLP-1 where n=2) with each peptide concentration performed in duplicate or triplicate during an individual experiment. Errors shown are $\pm$ SEM.

IC ₅₀	GCGR Control	GCGR + RAMP2
Glucagon	1.403 nM $\pm$ 0.21	0.768 nM $\pm$ 0.15
GLP-1	>10000 nM	>10000 nM
OXM	10.43 nM $\pm$ 2.59	3.873 nM $\pm$ 0.93
G(X)	3.381 nM $\pm$ 1.07	3.984 nM $\pm$ 1.81

Table 4.2: Binding affinities of A) glucagon, B) GLP-1, C) oxyntomodulin and D) analogue G(X) to the human glucagon receptor. Whole CHO-K1-GCGR cells $\pm$ RAMP2 were used. I¹²⁵-glucagon was used as the competing peptide in all assays and IC₅₀ values were calculated as a mean of four separate experiments (except for GLP-1 where n=2) with each peptide concentration performed in duplicate or triplicate during an individual experiment. Errors shown are $\pm$ SEM

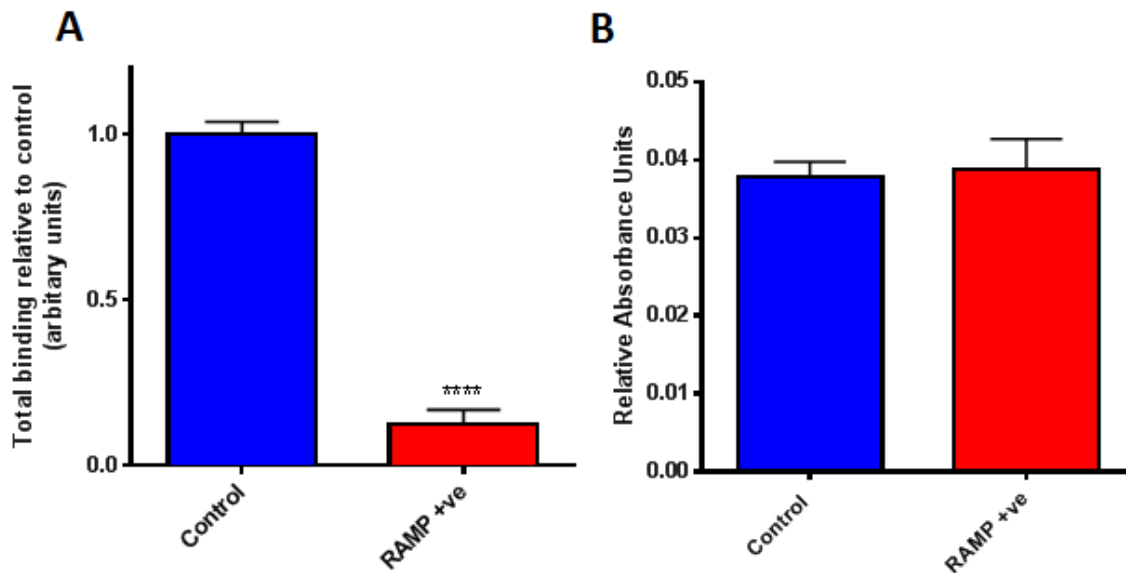


Figure 4.6 A: Total binding of I-125-glucagon to the GCGR in whole CHO-K1-GCGR cells $\pm$ RAMP2 (p<0.0001). B: The protein content performed by Bradford assay (used here as a surrogate marker for the number of cells) for whole CHO-K1-GCGR cells $\pm$ RAMP2. Errors shown are $\pm$ SEM.

4.4.4 cAMP accumulation assay for activation of adenylyl cyclase

In order to assess whether RAMP2 affected the $G_{\alpha s}$ pathway, cAMP accumulation was measured in its presence/absence (Figure 4.7). In control cells, the highest concentrations of peptide resulted in cAMP accumulation lower than the EMax which is a well described effect known as desensitisation. In the presence of RAMP2, glucagon, oxyntomodulin and analogue G(X) increased the EC50 i.e. the presence of RAMP2 reduced the potency of these ligands for GCGR. When the GCGR was stimulated by oxyntomodulin or analogue G(X), the EMax (efficacy) was increased in the presence of RAMP2. It was not possible to calculate EC50 for GLP-1 as EMax was not achieved at the doses used. EC50 and EMax data are summarised in table 4.3.

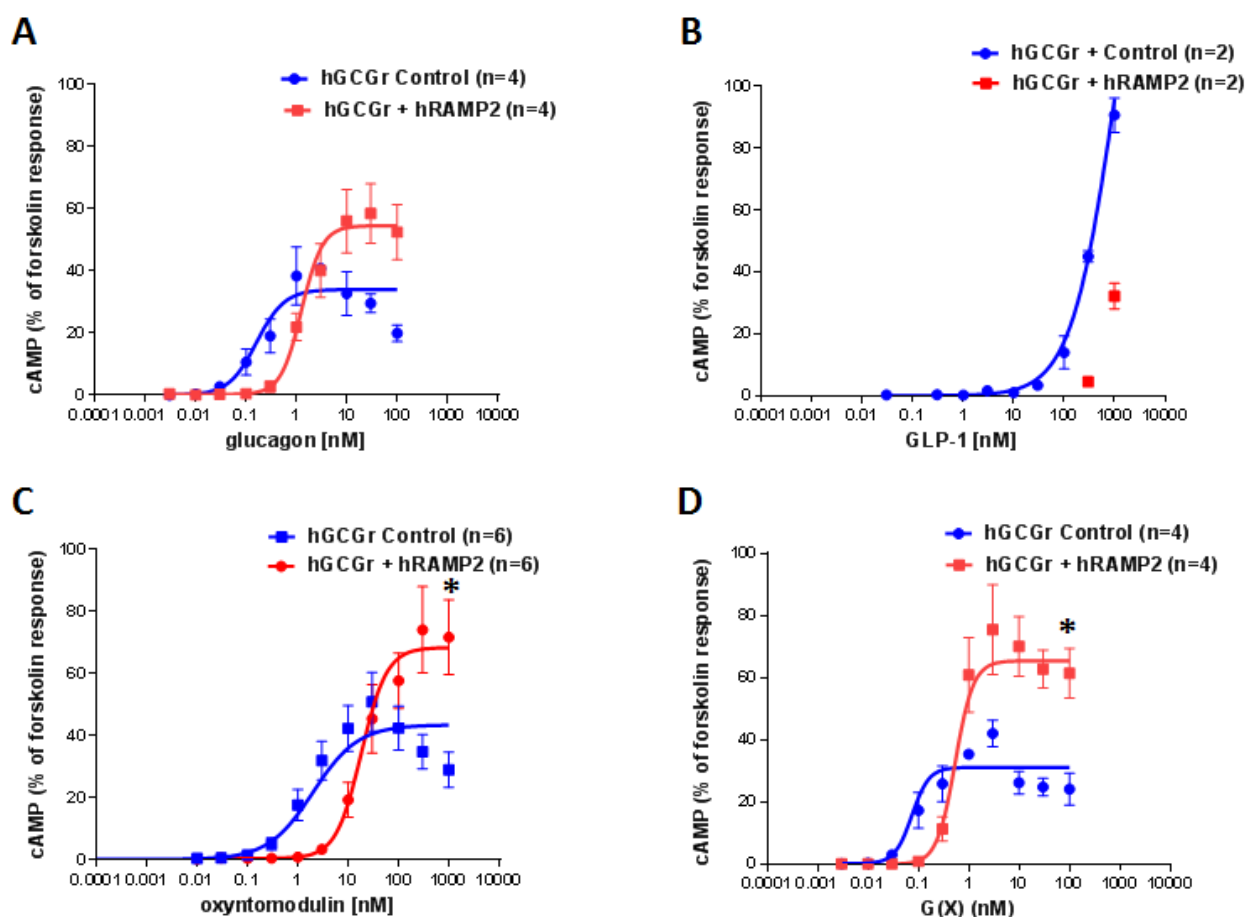


Figure 4.7: Human glucagon receptor-mediated cAMP accumulation in CHO-K1-GCGR cells $\pm$ RAMP2 by ligands A) glucagon, B) GLP-1, C) oxyntomodulin and D) analogue G(X). Each peptide concentration was tested in duplicate or triplicate in each experiment. Values calculated as a mean from a minimum of four separate experiments (unless stated otherwise). * $p < 0.05$ comparing the EMax for CHO-K1-GCGR cells $\pm$ RAMP2. Errors shown are $\pm$ SEM.

		CHO-K1-GCGR cells	CHO-K1-GCGR cells +RAMP2
Glucagon	EC50 (nM)	0.161 ± 0.063	1.263 ± 0.289*
	E_{max} (%)	34.04 ± 6.897	54.50 ± 9.781
GLP-1	EC50 (nM)	N/A	N/A
	E_{max} (%)	N/A	N/A
Oxyntomodulin	EC50 (nM)	1.089 ± 0.382	12.97 ± 8.544*
	E_{max} (%)	34.69 ± 6.815	46.23 ± 7.409*
Analogue G(X)	EC50 (nM)	0.074 ± 0.056	0.538 ± 0.065*
	E_{max} (%)	31.11 ± 3.578	65.43 ± 7.027*

Table 4.3. Summary of EC50 and E_{Max} cAMP accumulation data for glucagon, GLP-1, oxyntomodulin and analogue G(X) at the glucagon receptor. EC50 is defined as the concentration of agonist required to cause 50% of the maximal possible effect of that agonist. E_{max} is the maximal cAMP response of the agonist expressed as a percentage of forskolin response. Values calculated as a mean from a minimum of four separate experiments (except for GLP-1 n=2). Values shown as mean ± SEM. * p<0.05 comparing CHO-K1-GCGR cells ± RAMP2.

4.4.5 Intracellular calcium flux

To assess the effect of RAMP2 on the Gq pathway, intracellular calcium flux was measured in real time. Figure 4.8 shows the effect of injection of increasing concentrations of glucagon on calcium flux in the absence (A) and presence (B) of RAMP2. For glucagon and oxyntomodulin, the calcium response was attenuated when cells expressing the glucagon receptor were co-expressed with RAMP2, as demonstrated by a significantly lower Emax (Figure 4.9). This also appears to be true for G(X) however, as maximal calcium response was not achieved with cells expressing GCGR alone, Emax could not be readily calculated. EC50 was unchanged in the presence of RAMP2 for all ligands (Table 4.4).

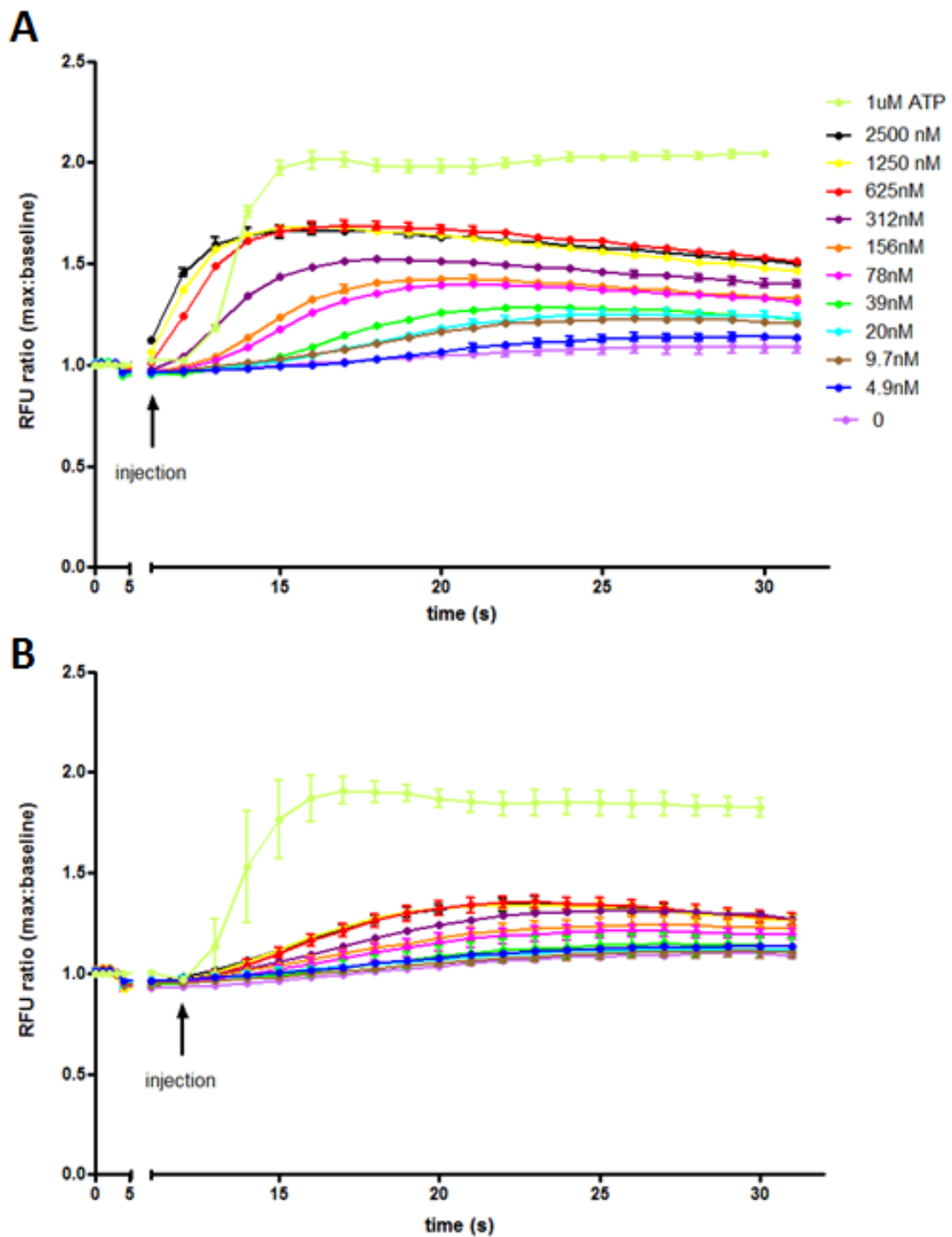


Figure 4.8. Intracellular calcium flux in response to varying doses of glucagon in real-time in CHO-K1-GCGR cells (A) without RAMP2 and (B) with RAMP2. Glucagon was injected into the cell media at time 5s. The fluorescence (measured in relative fluorescence units (RFU)), a function of intracellular calcium concentration, was monitored for 30s thereafter. 1uM ATP was used as a positive control.

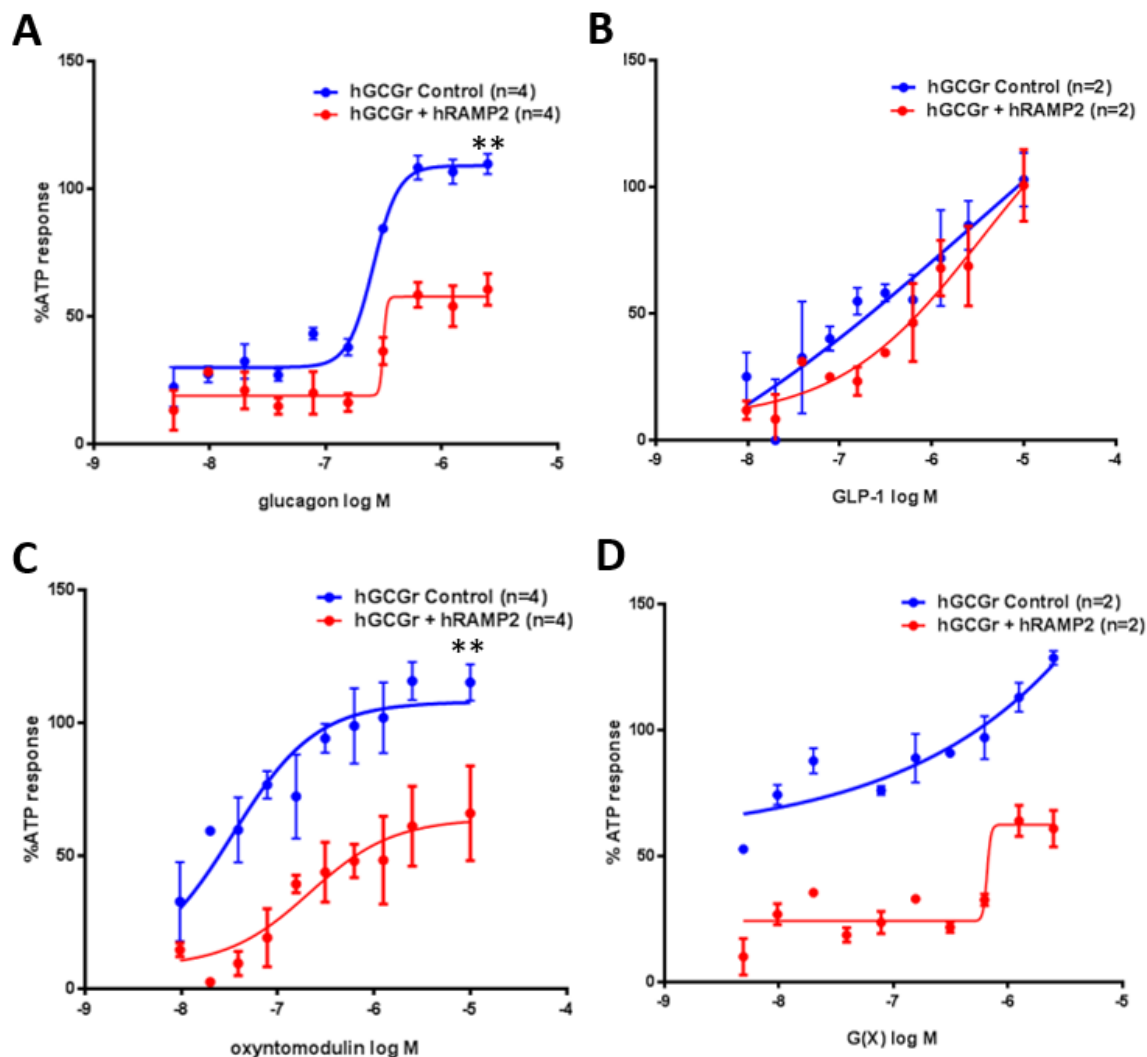


Figure 4.9: Human glucagon receptor-mediated calcium flux in CHO-K1-GCGR cells $\pm$ RAMP2 by ligands A) glucagon, B) GLP-1 and C) oxyntomodulin and D) analogue G(X). Each peptide concentration was tested in duplicate or triplicate in each experiment. The maximal RFU ratio for each peptide concentration is plotted as a percentage of maximal ATP response. Values calculated as a mean from a minimum of four separate experiments (unless stated otherwise). ** $p < 0.01$ comparing the EMax for CHO-K1-GCGR cells $\pm$ RAMP2. Errors shown are $\pm$ SEM.

		CHO-K1-GCGR cells	CHO-K1-GCGR cells +RAMP2
Glucagon	EC50 (nM)	256.5 ± 27.46	314.1 ± 37.03
	E_{max} (%)	109.0 ± 2.215	57.7 ± 1.313**
GLP-1	EC50 (nM)	N/A	N/A
	E_{max} (%)	N/A	N/A
Oxyntomodulin	EC50 (nM)	109.6 ± 11.5	156.8 ± 43.21
	E_{max} (%)	108 ± 9.28	64 ± 12.3**
Analogue G(X)	EC50 (nM)	N/A	656.6 ± 35.0
	E_{max} (%)	N/A	62.5 ± 6.06

Table 4.4. Summary of EC50 and E_{Max} calcium data for glucagon, GLP-1, oxyntomodulin and analogue G(X) at the glucagon receptor. EC50 is defined as the concentration of agonist required to cause 50% of the maximal possible effect of that agonist. E_{max} is the maximal calcium response of the agonist expressed as a percentage of ATP response. Values calculated as a mean from a minimum of four separate experiments (except for GLP-1 n=2). Values shown as mean ± SEM. ** p<0.01 comparing CHO-K1-GCGR cells ± RAMP2.

4.4.6 β -Arrestin recruitment

For all ligands (glucagon, GLP-1, oxyntomodulin and analogue G(X)), β -Arrestin recruitment did not occur in the cells expressing both GCGR and RAMP2 (see Figure 4.10).

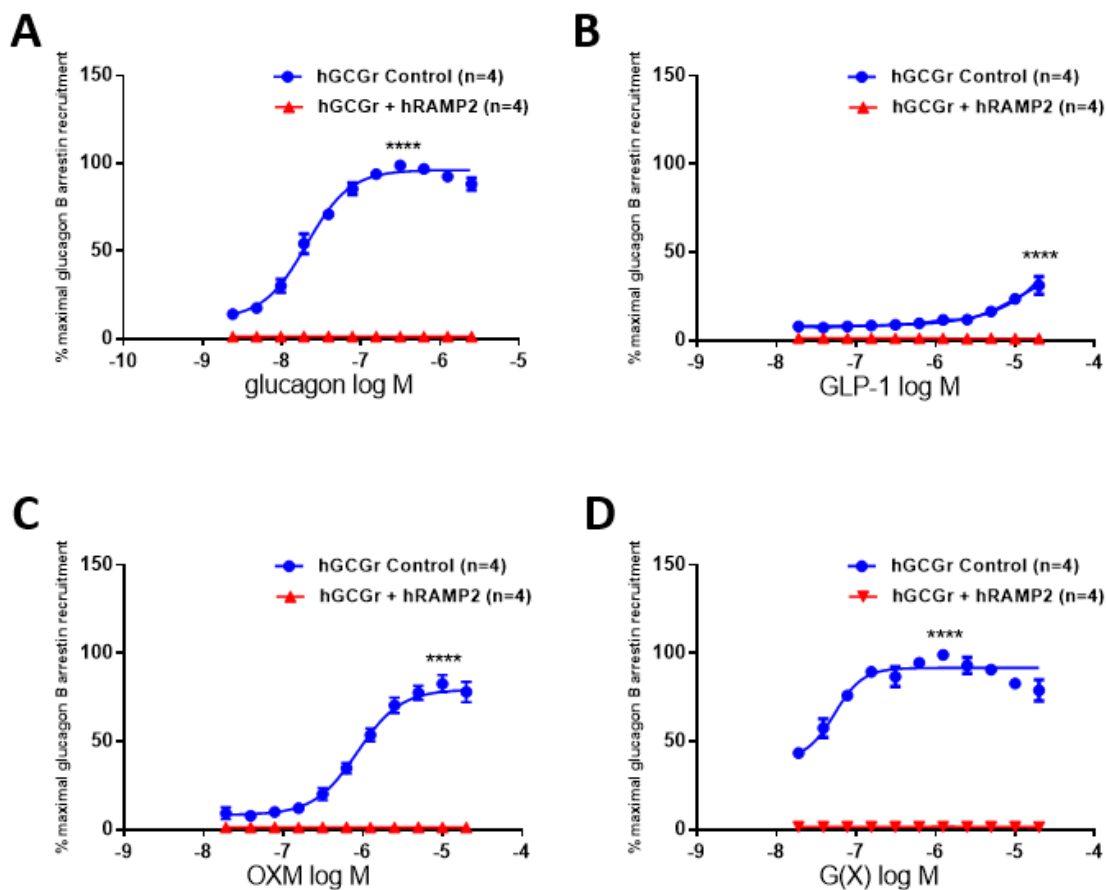


Figure 4.10: Human glucagon receptor-mediated β -Arrestin recruitment in CHO-K1-D-GCGR cells $\pm$ RAMP2 by endogenous ligands A) glucagon, B) GLP-1 and C) oxyntomodulin and D) analogue G(X). Each peptide concentration was tested in duplicate or triplicate in each experiment. Results are expressed as a percentage of maximal glucagon-mediated β -Arrestin recruitment. Values calculated as a mean from a minimum of four separate experiments. ** p<0.0001 comparing the EMax for CHO-K1-D-GCGR cells $\pm$ RAMP2. Errors shown are $\pm$ SEM.**

4.4.7 SiRNA knockdown

Efficient siRNA knockdown of RAMP2 was achieved with both 10 nM and 50 nM siRNA pools (Figure 4.11). This was confirmed in both cell lines (CHO-K1-GCGR-RAMP2 and CHO-K1-D-GCGR-RAMP2). The CHO-K1-GCGR-RAMP2 cell line was used for cAMP accumulation and calcium flux experiments while CHO-K1-D-GCGR-RAMP2 cells, containing the β -arrestin recruitment reporter signal, were used in the β -arrestin recruitment assays. As expected, scrambled siRNA did not affect RAMP2 gene expression.

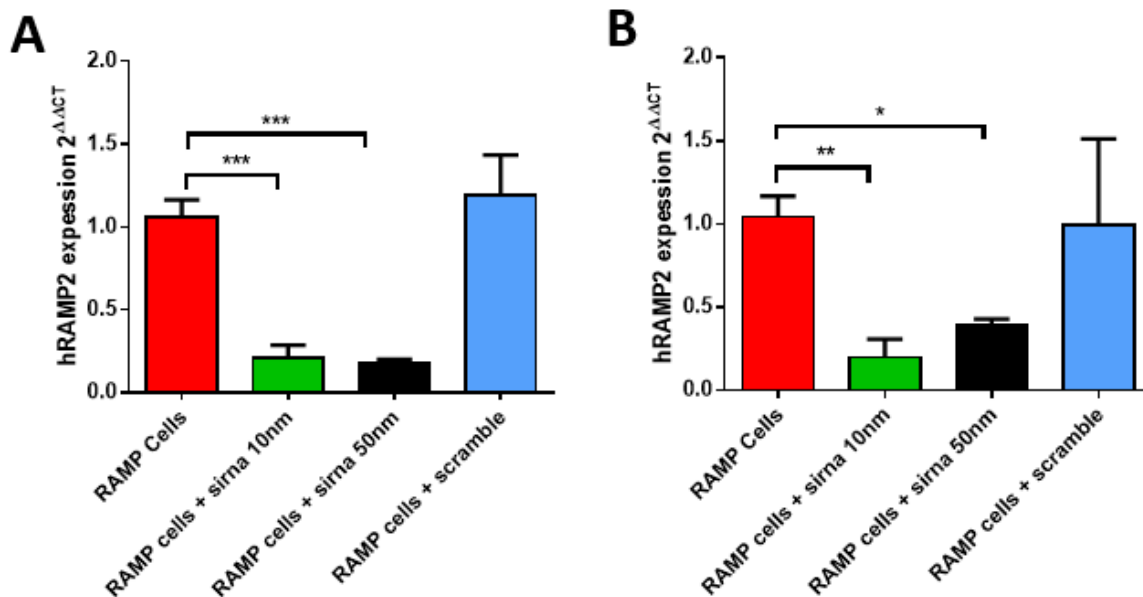


Figure 4.11. Effect of siRNA knockdown of RAMP2 in two different cell lines A) CHO-K1-GCGR-RAMP2 cells and B) CHO-K1-D-GCGR-RAMP2 cells. The CHO-K1-GCGR-RAMP2 cell line was used for cAMP accumulation and calcium flux experiments while CHO-K1-D-GCGR-RAMP2 cells, containing the β -arrestin recruitment reporter signal, were used in the β -arrestin recruitment assays. Values calculated as a mean from a minimum of two separate experiments. *p<0.05 **p<0.01 *p<0.001. Errors shown are $\pm$ SEM.**

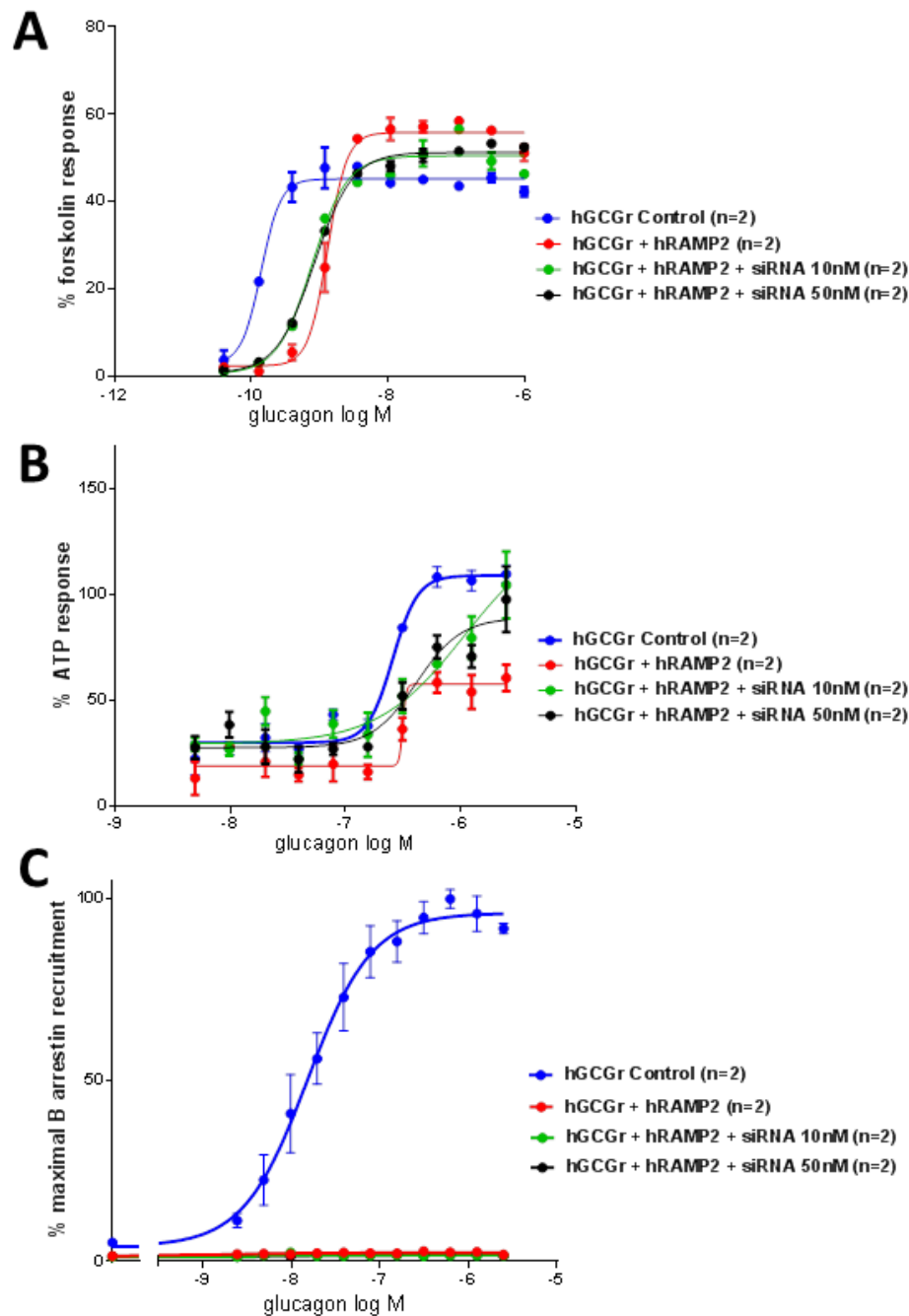


Figure 4.12. Effect of siRNA knockdown of RAMP2 on A) cAMP accumulation B) calcium flux and C) β -arrestin recruitment in response to glucagon. The CHO-K1-GCGR $\pm$ RAMP2 cell lines were used for cAMP accumulation and calcium flux experiments while CHO-K1-D-GCGR $\pm$ RAMP2 cells, containing the β -Arrestin recruitment reporter signal, were used in the β -Arrestin recruitment assays. Each peptide concentration was tested in duplicate or triplicate in each experiment. Values calculated as a mean from a minimum of two separate experiments. Errors shown are $\pm$ SEM.

SiRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 cells resulted in partial restoration of cAMP EC₅₀ and E_{max} to levels seen with control cells (CHO-K1-GCGR cells) (Figure 4.12A). A similar finding was demonstrated for calcium flux (Figure 4.12B). However, there was no restoration of β -arrestin recruitment in the presence of 10 or 50 nM siRNA to RAMP2 (Figure 4.12C). To further investigate this discrepancy between cell lines in responsiveness to siRNA knockdown, the gene expression level of RAMP2 was compared between CHO-K1-GCGR-RAMP2 and CHO-K1-D-GCGR-RAMP2 cells (Figure 4.13). It was found that RAMP2 expression was forty-two fold higher in CHO-K1-D-GCGR-RAMP2 cells compared to CHO-K1-GCGR-RAMP2 cells, suggesting that even with knock down of RAMP2 by siRNA, sufficient RAMP2 mRNA remained to suppress β -arrestin recruitment

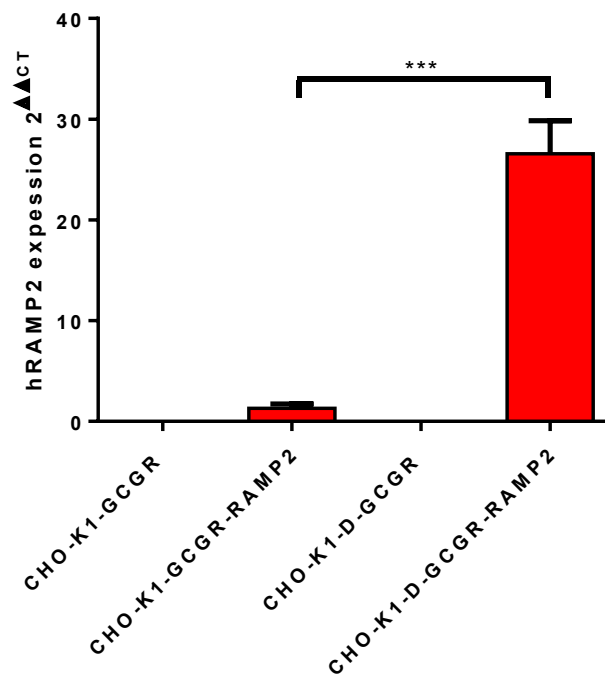


Figure 4.13. Gene expression of RAMP2 in CHO-K1-GCGR, CHO-K1-GCGR-RAMP2, CHO-K1-D-GCGR and CHO-K1-D-GCGR-RAMP2 cell lines. CHO-K1-GCGR±RAMP2 cells were used for cAMP accumulation and calcium flux experiments while CHO-K1-D-GCGR±RAMP2 cells, containing the β -Arrestin recruitment reporter signal, were used in the β -Arrestin recruitment assays. Values calculated as a mean from a minimum of two separate experiments. ***p<0.001. Errors shown are $\pm$ SEM.

4.4.8 Confocal microscopy

Visualisation of the cells using confocal microscopy revealed that all cells expressed GFP-GCGR. This was expected as these cells had been stably transfected. Approximately 20% of cells also expressed CFP-RAMP2 which reflected the transient transfection. Where both GCGR and RAMP2 were co-expressed, they colocalised to a very high degree (see Figure 4.14).

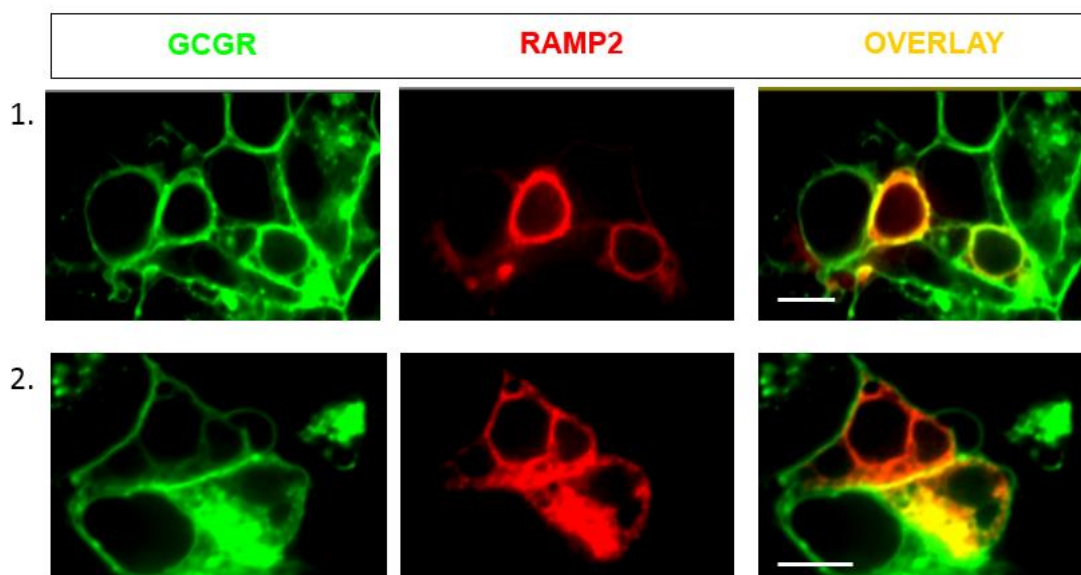


Figure 4.14. The GCGR and RAMP2 colocalise. Cells expressing GFP-GCGR (left) are excited by the 488 nm laser shown in green. Visualising the same two groups of cells (1 and 2), cells expressing CFP-RAMP2 (middle) are excited by the 405 nm laser shown in red. Cells expressing both GFP-GCGR and CFP-RAMP2 (right) are shown in yellow. Rows 1 and 2 are examples of two different groups of cells and both serve to show the same finding. The figure shown is of nonpermeabilized cells and is representative of at least two independent experiments.

In order to be certain that GFP was not also being excited by the 405 nm laser, cells that were excited by the 488 nm laser (used to visualise GFP) were then excited by the 405 nm laser (used to visualise CFP). No signal was detected with the 405 nm laser indicating that GFP was not also being excited by the 405 nm laser and that CFP could reliably be measured using the 405 nm laser (see figure 4.15).

In the presence of RAMP2, GCGR appeared to be more internalized as the GFP signal line around the cells appeared thicker in presence of RAMP2 (Figure 4.16). When examined quantitatively through measurement of GFP intensity per pixel at the membrane, less GCGR was expressed at the membrane in cells expressing RAMP2 compared to RAMP2 negative cells ($p < 0.01$) (Figure 4.17).

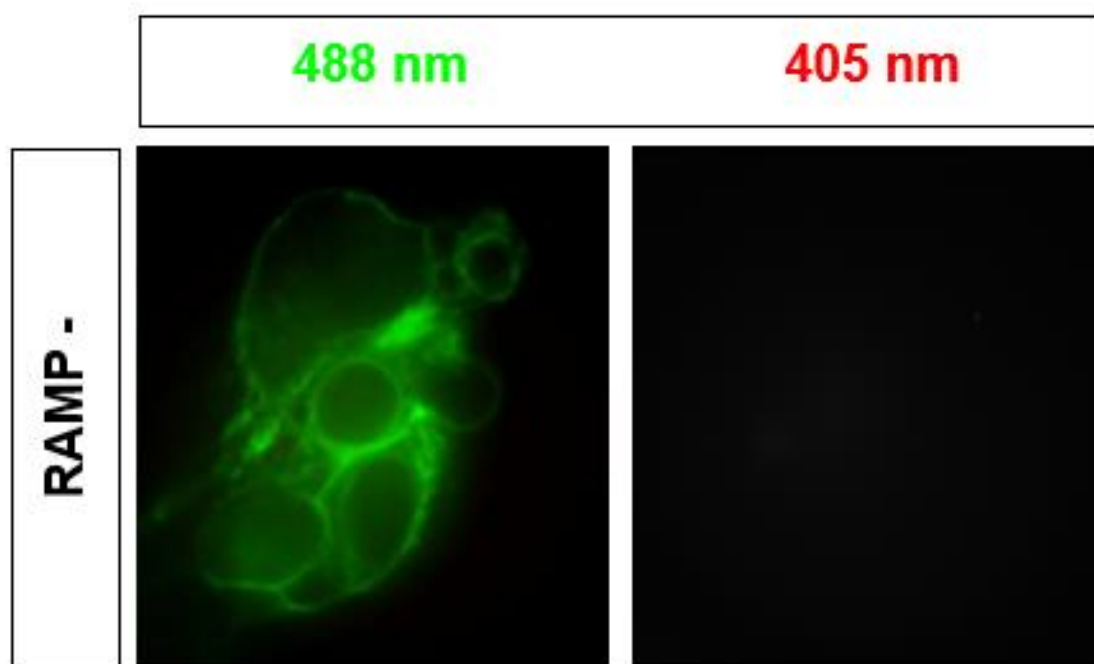


Figure 4.15: A negative control indicating that the 405 nm laser did not excite GFP and could be reliably used to measure CFP intensity. On the left, the 488 nm laser is used to excite GFP. On the right, the 405 nm laser is used with no resulting excitation of GFP.

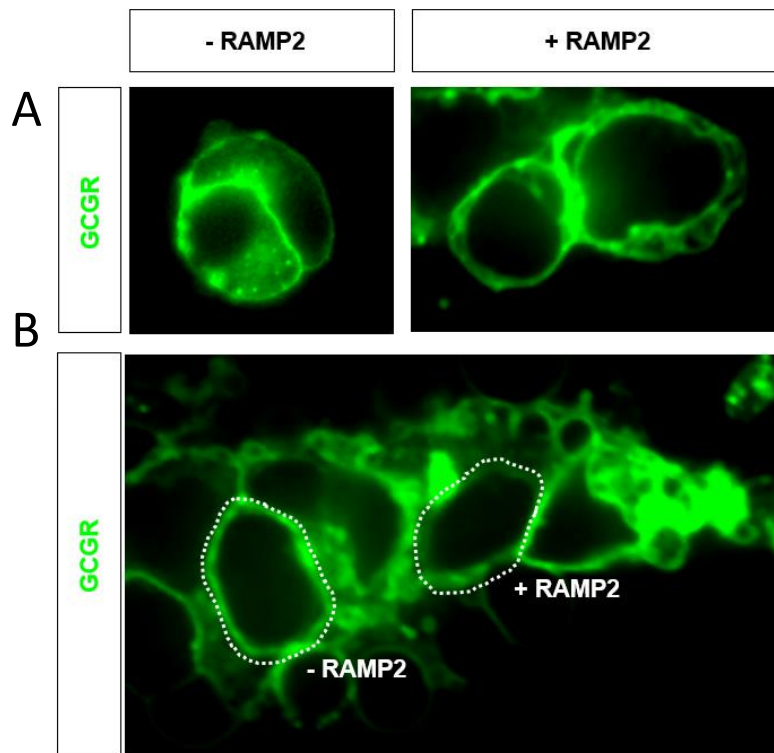


Figure 4.16: RAMP2 causes GCGR internalisation A) Cell surface expression of the GCGR in RAMP2 negative cells (top left) and RAMP2 positive cells (top right). The GFP signal line around the cells is thicker in the presence of RAMP2 B) GFP intensity was measured for cells in pairs therefore each RAMP2 positive cell had a RAMP2 negative cell as a control captured in the same image.

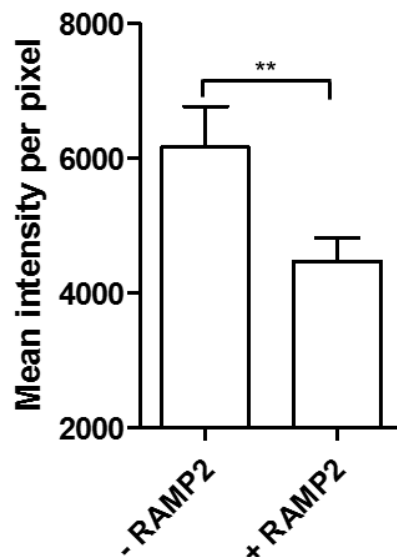


Figure 4.17: Measurement of cell surface expression. Mean GFP intensity per pixel measured at the membrane for RAMP + cells and RAMP- cells. GFP intensity was measured for cells in pairs therefore each RAMP+ cell had a RAMP- cell as a negative control captured in the same image (n=8 per group) **p<0.01. Errors shown are $\pm$ SEM.

4.5 Discussion

It has previously been demonstrated by immunofluorescence confocal microscopy that RAMP2 interacts with the glucagon receptor. In this chapter, I have aimed to investigate the functional effect of this interaction by looking specifically at the effect of RAMP2 on 1) ligand binding at the GCGR, 2) GCGR cell signalling and 3) GCGR subcellular distribution. Here I demonstrate that co-expression of RAMP2 with GCGR does not alter the binding affinity of glucagon or its related peptides. However, the presence of RAMP2 has a marked effect on signalling via the $G_{\alpha s}$ and G_q pathways, as well as β -arrestin recruitment. Furthermore, RAMP2 appears to co-localise with the GCGR and influence its sub-cellular distribution.

4.5.1 RAMP2, the glucagon receptor and ligand specificity

The most remarkable pharmacological consequence of RAMP interaction with receptors for the calcitonin family is the generation of unique receptor pharmacological profiles which affects both ligand binding and cell signalling properties (McLatchie et al., 1998, Christopoulos et al., 1999, Tilakaratne et al., 2000). Unlike its interaction with either the CTR or CRLR receptor, the association of RAMP2 with the glucagon receptor did not cause a significant alteration in the binding affinity of glucagon and its related peptides to the glucagon receptor in whole cells. Although not statistically significant, IC₅₀ values were lower for glucagon and OXM in the presence of RAMP2 and further work is required to investigate this. Additionally, saturation binding experiments using ¹²⁵I-GCG as the radioligand revealed that co-expression of RAMP2 resulted in a ten-fold reduction in GCGR binding sites when compared with those determined in the absence of RAMP. This reduction in total binding of glucagon may be due to reduced receptor expression on the cell surface. This may be a direct effect of the interaction of RAMP2 and the GCGR resulting in internalisation. Alternatively, this may be an indirect effect, for example, RAMP2 may influence GCGR cell surface expression via its effect on β -arrestin recruitment.

4.5.2 RAMP2, the glucagon receptor and cell signalling

Co-expression with RAMP2 did not bias signalling for the different ligands via the $G_{\alpha s}$ and G_q pathways. RAMP2 seems to affect glucagon and its related ligands in the same

way. With regards to the $G_{\alpha s}$ pathway, the presence of RAMP2 caused a reduction in potency and increase in efficacy. Whether this is a function of the reduced availability of binding sites is yet to be determined. In receptor pharmacology, four factors can control the agonist potency for a G-protein-coupled receptor (Conn, 2013). Two are system-cell dependent and the other two are receptor dependent. Receptor density and the efficiency of coupling to the G proteins are the system-dependent factors; affinity and intrinsic efficacy are the receptor-dependent factors. Thus, on the basis of classical receptor theory, variance in the relative potency of ligands at the GCGR with/without RAMP2 may be due to differences in receptor density.

The increase in efficacy of cAMP production observed with RAMP2 is more intriguing. This enhancement in cAMP response is all the more striking as it is in face of reduced cell surface expression of GCGR. The simplest interpretation is that by some mechanism, RAMP2 increases the accessibility of the receptor to the G-protein (Morfis et al., 2003). Alternatively, RAMP2 may inhibit the desensitisation response that is classically seen with the GCGR. Desensitisation is a phenomenon whereby prolonged agonist stimulation attenuates signalling through GPCRs, protecting them from acute and chronic over-stimulation (Ferguson, 2001, Moore et al., 2007). This involves phosphorylation of receptors by GPCR kinases (GRKs) and binding of β -arrestins which uncouple receptors from G-proteins (Shenoy et al., 2006). It is reported that glucagon receptors are internalised upon 30 minutes of glucagon stimulation (Buggy et al., 1997, Krilov et al., 2008, Merlen et al., 2006) and this is mediated by GRKs as well as β -arrestins (Krilov et al., 2011). The receptor's carboxyl terminus is required for internalization (Krilov et al., 2008, Buggy et al., 1997) and phosphorylation of certain Ser residues is also necessary (Merlen et al., 2006). RAMP2 may potentially interfere with any of the aforementioned to prevent desensitisation of the GCGR. Interestingly, the mechanism of desensitisation of the CRLR depends on which RAMP is present in microvascular endothelial cells. Both CGRP (CRLR-RAMP1) and adrenomedullin (CRLR-RAMP2/3) receptors desensitize after prolonged agonist exposure but only adrenomedullin receptors internalise (Nikitenko et al., 2006). CGRP receptor desensitisation in the absence of a loss of cell surface expression is thought to be GRK-mediated (Penn et al., 2000, Aiyar et al., 2000) and therefore the adrenomedullin internalisation is deemed to be a β -arrestin driven process. Here, I speculate that the

loss of desensitisation seen with GCGR-RAMP2 may be due to the inhibition of β -arrestin recruitment.

On examination of the Gq pathway, intracellular calcium flux was found to be attenuated in the presence of RAMP2. Interestingly, preferential coupling to $G_{\alpha s}$ versus Gq has been reported for AMY1 and AMY3 receptors, but not AMY2 (Morfis et al., 2008). The finding that cAMP signalling is specifically augmented and calcium signalling attenuated by RAMP2 at the GCGR is highly relevant because the classic coupling pathway associated with GCGR activation has always been the stimulation of cAMP accumulation via coupling to $G_{\alpha s}$ proteins. In the 1980s, it was proposed that the cellular effects of glucagon may also be mediated a rise in intracellular calcium concentration via coupling to Gq proteins (Benedetti et al., 1989, Bygrave et al., 1993, Mine et al., 1988a, Mine et al., 1988b). Wakelam et al postulated the existence of two distinct hepatic glucagon receptors: one that couples to adenylate cyclase and the other to Gq proteins (Wakelam et al., 1986). This finding of a selective enhancement of the GCGR cAMP response in the presence of RAMP2 is one possible mechanism that could explain some of the contradictory results observed in other studies of glucagon receptor signalling. More specifically, the endogenous RAMP2 protein may exert a previously unappreciated impact on the signalling efficiency of the glucagon receptor. Whether this is tissue-specific and dependent on the prevailing physiological conditions is yet to be seen. These findings demonstrate that RAMP2 is able to bias the signalling pathways of glucagon and its related peptides and indicates the potential for selective modulation of receptor signalling through targeting of the RAMP/receptor interface.

The effect of co-expression of RAMP2 on β -Arrestin recruitment was also investigated. The presence of RAMP2 completely abolished β -Arrestin recruitment and this finding was consistent for glucagon as well as GLP-1, oxyntomodulin and G(X). Whether RAMP2 interacts with the GCGR at the same site as β -arrestin binds, thereby disrupting β -arrestin recruitment, is one possible theory. Krilov et al have shown that β -Arrestins are crucial for the recycling of the GCGR (Krilov et al., 2008) therefore loss of β -Arrestin recruitment may explain the reduced cell surface expression of the GCGR when RAMP2 is present. Alternatively, reduced cell surface expression of GCGR may be the primary effect of RAMP2 and this may in turn prevent β -Arrestin recruitment. β -Arrestin recruitment was not rescued through knockdown using RAMP2 siRNA. The fact that

these cells containing the β -arrestin recruitment reporter signal (CHO-K1-D-GCGR) had 42 fold greater RAMP2 expression compared to the CHO-K1-GCGR cells used in the cAMP and calcium experiment may account for the difference in response to siRNA.

4.5.3 RAMP2, the glucagon receptor and subcellular distribution

Visualisation of RAMP2 and the glucagon receptor using confocal microscopy revealed two key findings. Firstly, it is demonstrated for the first time that RAMP2 and the GCGR co-localise. Secondly, in the presence of RAMP2, there was reduced cell surface expression of the GCGR. This is consistent with the saturation binding experiments which found reduced binding of ^{125}I -GCG in the presence of RAMP2. As suggested previously, this may be a consequence of reduced β -arrestin recruitment. It would be interesting to examine whether siRNA knockdown of RAMP2 restored GCGR cell surface expression; this was not carried out as part of the work presented here due to time and technical limitations but is nonetheless important.

These findings appear to be at odds with the work done by Christopoulos et al which reported that, when co-expressed with GCGR, RAMP2 translocates to the cell surface. A number of differences exist in the experimental approach between this current study and that of Christopoulos. Firstly, in their study only the RAMPs, and not the GCGR, were tagged so it was not possible to comment on where the receptor was trafficked to. Secondly, in the Christopoulos study, RAMP2 was N-terminally tagged with haemagglutinin whereas here C-terminally CFP-tagged RAMP2 was utilised. It is the N-terminal that contains the natural, predicted signal peptide sequence of RAMP2 and therefore this may have had a bearing on expression of RAMP2. Furthermore, the choice of tag itself may also be a critical factor. It has previously been shown that use of a haemagglutinin or C-myc tag can affect CRLR transit and terminal glycosylation in the Golgi in the presence of RAMP2/3 (Hilairt et al., 2001).

A previous study of unglycosylated murine RAMPs and glucagon receptors in *Xenopus* oocytes failed to reveal the association between the two (Flahaut et al., 2002). This discrepancy may be due to a species difference in RAMP-receptor interaction or the effect of removal of the consensus glycosylation sites. However, more likely it is a consequence of the highly divergent cellular backgrounds. It has previously been shown that interaction of the CTR with RAMPs, especially RAMP2, is sensitive to the cellular

background in which it is expressed. This suggests that other cellular components, such as G proteins, are likely to contribute to RAMP-receptor interaction (Tilakaratne et al., 2000).

4.5.4 Further work

Taken together, this work demonstrates that RAMP2 can affect the cell signalling pathways of the GCGR as well as its trafficking within the cell. A proposed model of RAMP2 action on the GCGR is shown in figure 4.18. There are two possible mechanisms by which RAMP2 could influence GCGR pharmacology. A direct contribution of binding epitopes to the relevant ligands is possible. Alternatively, RAMP2 could act indirectly by altering the conformation of the GCGR. For the CTR and CRLR, there is strong experimental support for interactions between the long N-termini of the RAMP and the respective receptor determining the specific pharmacology of each receptor complex (Fraser et al., 1999, Hay et al., 2006b, Hay et al., 2006a, Zumpe et al., 2000, Fitzsimmons et al., 2003, Udawela et al., 2006, Steiner et al., 2002, Qi et al., 2008). Further work, possibly using approaches such as site-directed mutagenesis and the creation of RAMP chimeras, will be useful in determining the critical domains and residues required for RAMP2-GCGR interaction.

The effect RAMP2 on the GCGR and how this translates in vivo is yet to be determined. The work conducted thus far has been in overexpressing cell lines. The logical next step would be to use primary cells in tissue relevant to glucagon receptor physiology. Additionally, endogenous tissue co-expression of RAMP2 and GCGR has not yet been investigated. RAMP mRNA tissue expression using northern blot analysis was reported initially by McLatchie et al on their first discovery of RAMPs (McLatchie et al., 1998), however, GCGR-relevant tissues such as brown adipose tissue, hypothalamus and the nodose ganglion were not specifically examined.

An additional question is whether the RAMP2-GCGR interaction is controlled in a physiological setting. It would be important to determine what process controls this and what effect it has on glucagon signalling. Co-expression may occur in some tissues under certain conditions and not others as expression of RAMP2 may be controlled by the prevailing physiological conditions, for example, glucose and insulin levels.

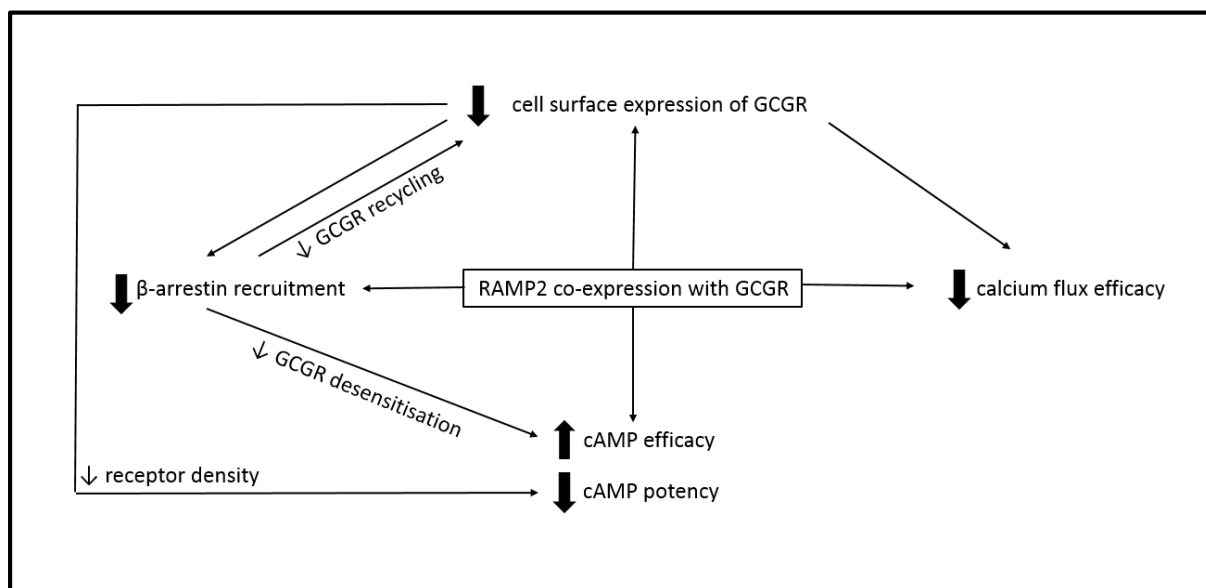


Figure 4.18. Proposed model of the effect of RAMP2 on the GCGR. Presence of RAMP2 results in 1) a reduction in cell surface expression of GCGR as demonstrated by saturation binding experiments and confocal microscopy, which may be related to reduced β -arrestin recruitment causing decreased GCGR recycling 2) an increase in efficacy (E_{max}) of the cAMP response which may be driven by reduced β -arrestin recruitment causing less desensitisation 3) a reduction in potency (EC_{50}) of the cAMP response which may reflect a reduction in receptor density at the cell surface 4) decreased efficacy of calcium flux and 5) complete obliteration of β -arrestin recruitment.

The work presented here has focussed on the effect of RAMP2 on the GCGR, based on the work done by Christopoulos et al. The effects of RAMP1 and RAMP3 have not yet been tested functionally and although they may not necessarily be physiologically relevant, they are still of interest for the purposes of potential pharmacological exploitation. Of ultimate interest is the effect of RAMP2 *in vivo*. The generation of RAMP2 knockout mice may be of use in understanding its effect on glucagon receptor signalling.

4.5.5 Conclusion

This work has added to our understanding of GCGR's physiological function and how this is modified by a recently discovered allosteric modulator, RAMP2. In the future, it could suggest new therapeutic avenues for obesity and diabetes. Allosteric modulation through the RAMP2 system may allow 'biasing' of the signalling pathways to exploit the desirable downstream effects. This could inform the construction of new peptide analogues with selective agonist activities, incorporating therapeutically desirable properties such as appetite suppression and increase in energy expenditure, without undesired properties such as increasing hepatic glucose output and provoking hyperglycaemia. It may also be possible to devise or screen for small molecules that are able to modify the interaction of RAMP2 with GCGR and therefore modify the physiological effects of GCG in order to obtain desired therapeutic outcomes.

CHAPTER 5:

GENERAL DISCUSSION

5.1 Obesity is a global challenge

The increasing prevalence of obesity worldwide is due to a combination of genetic factors which predispose to weight gain as well as environmental factors including Western sedentary lifestyles and easy availability of high calorie food. The complications and risks that accompany increased body weight and the continued failure of lifestyle intervention therapy to cause sustained weight loss mean that there is great demand for novel approaches to tackle obesity. Current pharmacological approaches to combat obesity are hampered by limited efficacy or considerable adverse effects. Therefore the development of safe and efficient anti-obesity agents is an increasing global priority.

The most effective treatment for obesity is bariatric surgery (Gilbert and Wolfe, 2012), but it is impractical to apply to the general obese population due to its cost and risk of mortality (Jackson and Hutter, 2012). Recent research has suggested that increased circulating GLP-1 levels contribute, at least in part, to the reduced appetite and body weight gain observed after bariatric surgery (le Roux et al., 2007, Xiong et al., 2015, Lund et al., 2015). GLP-1 has multiple effects that could contribute to weight loss and improvements in glucose tolerance, including a reduction in appetite, a delay in gastric emptying and an incretin effect (Kreymann et al., 1987, Verdich et al., 2001, Zander et al., 2002, Willms et al., 1996).

Additionally, after Roux-en-Y gastric bypass surgery, there is a post prandial elevation in glucagon levels, with peak levels double those during the fasting state (Jacobsen et al., 2012, Nannipieri et al., 2013, Falken et al., 2011). This is paradoxical to the reduction in glucagon levels normally observed after a meal. As well as increasing blood glucose, glucagon is known to exert a reduction in food intake and an increase in resting energy expenditure (Geary et al., 1992, Nair, 1987). As RYGB patients have improved carbohydrate tolerance it would suggest that glucagon does not cause an elevation in post prandial glycaemia, or that the observed raised levels of GLP-1 balance out any glucagon induced hyperglycaemia. This suggests that mimicking the post-bariatric state by peripherally administering glucagon and GLP-1 to the obese may be a logical, cost effective therapeutic approach. Strong appetite inhibition stimulates counter-regulatory mechanisms which act to prevent starvation, by increasing food seeking behaviour and

decreasing metabolic rate. Thus, by combining a therapy such as GLP-1 with the effects of glucagon, which may prevent the resulting hypoglycaemia from periods of low food intake and may increase energy expenditure, these mechanisms may be overridden.

Oxyntomodulin further exemplifies this dual approach to weight loss. Animal and human studies have demonstrated the efficacy of OXM in modulating energy homeostasis and decreasing body weight (Baggio et al., 2004, Dakin et al., 2001, Cohen et al., 2003, Wynne et al., 2006, Laferrere et al., 2010, Dakin et al., 2004, Kosinski et al., 2012, Wynne et al., 2005, Derosa and Maffioli, 2012, Schjoldager et al., 1988) and phase I trials in man are ongoing (Pfizer., 2012). Although OXM binds to both the GLP-1R and GCGR, it does so with lower affinity than the cognate hormones. Furthermore, the mechanisms through which OXM exerts its beneficial effects on body weight are not fully understood. Therefore, this thesis investigates glucagon and GLP-1 co-administration and energy homeostasis, focusing on food intake and energy expenditure as a dual approach to weight loss.

This thesis is divided into 2 sections. In the first section (Chapters 2 and 3), I aimed to elucidate whether co-infusion of the native hormones glucagon and GLP-1 had an effect on energy intake, expenditure and glucose homeostasis in overweight humans. In the second section (Chapter 4), I investigated the possibility of allosterically modulating glucagon receptor function by overexpressing RAMP2 in cell lines. The effect of RAMP2 on GCGR function was examined for glucagon, GLP-1, oxyntomodulin and analogue G(X), a GLP-1 and GCG co-agonist developed in our laboratory (Department of Investigative Medicine, Imperial College London). Overall, this thesis investigated the role of glucagon and GLP-1 in energy homeostasis by elucidating their involvement in food intake, energy expenditure and glucose homeostasis and by further understanding their functionality at a receptor level.

5.2 Dual agonism at the glucagon and GLP-1 receptors

The data from chapters 2 and 3 supports the idea that dual agonism leads to greater food intake reduction without deleterious effects on glycaemia. In 2009, two separate groups reported the discovery of a GLP-1/glucagon co-agonist and its ability to

eliminate obesity in rodents (Day et al., 2009, Pocai et al., 2009). Since then, other groups have demonstrated the beneficial effects of dual agonism on other metabolic parameters. With regards to lipid metabolism, co-agonist treatment decreased triglyceride levels in serum and liver and reduced serum cholesterol, mainly due to reduction in low-density lipoprotein (LDL) cholesterol. These changes were not seen with pair-fed controls (Patel et al., 2013). Co-agonist treatment also improved glucose tolerance and increased insulin sensitivity, as observed during glucose and insulin-tolerance test, hyperinsulinemic clamp, and reduced gluconeogenesis, as observed in pyruvate-tolerance test (Patel et al., 2013). Additionally, GLP-1/glucagon co-agonism restores leptin responsiveness in mice maintained on a high fat diet (Clemmensen et al., 2014).

Finan et al have taken the dual agonism approach one step further (Finan et al., 2015). In January 2015, they reported the discovery of a new monomeric peptide that reduces body weight and diabetic complications in rodent models of obesity by acting as a triple agonist: simultaneously at the GLP-1, glucagon and glucose-dependent insulintropic polypeptide (GIP) receptors. They demonstrate that these individual constituent activities harmonize to govern the overall metabolic efficacy, which predominantly results from glucagon action to increase energy expenditure, GLP-1 action to reduce caloric intake and improve glucose control, and GIP action to potentiate the incretin effect and buffer against the diabetogenic effect of inherent glucagon activity. The resulting effect was reduced body weight, enhanced glycaemic control and reversal of hepatic steatosis in relevant rodent models. These studies emphasise the translational value of polypharmacotherapy for the treatment of obesity and diabetes.

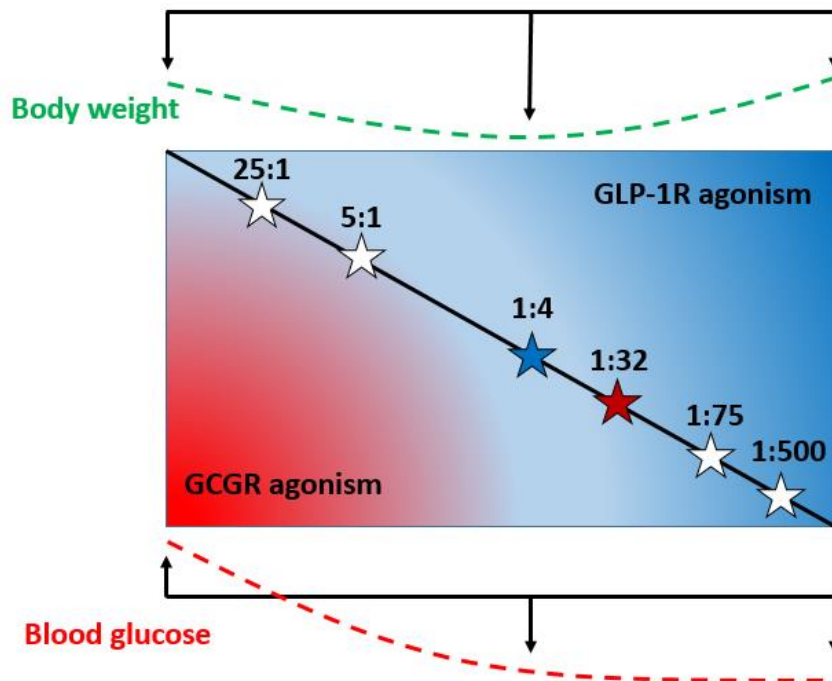


Figure 5.1: Schematic adapted from Day et al. 2009 showing the hypothesised effects of different ratios of GLP-1 receptor and glucagon receptor action, as calculated by glucagon and GLP-1 receptor-mediated cAMP synthesis (Day et al., 2009). The blue and red stars represent glucagon /GLP-1 co-agonists which have been demonstrated to cause a reduction of body weight through decreased food intake and increased energy expenditure.

What is the the ideal ratio of GLP-1R/GCGR agonism? Day et al have previously proposed that GLP-1R agonism should be four-fold higher than that of GCGR agonism, and at this point maximum weight loss is achieved without the detrimental effects on blood glucose (Figure 5.1). It is postulated that the intrinsic GLP-1R agonism of these molecules opposes and potentially neutralizes any glucagon receptor-mediated diabetogenic effects. Additionally, the considerable decrease in fat mass provides strong metabolic benefits that synergise to dominate any hyperglycemic drive. However, if the ratio of GCGR agonism is too low, then body weight loss may be no greater than the modest weight loss seen with GLP-1 receptor agonists already licensed as diabetes therapies. It has yet to be determined if the effects of a dual agonist shown in rodents are of clinical significance, although the weight loss obtained with native OXM in overweight subjects is encouraging (Wynne et al., 2005).

The studies detailed above have predominantly been carried out in rodent models. Little data exists as how these ratios translate into humans. In Chapter 2, I demonstrate for the first time that co-infusion of native glucagon and GLP-1 at low doses reduces food intake additively in non-diabetic, overweight human volunteers. However, the increase in nausea is likely to limit the dose at which glucagon/GLP-1 co-agonists can be administered. The molar ratio of glucagon to GLP-1 administered in Chapter 2 was 7:1, considerably more glucagon 'heavy' than the ratios suggested by Day et al. At the doses given, there was a non-significant increase in EE and no significant deterioration in glucose control over the infusion period. In a previous study conducted in this department, a glucagon: GLP-1 ratio of 35:1 was administered and this caused a significant increase in energy expenditure (Tan TM et al., 2012). That study, however, was conducted in the fasting state and this is likely to be the reason that no nausea was experienced. In Chapter 3, I show that co-infusion of native glucagon and GLP-1 at low doses results in improved carbohydrate tolerance relative to placebo and glucagon alone and was comparable to the GLP-1 arm. Here the glucagon: GLP-1 ratio was 3.5:1. Thus it is clear that different ratios of glucagon to GLP-1 will confer diverging beneficial effects, especially with regards to energy expenditure (glucagon-mediated) vs improved glycaemia (GLP-1-mediated). The findings presented here represent acute infusion studies and it is clear that longer term studies are required to assess whether the beneficial effects of dual infusion are sustained. The fact that OXM has been shown to be effective over 28 days supports this notion (Wynne et al., 2005). A chronic combination infusion study is currently underway in the department. Taken together, these findings suggest that drug therapy aimed at glucagon and GLP-1 receptor co-agonism could potentially result in weight loss through the dual mechanism of reduced food intake and increased EE, without detrimental effects on glucose homeostasis.

5.3 Allosteric modulation of glucagon receptor function by RAMP2

Recently, the crystal structure of the 7-transmembrane domain of the glucagon receptor was solved (Siu et al., 2013). Current knowledge about the structure and function of class B GPCRs suggests that through binding with the extra-cellular domain and the 7-transmembrane domain, the peptide ligand stabilizes conformational changes in the 7-transmembrane domain that facilitate receptor activation and signalling via G-proteins,

arrestins or other factors (Parthier et al., 2009, Hollenstein et al., 2014, Pal et al., 2012). It is possible that changes in cellular homeostatic mechanisms, for example due to disease, are mediated in part by alterations in the type and/or level of endogenous signaling molecules that interact with GPCRs in an allosteric manner. The work presented in chapter 4 sought to assess if RAMP2, an endogenous allosteric modulator known to interact with the GCGR, could affect the phenotype of the GCGR.

In Chapter 4, I demonstrate that co-expression of RAMP2 and the GCGR does not alter the binding affinity of glucagon or its related peptides. However, saturation binding experiments showed that co-expression of RAMP2 resulted in a ten-fold reduction in GCGR binding sites when compared with those determined in the absence of RAMP2. This result was confirmed by confocal work which demonstrated that RAMP2 colocalises with the GCGR and causes significant GCGR internalisation. Furthermore, the presence of RAMP2 has a marked effect on signalling down the $G\alpha_s$ and Gq pathways, as well as β -arrestin recruitment. Given that RAMPs have been shown to affect the ligand-receptor interaction for other GPCRs (Hay et al., 2006a, Watkins et al., 2014), it is postulated that the RAMP2-GCGR complex subtly changes the arrangement of key residues involved in G-protein coupling and β -arrestin recruitment. Further experiments looking at β -arrestin recruitment, using alternative cell lines and by tagging and visualising the RAMP2- β -arrestin interaction are warranted. Additionally, the ability of RAMPs to affect cell trafficking (Bomberger et al., 2005a, Bomberger et al., 2005b) also appears to be borne out with the RAMP2-GCGR interaction. Thus, it is clear that RAMP2 influences the function of the GCGR.

The work presented here highlights the importance of considering all cellular components when designing new therapeutic agents. Furthermore, the effects of RAMP2 on GCGR signalling could be exploited when designing biased drugs to engage therapeutically beneficial pathways and minimise signalling down pathways leading to unwanted effects such as hyperglycaemia. The dual GCG/GLP-1 analogue G(X) used in Chapter 4 was selected for its ability to bind to both the GCGR and GLP-1 R. Although no ligand bias was uncovered for G(X) compared to glucagon activity at the GCGR, the work presented here gives impetus to screen further analogues to determine whether specific ligand bias exists.

Given the profound effect that RAMPs can exert on GPCR signalling, RAMPs themselves are potential drug targets for the treatment of diseases where aberrant activation of these receptor complexes may contribute to disease pathogenesis. The RAMP1/CRLR complex, the receptor for CGRP, is currently being targeted for the treatment of migraine headache (BIBN4096BS (Olcegepant) and MK0974 (Telcagepant)) (Salvatore et al., 2008, Mallee et al., 2002, Doods et al., 2000, Doods et al., 2007, Ho et al., 2008). Several small molecule non-peptide antagonists have also been described for the CGRP receptor, some of which have binding sites on RAMP1. RAMPs could also therefore be targeted directly, either by traditional small molecule inhibitors, by developing therapeutic RNAi, or antibodies which interact directly with RAMPs. However, as RAMPs are present at the cell surface as part of a receptor complex, it may be a challenge to secure suitable epitopes that could be targeted with antibody-based therapies.

Although RAMPs were initially discovered as accessory proteins to the CRLR, their potential impact on drug development could be significant, given the multitude of receptors that RAMPs interact with. However, structure-based drug design targeting RAMPs is still limited due to incomplete structural data. The extracellular portions of the RAMP/GPCR complex are likely to receive the most attention for drug development, however, drugs that bind at the C-terminus of the RAMP may usefully alter receptor trafficking or downstream signalling profiles. Although challenging to produce, agents that prevented RAMP-receptor association in the endoplasmic reticulum could also prevent the appearance of such complexes at the cell surface. It has recently been found that, in addition to receptors, RAMPs can also interact with other proteins such as tubulin (Kunz et al., 2007). The physiological significance of this is still unknown, however, this interaction could represent a mechanism for compartmentalization of RAMP-receptor complexes or novel receptor-independent functions.

Targeting RAMPs alone could have multiple effects depending on the specific RAMP targeted. This may lead to unforeseen consequences due to the widespread distribution of these proteins and our limited understanding of the potential breadth of RAMP function. Nevertheless, RAMP-targeted drugs would be of great utility in understanding

the physiology of RAMPs and could be empirically investigated for therapeutic potential.

5.4 Conclusion

Dual agonism of both the glucagon and GLP-1 receptors is a rational approach to the development of a new therapy for obesity and diabetes, through a simultaneous reduction in food intake and increased energy expenditure, whilst maintaining or even improving glucose homeostasis. Furthermore, understanding the cell signalling pathways of the glucagon receptor and how they can be manipulated allosterically by RAMP2 may inform and optimise the development of future GCGR/GLP1 dual agonists.

5.5 Future work

The Department of Investigative Medicine, Imperial College London is currently undertaking a drug discovery programme of dual glucagon/GLP-1 analogue development. These analogues will allow the study of chronic glucagon and GLP-1 receptor agonism in humans through a prolonged pharmacokinetic profile compared to the native hormones. I aim to conduct a multiple ascending dose finding study in human volunteers using a selected glucagon/GLP-1 analogue. The analogue will be selected through a screening programme, based on *in vitro* work demonstrating the ability of the analogue to bind and activate both the GCGR and GLP-1R. The selected analogue will be administered to rodents and dogs to study its effects on food intake, energy expenditure and glucose levels and to measure its pharmacokinetic profile. In man, I will measure breakdown products of this glucagon/GLP-1 analogue and study its pharmacokinetic profile following administration. In addition, I will investigate the effects of this analogue on glucose, insulin, food intake and perceived feelings of nausea. The results from this initial dose-finding study in humans will determine the dose of glucagon/GLP-1 analogue that will be administered in a subsequent phase 1 clinical trial.

A co-ordinated plan of work further assessing the effect of RAMP2 on the GCGR is ongoing in the department. The work conducted thus far has been in overexpressing cell lines and future work will examine whether the findings presented here are replicated

in primary cells in tissue relevant to glucagon receptor physiology. Additionally, endogenous tissue co-expression of RAMP2 and GCGR has not yet been investigated. Of ultimate interest is the effect of RAMP2 in vivo. The generation of RAMP2 knockout mice may be useful in understanding its effect on glucagon receptor signalling.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

Appendix A: Consent form

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Ref. No. 10/H0707/80	Version 02	Submission date 2 nd Nov 2011
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The effect of glucagon and GLP-1 on food intake and carbohydrate tolerance

PARTICIPANT CONSENT FORM

The participant should complete the whole of this sheet him- or herself
(please initial each statement if it applies to you)

I have read the Information Sheet for Research Participants	☐
I have been given the opportunity to ask questions and discuss this study	☐
I have received satisfactory answers to all my questions	☐
I have received enough information about the study	☐
The study has been explained to me by Dr _____	
I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care	☐
I agree to take part in this study	☐
I agree that my GP will be informed that I am taking part in the study	☐
I understand that the NHS and College as sponsor may also review records as part of audit process	☐
I agree that my blood samples will be kept indefinitely and may be used for further analysis or in other ethically approved research	☐

Signed _____	Date _____
(NAME IN BLOCK CAPITALS) _____	
Investigator's signature _____	Date _____
(NAME IN BLOCK CAPITALS) _____	

Appendix B: Visual analogue scale

Initials	Date:
Code	Visit number:
Time:	T =

How hungry do you feel right now?

NOT AT ALL  EXTREMELY

How sick do you feel right now?

NOT AT ALL  EXTREMELY

How pleasant would it be to eat right now?

NOT AT ALL  EXTREMELY

How much do you think you could eat right now?

NOTHING  LARGE AMOUNT

How full do you feel right now?

NOT AT ALL  EXTREMELY

Due to the commercial sensitivity of this data, the amino acid sequence of analogue G(X) has been removed from this thesis

Figure 4.1. Amino acid sequences of glucagon, analogue G(X) and GLP-1. Fully conserved regions are depicted in pink. Abbreviations: glucagon-like peptide-1 (GLP-1). Amino acids: alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), tyrosine (Tyr)

Appendix D: Solutions used in this thesis

LB

10g sodium chloride, 10g tryptone and 5g yeast extract mixed in GDW. pH adjusted to 7.5 with NaOH. Sterilised immediately by autoclaving.

TFB I

Dissolve 589 g CH₃COOK, 2.418 g RbCl, 1.979 g CaCl₂·2H₂O and 438 mg MnCl₂·4H₂O in a/c water. Add 37.5 ml 80% glycerol and top up to 200 ml with water. Sterilise by passing through 0.2µm filter.

TFB II

Dissolve 418 mg MOPS, 3.28 g CaCl₂·2H₂O and 242 mg RbCl in a/c water. Add 37.5ml 80% glycerol and make up to 200 ml with water. Sterilise by passing through 0.2µm filter.

Alkaline SDS

2 ml sodium hydroxide and 5 ml 20% SDS mixed in 93 ml a/c GDW.

GTE

2.5 ml 1 M Tris-HCl, pH 8.0, 2 ml 0.5M EDTA and 5 ml 18% glucose mixed in GDW to a final volume of 100 ml. Sterilised by passing through a 0.2µm filter

2M Sodium acetate pH 4.6

246.15 g CH₃COONa dissolved in 1200 ml a/c water, adjust to pH 4.6 with glacial acetic acid.

TES buffer

Add 5ml of 1M Tris pH 8.0 to 50ml distilled water, add 2ml of 0.25M EDTA. Add 0.3g NaCl and make up to 100ml with distilled water.

50x TAE

242 g Trizma base dissolved in 843 ml GDW and 57 ml of glacial acetic acid and 100ml 0.5 M EDTA.

Gel loading buffer

Mix 3.125 ml 80% glycerol, 50 µl 0.5M C₁₀H₁₄H₂O₈Na₂·2H₂O and 6.075 ml a/c water. Then add 10 mg orange G.

Cesium chloride saturated propan-2-ol

Vigorously mix 100 g CsCl₂, 100 ml a/c water and 1000 ml propan-2-ol and leave to settle.

100x TE

121.1 g Trizma base and 3.7 g EDTA dissolved in 800 ml GDW, pH adjusted to 7.5 with HCl and make up to 1L.

0.1M 25kD PEI pH 7.0

Dissolve 450 mg PEI in 80 ml GDW. Adjust to pH 7.0 with HCl. Make up to 100 ml with water. Filter solution.

Appendix E: Publication arising from Chapter 2



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Dr. Jaimini Gega
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Coinfusion of Low-Dose GLP-1 and Glucagon in Man Results in a Reduction in Food Intake

Diabetes 2014;63:3711–3720 | DOI 10.2337/db14-0242

Obesity is a growing epidemic, and current medical therapies have proven inadequate. Endogenous satiety hormones provide an attractive target for the development of drugs that aim to cause effective weight loss with minimal side effects. Both glucagon and GLP-1 reduce appetite and cause weight loss. Additionally, glucagon increases energy expenditure. We hypothesized that the combination of both peptides, administered at doses that are individually subanorectic, would reduce appetite, while GLP-1 would protect against the hyperglycemic effect of glucagon. In this double-blind crossover study, subanorectic doses of each peptide alone, both peptides in combination, or placebo was infused into 13 human volunteers for 120 min. An ad libitum meal was provided after 90 min, and calorie intake determined. Resting energy expenditure was measured by indirect calorimetry at baseline and during infusion. Glucagon or GLP-1, given individually at subanorectic doses, did not significantly reduce food intake. Coinfusion at the same doses led to a significant reduction in food intake of 13%. Furthermore, the addition of GLP-1 protected against glucagon-induced hyperglycemia, and an increase in energy expenditure of 53 kcal/day was seen on coinfusion. These observations support the concept of GLP-1 and glucagon dual agonism as a possible treatment for obesity and diabetes.

Glucagon is a counterregulatory hormone, secreted at high levels during hypoglycemia and fasting. It promotes glycogenolysis and gluconeogenesis, as well as hepatic fatty acid β -oxidation and ketogenesis (1). The glucagon

receptor is expressed in a broad range of tissues, including the liver, kidney, adipose tissue, pancreas, heart, brain, gastrointestinal tract, and adrenal glands (2). Its effects may therefore be more widespread than just control of glucose metabolism, and it is increasingly recognized that glucagon plays a key role in general energy homeostasis. Glucagon has been shown to potently increase satiety and acutely reduce food intake in humans (3). Additionally, glucagon has the ability to significantly increase energy expenditure during infusion in man (4,5) and has been reported to promote nonshivering thermogenesis in brown adipose tissue in rodents (6). Appetite inhibition classically results in defense of body weight by a reduction of energy expenditure (7,8). The increased energy expenditure in association with anorexia induced by glucagon thus potentially enhances its usefulness as an antiobesity therapy.

The prohormone for glucagon, proglucagon, is processed to GLP-1 in the gut. GLP-1 is secreted postprandially in response to direct stimulation of mucosal L cells by nutrients within the gut lumen and indirectly via neuronal pathways within the enteric nervous system. GLP-1 binds to the G-protein-coupled GLP-1 receptor found in pancreatic islet cells as well as brain, heart, and lung tissue (9) and exerts an incretin effect, stimulating glucose-dependent insulin release by β -cells (10). Acute intravenous injection of GLP-1 has also been shown to also reduce appetite and calorie intake (11), an effect that has been observed in lean, obese, and type 2 diabetic volunteers. As a result, GLP-1 is capable of achieving a modest reduction in body weight (12). GLP-1 also causes nausea and delayed gastric emptying,

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J.Ce. and R.C.T. contributed equally to this study.

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which limits the dose that can be used clinically (13). At present, GLP-1 analogs, such as exenatide and liraglutide, are licensed for the treatment of type 2 diabetes, improving glucose control and resulting in mild weight loss (14).

Obesity is a growing global epidemic. By 2015, projections suggest that 4 billion adults will be overweight and >700 million will be obese (15). It is therefore clear that new strategies are urgently needed to tackle obesity. Both glucagon and GLP-1 are apparently involved in physiological regulation of appetite and are consequently attractive targets for the development of drugs for weight loss. GLP-1 analogs produce only a small weight loss in diabetic subjects or obese patients (14). Moreover, glucagon would be expected to cause hyperglycemia, an undesired effect, especially in patients with diabetes. Dual administration of glucagon and GLP-1, or analogs thereof, could provide additional benefit over GLP-1 analogs alone. We have previously shown that the proglucagon derivative and gut hormone oxyntomodulin (OXM), which is an agonist at both the glucagon and GLP-1 receptors, is able to reduce body weight and increase energy expenditure without causing hyperglycemia in man (16). Interestingly, the anorectic effect of OXM is abolished in *Glp1r*^{-/-} mice (17), suggesting that OXM exerts its effect on food intake via the GLP-1 receptor. However, it is a relatively weak agonist for the GLP-1 receptor, being less potent by two orders of magnitude compared with GLP-1 (18). This calls into question the mechanism of OXM's anorectic effect, as OXM is secreted postprandially at concentrations that are in the same order as GLP-1 itself (19). We propose that this unexpectedly strong inhibition of appetite by OXM might be due to its combined action on both the glucagon and GLP-1 receptor. Others have demonstrated that dual agonism at both the GLP-1 and glucagon receptors augments appetite reduction and weight loss in rodents and improves glucose homeostasis in animal models of diet-induced obesity and diabetes (20,21). This approach could combine the appetite-suppressive effects of GLP-1 and glucagon with the energy expenditure-increasing effects of glucagon. Recent work by our group has also demonstrated that the combination of glucagon and GLP-1 does indeed increase energy expenditure and that the hyperglycemic effects of glucagon are counterbalanced by the action of GLP-1 in humans (5).

We hypothesized that the combination of glucagon and GLP-1 might enhance the reduction of food intake over that observed when the respective hormones are given alone. The current study was therefore designed to demonstrate the acute effects of intravenous infusion of GLP-1 and glucagon when given at low doses, both alone and in combination, on food intake (22), glucose homeostasis, and energy expenditure in humans. This approach was taken, instead of using OXM, as it gave us the flexibility to determine the subanorectic doses of GLP-1 and glucagon individually.

RESEARCH DESIGN AND METHODS

This study was reviewed and approved by the West London Research Ethics Committee (10/H0707/80) and carried out according to the principles of Good Clinical Practice and the Declaration of Helsinki.

Sixteen nondiabetic, overweight volunteers with a mean BMI of 27 kg/m² (range 24–32.9) were recruited by advertisement. All participants underwent health screening including medical history, physical examination, biochemical and hematological testing, and 12-lead electrocardiogram. Any abnormal eating behavior was assessed using the Dutch Eating Behavior Questionnaire (23) and the SCOFF questionnaire (24). Female volunteers were premenopausal, with regular menstrual cycles, and were not taking hormonal contraceptives. Smokers were excluded. Informed and written consent was obtained. Of the initial 16 recruited, 3 were excluded from final analysis, prior to unblinding, due to abnormal eating behavior not picked up by the standard questionnaires. One participant ate less than the minimum required 300 kcal at the acclimatization visit, one participant did not finish eating within the allotted time (20 min), and the third demonstrated progressive aversion to his chosen meal throughout the course of the study as demonstrated by visual analog scores (VAS). Participants' demographics are described in Table 1.

Study Design

An initial dose-finding phase was undertaken in order to establish a subanorectic dose of glucagon. We used a known subanorectic dose of GLP-1 (11,25). After the dose-finding phase, participants attended for five study visits. The first visit was an unblinded acclimatization visit, during which participants were infused with placebo alone (Gelofusine; B. Braun, Crawley, U.K.) in order to

Table 1—Demographics of study participants

Volunteer	BMI (kg/m ²)	Age	Sex
F1	24.0	25	M
F2	24.2	41	M
F3	24.8	32	M
F4	26.3	40	F
F5	26.6	33	M
F6	26.8	21	F
F7	26.8	40	F
F8	26.3	35	M
F9	32.9	28	F
F10	29.4	23	M
F11	27.6	39	M
F12	28.5	32	M
F13	27.4	22	M
All (mean or n)	27.0	31.6	9 M, 4 F

F, female; M, male.

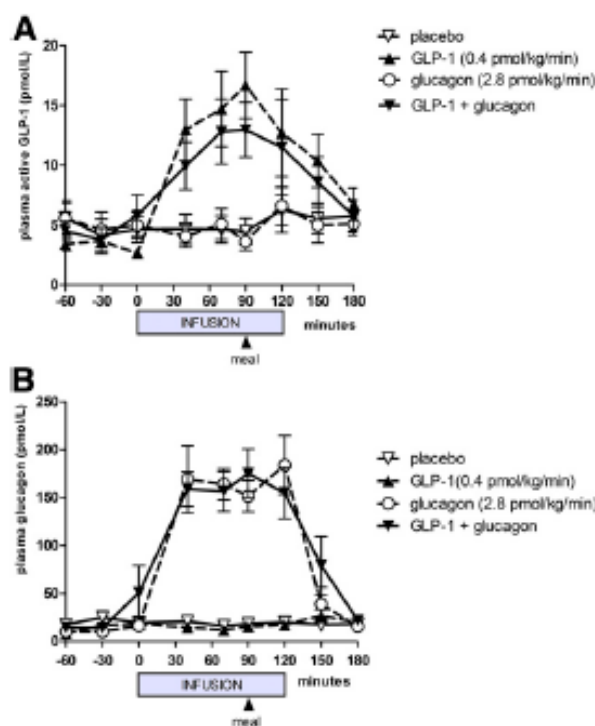


Figure 1—Plasma active GLP-1 (A) and glucagon (B) levels after hormone infusion. Mean $\pm$ SEM plasma levels plotted. Infusion denoted by gray bar; meal served at the arrow.

become familiar with study protocol. The four subsequent studies were conducted in a double-blind, four-way cross-over, randomized, controlled manner at least 2 days apart. Infusions consisted of 1) placebo (Gelofusine), 2) GLP-1₇₋₃₆ amide (0.4 pmol/kg/min; Clinalfa Basic, Bachem, Switzerland), 3) glucagon (2.8 pmol/kg/min; Novo Nordisk, Crawley, U.K.), or 4) combined GLP-1 and glucagon at the above doses. Gelofusine was used as the vehicle for hormone infusions in order to minimize adsorption of peptides to infusion lines and syringes (26).

Study Protocol

Volunteers attended the clinical research facility at 0830 h, having fasted from 2200 h the night before, and refrained from alcohol and strenuous exercise for the preceding 24 h. The study room was kept at a consistent temperature of 21°C, which was centrally controlled.

The study commenced at -60 min with the placement of two venous cannulae: one for blood sampling and one for intravenous hormone infusion. After cannulation, volunteers were encouraged to relax and were seated in reclining chairs. They were permitted to watch television or listen to music. After 30 min (-30 min), they were placed under an indirect calorimeter canopy (Gas Exchange Monitor; GEMNutrition, Daresbury, U.K.). Prior to measurement of energy expenditure, the calorimeter was

calibrated with "zero" (0.000% O₂, 0.000% CO₂) and "span" (20% O₂, 1.125% CO₂) gases (BOC Gases, Surrey, U.K.). Indirect calorimetry measurement was performed as previously described (5) for 30 min, allowing time for initial stabilization of readings, with the last 10 min of measurements used for analysis. Resting energy expenditure (REE), respiratory quotient (RQ), and carbohydrate and fat oxidation rates were calculated from the VO₂ and VCO₂ measured at 1-min intervals, and adjusted for urinary nitrogen excretion (27,28). After 30 min of calorimetry, the canopy was removed, and infusion of hormones was commenced (0 min). The infusion was initially ramped in order to rapidly achieve a steady-state plasma concentration of hormone. Ramping was carried out at four times the nominal infusion rate for 5 min and then twice the nominal infusion rate for a further 5 min and then reduced to the nominal rate for the remaining 120 min. At 40 min, further 30-min measurements of REE and substrate oxidation rates were made, still in the fasting phase. At 90 min, an ad libitum meal of known specific calorific value was served (spaghetti bolognese 188 kcal/100 g, chicken tikka masala 178 kcal/100 g, or macaroni cheese 194 kcal/100 g; Sainsbury's Supermarkets Ltd, London, U.K.). Participants had tasted their chosen meal during the acclimatization visit, and all deemed it to be palatable. The same meal was served at all five visits. Participants were allowed 20 min to eat and instructed to eat until comfortably full and then stop. The hormone infusion continued for 120 min in total and was terminated 10 min after the end of the meal. Participants remained in the study room for 60 min after termination of the infusion. At 180 min, the cannulae were removed, and the participants emptied their bladders. Urinary volume was measured in order to calculate urinary nitrogen excretion for estimation of protein oxidation. The participants were then discharged home.

During the study, pulse and blood pressure were measured at -60, -30, 0, 40, 70, 90, 120, 150, and 180 min. At these times, blood samples were also taken for measurement of glucose, insulin, glucagon, and active GLP-1. Glucose and insulin levels were measured by the Department of Chemical Pathology, Imperial College Healthcare National Health Service Trust (coefficient of variation [CV] <5% and <10%, respectively, across the working range). Samples for active GLP-1 and glucagon were collected in lithium heparin tubes containing 1,000 kallikrein inhibitor units. Active GLP-1 and total ghrelin were measured using commercially available ELISA kits (Millipore, Livingston, U.K.) according to the manufacturer's instructions (CV <7% and 8%, respectively), as was acylated ghrelin (BioVendor, Brno, Czech Republic) (CV <7%). Glucagon and total peptide YY (PYY) were assayed according to established immunoassay protocols by in-house radioimmunoassay (10,29) (CV <10% and <15%, respectively). At each of the above time points, a VAS was completed by the participant for assessment of nausea and satiety.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). Two-way repeated-measures ANOVA with Bonferroni post hoc test was used to compare differences in glucose, insulin, PYY levels, blood pressure, pulse, and VAS. One-way repeated-measures ANOVA with Newman-Keuls and Bonferroni post hoc tests was used to compare food intake, change in REE, and substrate oxidation rates between groups. Area under the curve (AUC) was calculated using the trapezoidal rule, and differences between treatment groups were compared using one-way repeated-measures ANOVA with Tukey post hoc test. Paired Student *t* test was used to compare ghrelin levels at baseline and during infusion. Data are reported mean $\pm$ SEM unless otherwise stated.

RESULTS

Baseline plasma active GLP-1 levels at -30 min were $4\text{--}5$ pmol/L. In the experimental groups receiving GLP-1 alone

or GLP-1 with glucagon, levels rose to $11\text{--}16$ pmol/L at 70 min postinfusion (Fig. 1A). Although active GLP-1 plasma levels appeared to be lower during the combination infusion compared with the GLP-1-only infusion, AUC_{GLP-1} was not significantly different during the infusion period ($0\text{--}120$ min [Supplementary Fig. 1A]). Mean plasma glucagon levels at -30 min were $14\text{--}19$ pmol/L, rising to $147\text{--}173$ pmol/L at 70 min in those groups receiving glucagon infusion (Fig. 1B).

Plasma glucose and serum insulin responses to placebo, glucagon, GLP-1, or combination infusions are shown in Fig. 2. In the placebo group, glucose and insulin remained constant during infusion and, as expected, rose in response to the meal served at 90 min. Glucagon infusion caused a rise in glucose from 4.8 ± 0.08 mmol/L to a peak of 6.5 ± 0.3 mmol/L at 40 min, with a corresponding rise in insulin to 31.2 ± 3.8 mU/L. GLP-1 infusion reduced plasma glucose during infusion from 4.9 ± 0.1 mmol/L to 4.1 ± 0.3 mmol/L at 40 min, with serum insulin levels

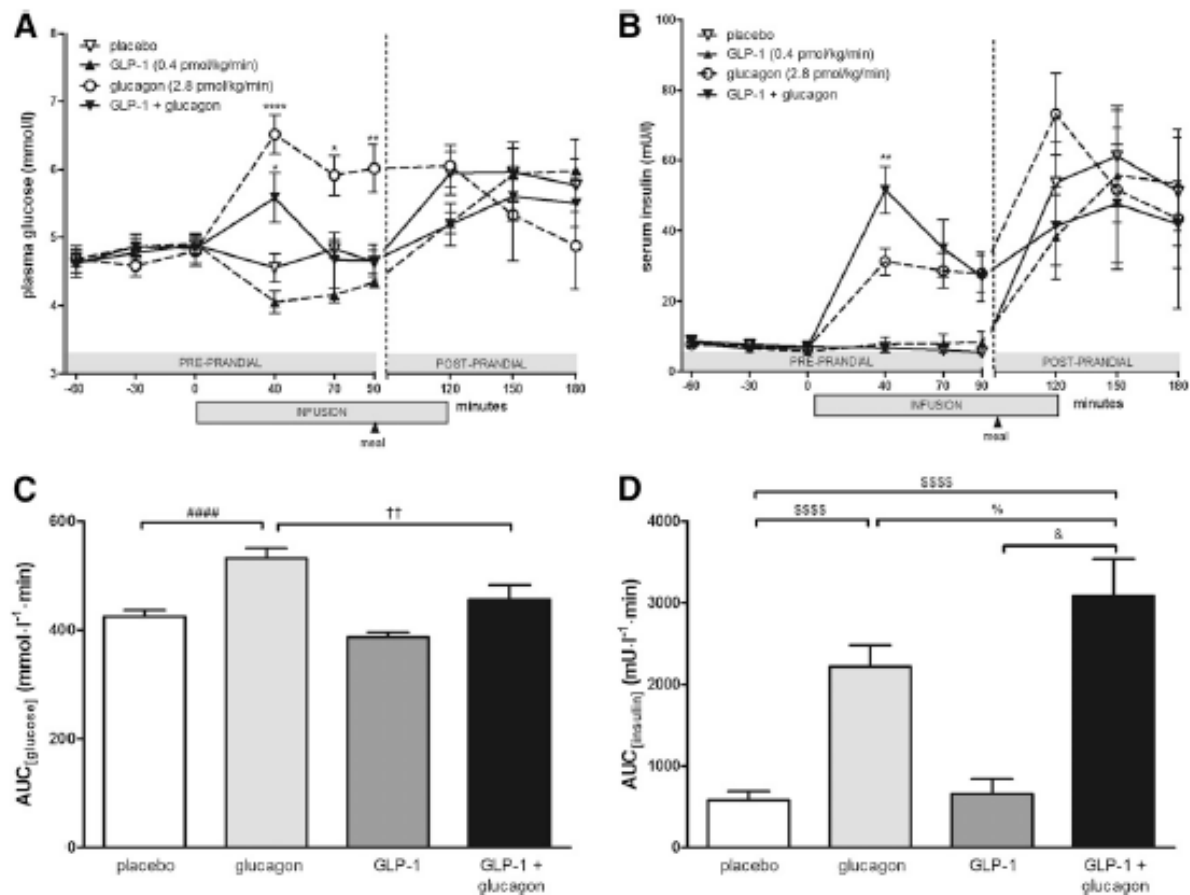


Figure 2—The effects of glucagon and GLP-1, alone and in combination, on plasma glucose (A and C) and serum insulin (B and D). A and B: Mean $\pm$ SEM plasma glucose and serum insulin levels. Infusion denoted by gray bar; meal served at the arrow. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 compared with placebo. C: AUC for glucose levels from 0 to 90 min (from the start of the infusion until just before the meal is served). ####*P* < 0.0001 compared with placebo; ††*P* < 0.01 compared with glucagon. D: AUC for insulin levels from 0 to 90 min. \$\$\$\$*P* < 0.0001 compared with placebo; %*P* < 0.01 compared with glucagon; &*P* < 0.0001 compared with GLP-1.

similar to that observed in the placebo arm. After glucagon and GLP-1 coadministration, AUC_{glucose} was similar to that seen with placebo and significantly lower than with glucagon alone (Fig. 2C). There was a significant increase in insulin during the glucagon/GLP-1 coinfusion of greater magnitude than seen with GLP-1 or glucagon alone (Fig. 2D). Postmeal, where calorie intake differed between treatment groups, glucose and insulin levels were not significantly different between groups.

As expected, glucagon alone and GLP-1 alone, at the doses given, did not significantly reduce food intake. However, glucagon and GLP-1 coinfusion significantly reduced food intake by 13% at the study meal compared with placebo ($P < 0.05$), which was also a significantly greater reduction than seen during infusion of glucagon alone ($P < 0.05$) or GLP-1 alone ($P < 0.05$) (mean energy intake at study meal: $1,086 \pm 110.1$ kcal [placebo], $1,086 \pm 96.9$ kcal [glucagon], $1,052 \pm 81.3$ kcal [GLP-1], and 879 ± 94.2 kcal [combined glucagon plus GLP-1]) (Fig. 3).

Neither the palatability of the buffet meal nor other satiety-related VAS responses were altered significantly by any of the infusions (Fig. 4A, C, and D). The nausea score significantly increased postmeal (120 min) during the combined infusion of glucagon and GLP-1 (Fig. 4B). Three participants reported mild nausea after the combined infusion, and two participants vomited after glucagon infusion. In all cases, this occurred postmeal between 120 and 160 min.

There were no significant differences in baseline REE between groups: $1,336 \pm 65.8$ kcal/day (placebo), $1,314 \pm 53.0$ kcal/day (glucagon), $1,330 \pm 71.9$ kcal/day (GLP-1), and $1,341 \pm 56.6$ kcal/day (combined glucagon plus GLP-1); $P = 0.7275$. The mean within-subject CV was $4.1 \pm 1.3\%$. After infusion, there was a trend toward higher REE in response to glucagon alone and glucagon/GLP-1 coadministration by a mean of 66.8 and 52.5 kcal/day, respectively (Fig. 5A).

RQ values and carbohydrate oxidation rates at baseline were similar in all treatment groups, and RQ did not change after GLP-1 infusion. A significant increase in RQ and carbohydrate oxidation was observed with both the glucagon and combination infusions (Fig. 5B and C). Glucagon alone and in combination with GLP-1 significantly reduced fat oxidation rates (Fig. 5D). Protein oxidation rate was calculated over the entire study period for each infusion, and none of the treatment arms were significantly different from placebo (data not shown). There were no significant changes in pulse or systolic or diastolic blood pressure (Supplementary Fig. 2) with any of the treatment groups.

Infusion of GLP-1 or glucagon alone did not affect total or acylated ghrelin levels. However, coinfusion led to a significant fall in both total ($P < 0.05$) and acylated ($P < 0.05$) ghrelin (Fig. 6A and B). Plasma PYY levels were unaffected by GLP-1 and glucagon individually or in combination (Fig. 6C).

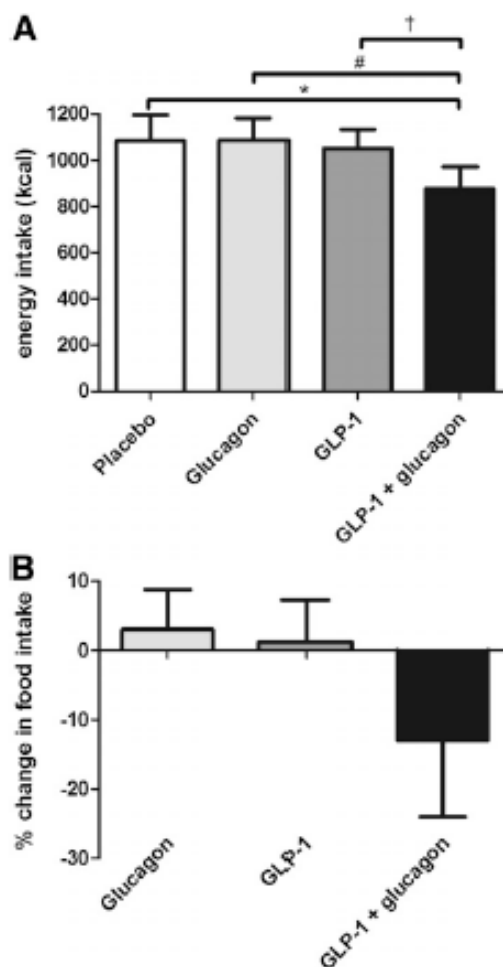


Figure 3—Energy intake at the study meal at 90 min. A: Mean $\pm$ SEM absolute energy intake. * $P < 0.05$ compared with placebo; # $P < 0.05$ compared with glucagon; † $P < 0.05$ compared with GLP-1. B: Energy intake as a percentage change of the placebo visit for individual volunteers.

DISCUSSION

This study shows that dual infusion of GLP-1 and glucagon reduces food intake significantly, whereas the same low doses of glucagon and GLP-1, when administered separately, do not exert a similar anorectic effect.

The dose of glucagon used in this study (2.8 pmol/kg/min) was established in a dose-finding study to be subanorectic. It is higher than the dose used previously by Geary et al. (3) (0.84 pmol/kg/min), which was demonstrated to reduce food intake. Our intention was to examine the effect of raised preprandial levels of glucagon and GLP-1 on food intake in a fasting state. In contrast, the study by Geary et al. examined the effect of elevated postprandial levels of glucagon after consumption of 500 g of tomato soup. The soup would be expected to stimulate anorectic gut hormone secretion (e.g., PYY, GLP-1) and suppress ghrelin secretion, explaining the differences in glucagon doses.

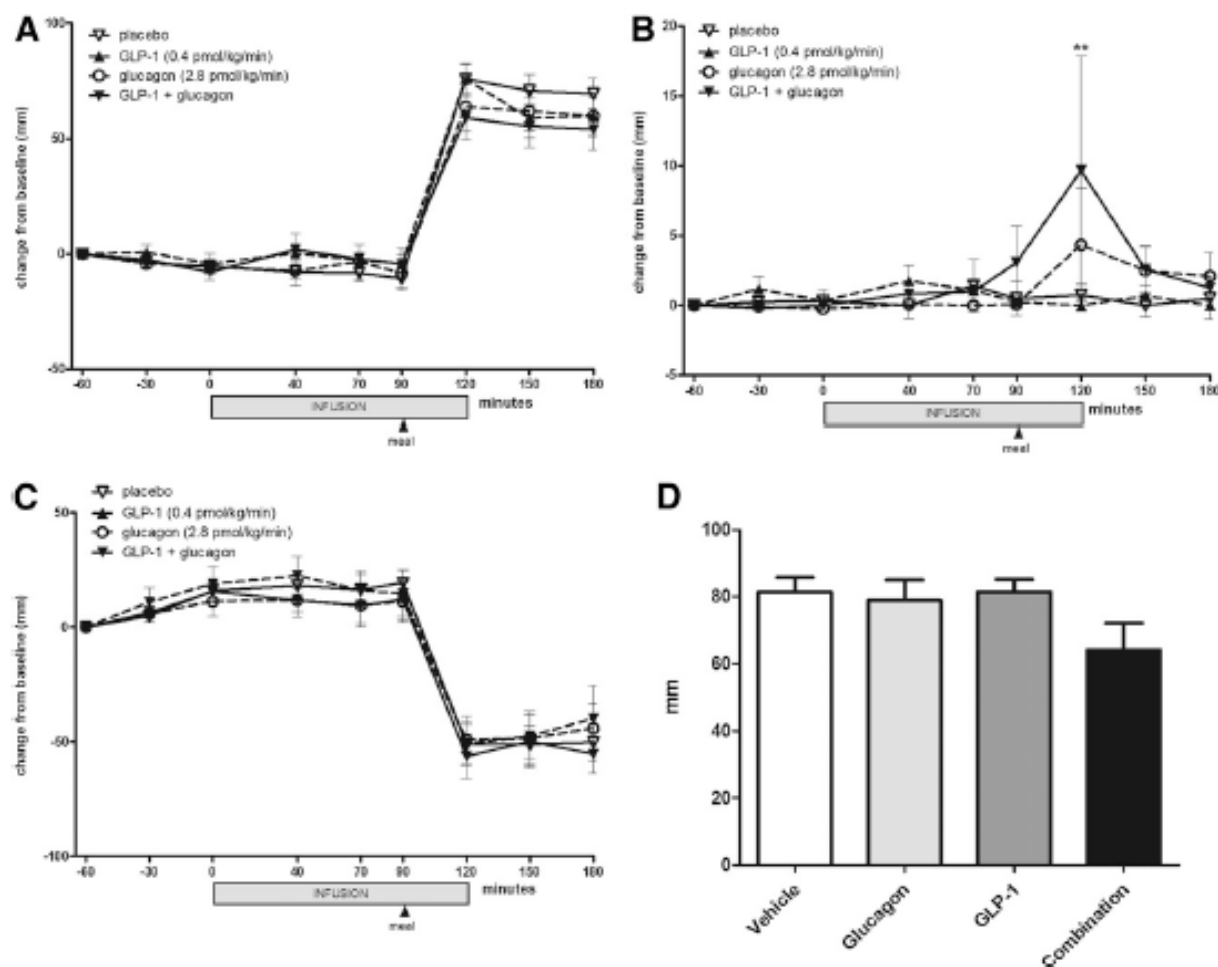


Figure 4—Subjective rating of satiety (A), nausea (B), hunger (C), and palatability (D) as measured by VAS response. For A–C, scores are depicted as change from baseline value (millimeters). ** $P < 0.01$ compared with placebo. For D, an absolute value (millimeters) is given.

Three subjects receiving the combination infusion experienced nausea. This nausea only became apparent postprandially, with no significant nausea occurring during the 90 min of infusion before the meal was served. The nausea is therefore unlikely to explain the reduction in food intake that was noted. GLP-1-based therapies for diabetes can cause nausea (14), as can glucagon (30). However, the doses used in this study were far smaller than those administered in these clinical situations. The postprandial nausea seen in this study may instead be accounted for by a delay in gastric emptying triggered by glucagon and GLP-1 (31,32).

Despite the significant reduction in food intake with coinfusion, no differences were observed in perceived hunger and satiety scores. Palatability of the meal was reduced with coinfusion, although this difference did not reach statistical significance. This discrepancy between perceived appetite and energy intake highlights the importance of measuring energy intake in an ad libitum meal, a more robust end point than VAS, which can be

affected by other factors such as age, sex, and physical activity (33).

Multiple pathways are responsible for the food intake reduction observed with both hormones. The hypothalamus expresses both glucagon and GLP-1 receptors (34,35), and intracerebroventricular glucagon and GLP-1 are both capable of reducing food intake (36,37), suggesting that peripheral glucagon and GLP-1 could exert a direct effect on the hypothalamus after crossing the incomplete blood-brain barrier at the median eminence. A second mechanism might be via activation of vagal afferents to the brainstem, as vagotomy attenuates the anorectic effect of glucagon and GLP-1 after peripheral administration (38,39). A third mechanism for food intake reduction might be via indirect effects on other gut hormones. Coadministration of GLP-1 (0.8 pmol/kg/min) and glucagon (14 pmol/kg/min) causes a significant reduction in circulating levels of the orexigenic hormone ghrelin (5). The current study corroborates these findings at a lower dose of GLP-1 (0.4 pmol/kg/min) and a far

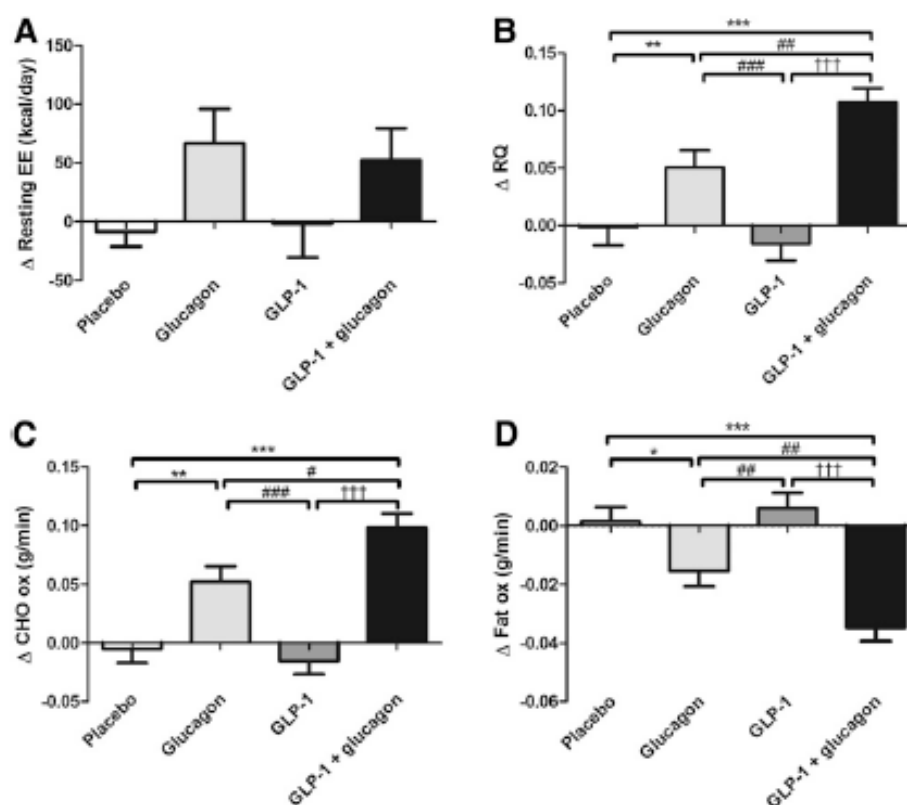


Figure 5—The effects of glucagon and GLP-1, alone and in combination, on REE (A), RQ (B), carbohydrate oxidation (CHO ox) (C), and fat oxidation (Fat ox) (D). Mean absolute change for each parameter (± SEM) between baseline and infusion phases plotted. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with placebo; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with glucagon; ††† $P < 0.001$ compared with GLP-1. EE, energy expenditure.

lower dose of glucagon (2.8 pmol/kg/min). In our study, neither GLP-1 nor glucagon, alone or in combination, affected plasma PYY levels. Näslund et al. (40) demonstrated a small inhibitory effect of GLP-1 on PYY secretion, where an infusion of 0.75 pmol/kg/min reduced postprandial PYY levels by 4–5 pmol/L. In contrast, our study examined fasting PYY levels and used a smaller dose of GLP-1. Thus, it appears that GLP-1 and glucagon, at the doses used here, can modulate ghrelin secretion but not PYY.

The hyperglycemic effect of glucagon is undesirable in patients with diabetes or impaired glucose tolerance, albeit the greatest element results from a one-off stimulation of glycogenolysis, which would be expected to decline with time. Coadministration with GLP-1 attenuates this hyperglycemia, consequent on enhanced insulin release and glucose disposal (5). Both GLP-1 and glucagon act directly on the β -cell to release insulin and, in addition, the hyperglycemia itself is a stimulus for insulin release (41). GLP-1's insulinotropic effects are dependent on the prevailing glucose level (10), which accounts for the relatively small insulinotropic response observed during GLP-1 infusion alone, as glucose levels

tend to decline after the start of the infusion (Fig. 2A). The insulinotropic response with the coinfusion is much larger in amplitude owing to the triple effect of GLP-1, glucagon, and hyperglycemia. The insulin level during the coinfusion is sustained even when glucose returns to ≤ 5 mmol/L at 70 min (Fig. 2A and B) because glucagon continues to exert an insulinotropic effect independent of the prevailing glucose level (42). Therefore, the addition of GLP-1 to glucagon in the doses used for our coinfusion is able to neutralize the undesirable hyperglycemic effect of glucagon alone.

Moreover, the reduction in food intake seen with the combination infusion is likely to contribute to the attenuated postprandial glycemic response. The postmeal glucose response to GLP-1 alone is attenuated compared with placebo. However, the rise in insulin is delayed with infusion of GLP-1, suggesting that this is not an incretin effect. This phenomenon may be related to delayed gastric emptying with GLP-1. Analysis of the glucose and insulin response to the meal is complex, as the subjects ate different amounts. Further studies examining the effect of glucagon and GLP-1 combination on the glucose and insulin response to a standardized calorie load are

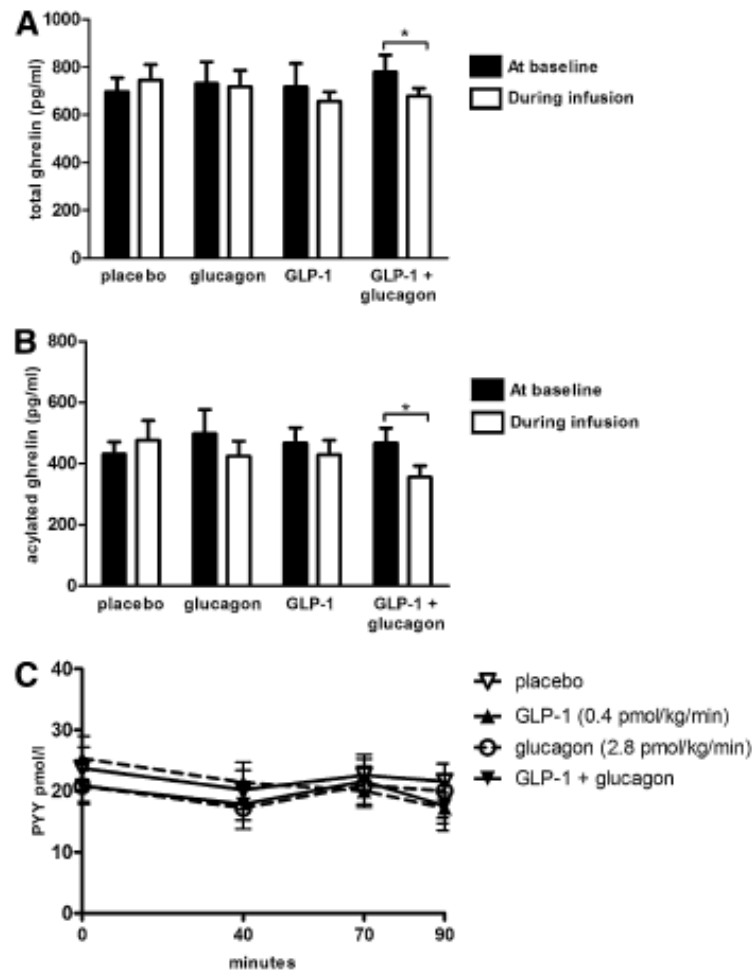


Figure 6—The effects of glucagon and GLP-1, alone and in combination, on total ghrelin (A), acylated ghrelin (B), and PYY (C) levels. A and B: Mean $\pm$ SEM plasma total and acylated ghrelin levels at baseline (0 min) and during infusion (90 min). * $P < 0.05$. C: Mean $\pm$ SEM plasma PYY levels at 0, 40, 70, and 90 min.

warranted in order to formally assess the effects on carbohydrate tolerance, particularly in diabetic patients who may have compromised β -cell reserve.

Consistent with our previous study, we demonstrated an increase in REE of ~ 50 kcal/day in both the glucagon alone and combination infusion groups, although this did not reach statistical significance. We also found a rise in RQ, rise in carbohydrate oxidation rate and fall in fat oxidation rate with glucagon alone and combination infusion, likely to be related to the relative substrate availabilities of glucose versus free fatty acid (5). The fact the rise in REE did not reach statistical significance was not unexpected in this current study, since the dose of glucagon used was a fifth that of our previous study (5). We speculate that the chronic sustained effects of this small increase in REE would have an important impact on body weight in the long-term when combined with the food intake reduction. The mechanism behind the increase in

REE mediated by glucagon remains unclear. This phenomenon could be mediated by increased thermogenesis in brown adipose tissue (6) and/or by futile substrate cycling (43). These effects may be direct, via tissue glucagon receptor (e.g., in brown adipose tissue), or indirect, via an increase in catecholamines (44).

We also found a rise in RQ, a rise in carbohydrate oxidation rate, and a fall in fat oxidation rate with glucagon alone consistent with our previous study and likely to be related to the relative substrate availabilities of glucose versus free fatty acids (5). In contrast, GLP-1 alone caused a small reduction in carbohydrate oxidation and a small increase in fat oxidation consistent with previous studies (45). Interestingly, coinfusion caused an increase in RQ, increase in carbohydrate oxidation, and decrease in fat oxidation with magnitudes approximately double those seen with glucagon alone. This phenomenon is consistent with our observation of a similar increase in

RQ, increase in carbohydrate oxidation, and decrease in fat oxidation with the combination compared with glucagon alone, although there was no significant statistical difference at the higher doses used in our previous study (5). It is possible that the combination of GLP-1 with glucagon may increase carbohydrate oxidation through a combined stimulation of glycolysis and glycogenolysis, and this requires further study.

In conclusion, this study reports that coadministration of glucagon and GLP-1, at doses which are individually subanorectic, significantly reduces food intake in humans. Furthermore, the coadministration of GLP-1 ameliorates the hyperglycemia of glucagon. These data are consistent with findings seen with acute infusion of OXM (16), suggesting that the anorectic and energy expenditure effects of OXM can be explained by costimulation of both the GLP-1 and glucagon receptors. These observations provide support for the further development of GLP-1/glucagon receptor coagonists as a therapeutic approach for obesity. This study has only examined the acute effects of GLP-1/glucagon coagonism, and further chronic studies need to be performed in humans to establish a therapeutically useful anorectic effect without exerting nausea as well as maintaining euglycemia. Establishing these effects will be the key to therapeutic exploitation.

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