

Type 2 Diabetes Mellitus and Glucagon Like Peptide-1 Receptor Signalling

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Abstract

It has been estimated that approximately 8.4% of the world population currently live with diabetes mellitus and type 2 diabetes is the most common form. Type 2 diabetes increases the risk of complications such as heart attacks, blindness, amputations and kidney failure. Glucagon like Peptide-1 (GLP-1) is an effective insulinotropic agent and therefore its effects on insulin secretion have been greatly examined for more than two decades. It is a polypeptide hormone secreted by the intestinal L-cells into the blood in response to food uptake. GLP-1 has a very short half-life *in vivo* due to the rapid proteolytic degradation by Dipeptidyl Peptidase IV (DPP-IV). Therefore DPP-IV resistant GLP-1 analogues, Exenatide and Liraglutide, have been developed and are currently being used in the treatment of type 2 diabetes. GLP-1 agonist functions by binding to its receptor, GLP1R, on the cell surface.

The GLP-1R belongs to the class B peptide receptor family based on its structure and function. The binding of GLP-1 to its receptor results in activation of Gas coupled adenylyl cyclase and the production of cyclic Adenosine Monophosphate (cAMP), which enhances glucose-induced insulin secretion. Continuous GLP-1R activation also causes insulin secretion and pancreatic islet β -cell proliferation and neogenesis. The GLP-1R is internalised following its activation, which regulates the biological responsiveness of the receptor. Structurally the GLP-1R contains a large N-terminal extracellular domain (TM1-TM7) joined by three intracellular loops (ICL1, ICL2, ICL3) and three extracellular loops (ECL1, ECL2, ECL3), and an intracellular C-terminal domain. These domains play critical roles in GLP-1R trafficking to the cell surface, and also in agonist dependent activation and internalisation of the receptor. This review is focused on type 2 diabetes, its treatment with GLP-1, GLP-1R structure and function, and the physiological affects resulting from GLP-1R activation.

Keywords: Diabetes mellitus; GLP-1; GLP-1R; GPCR; Insulin; Signalling

Introduction

The actions of Glucagon like peptide-1 (GLP-1) have been greatly examined over the last twenty years, due to the hormones effectiveness at lowering blood glucose levels and increasing insulin secretion in type 2 diabetic patients [1,2]. GLP-1 exerts its actions through the GLP-1 Receptor (GLP-1R), a family B G-Protein Coupled Receptor (GPCR) which mediates its effects through the Gas subunit, which in turn activates Adenylyl Cyclase (AC). The involvement of Gas and subsequent accumulation of cyclic Adenosine Monophosphate (cAMP) in glucose-induced insulin secretion is well established [3].

Type 2 Diabetes

Background

The World Health Organization describes diabetes mellitus as a “metabolic disorder of multiple aetiology characterised by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both” [4,5]. It was estimated that 366 million people (8.4% of the world’s adult population) lived with diabetes in 2011 [6]. This number will continue to rise and has been estimated to reach 439 million by 2030 [7]. Diabetes remains the leading cause of blindness, end stage renal disease, lower limb amputation, and cardiovascular disease [8,9]. Diabetes mellitus is classified into four categories, type 1, type 2, other specific types and gestational diabetes (Table 1) of which type 2 is the most common form [4,5,10].

Pathophysiology and causes

Insulin is a hormone that is secreted in response to food uptake to maintain glucose homeostasis. It is produced by the β -cells in the islet of Langerhans in the pancreas [11]. Synthesis occurs on the rough Endoplasmic Reticulum (ER) as preproinsulin, containing a signal peptide that is cleaved to form proinsulin (Figure 1). Proinsulin then

Types	Description and Subtypes
Type 1	β -cell destruction, usually leading to absolute insulin deficiency Autoimmune Idiopathic
Type 2	Ranging from predominantly insulin resistant with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance
Other Specific Types	Genetic defects of β -cell function Genetic defects in insulin action Diseases of the exocrine pancreas Endocrinopathies Drug- or chemical induced Infections Uncommon form of immune-mediated diabetes Other genetic syndromes sometimes associated with diabetes
Gestational Diabetes	Carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy

Diabetes mellitus is classified into four categories, type 1, type 2, other specific types and gestational diabetes

Table 1: Aetiological classification of disorders of glycaemia.

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Received October 08, 2013; Accepted October 25, 2013; Published November 01, 2013

Citation: Thompson A, Kanamarlapudi V (2013) Type 2 Diabetes Mellitus and Glucagon Like Peptide-1 Receptor Signalling. Clin Exp Pharmacol 3: 138. doi:10.4172/2161-1459.1000138

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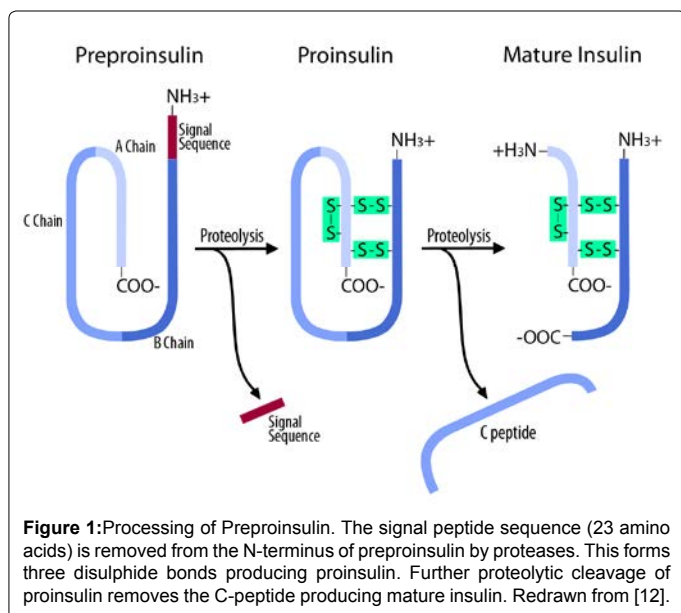


Figure 1: Processing of Preproinsulin. The signal peptide sequence (23 amino acids) is removed from the N-terminus of preproinsulin by proteases. This forms three disulphide bonds producing proinsulin. Further proteolytic cleavage of proinsulin removes the C-peptide producing mature insulin. Redrawn from [12].

traffics through the trans golgi network and packaged into secretory vesicles until required [12]. The hydrolysis of dietary carbohydrates such as starch or sucrose within the small intestines results in the production of glucose that is then absorbed into the blood. An increase in glucose concentrations in the blood stimulates the release of insulin. Insulin has different effects depending on the target tissue; it either facilitates the entry of glucose into adipose and muscle tissue or stimulates the liver to store glucose as glycogen. If insulin is absent or in low concentrations within the body, insulin sensitive cells are unable to absorb glucose and therefore use other fuel sources such as fatty acids for energy. When blood glucose levels are low, insulin is not produced and instead glucagon is secreted, broken down and released as glucose [13,14].

In normal individuals, glucose homeostasis keeps glucose levels under control and within the normal range of 80 to 120 mg/100 ml (4.4-6.7 mM). For patients with diabetes, insufficient insulin release results in hyperglycemia and high blood glucose levels [14,15]. An absolute lack of insulin producing β -cells in the pancreas results in the development of type 1 diabetes [4,5,16]. In contrast, type 2 diabetes is a result of insulin dependent cells not being able to respond to insulin effectively, also known as insulin resistance [4,5]. It is being noticed that type 2 diabetes only develops in patients who are insulin resistant with the onset of β -cell dysfunction [17].

It is estimated that 60-90% of patients with type 2 diabetes are obese, and obesity itself can cause or aggravate insulin resistance [18]. There is a greater than 90-fold increase in developing type 2 diabetes if you are obese [19]. Body Mass Index (BMI) is defined as the individual's body weight divided by the square of their height (kg/m^2), and a BMI greater than 25 is overweight and above 30 is obese [20]. It is suggested that patients with type 2 diabetes aim for a BMI of 25 or below [21]. However, there is still a 2.4-fold increased risk of developing type 2 diabetes in those who are of normal BMI (between 18.5 and 24.9) but have an increased percentage of body fat distributed in the abdominal region [22-24].

Diet, sedentary lifestyle and genetics all play a role in the development of type 2 diabetes [7,25,26]. The dietary intake of saturated fat, trans-fatty acids and total fats was considered risk factors in the development of type 2 diabetes. In contrast dietary fibres or

non-starch polysaccharides were considered protective factors [25]. Low-fat vegetarian and vegan diets have the potential to be used for the management of type 2 diabetes because they are associated with weight loss; improve cardiovascular health and increase insulin sensitivity [27,28]. A cohort study evaluated the association of multiple lifestyle factors, including diet, physical activity, alcohol use, smoking habits and adiposity measures, with the risk of developing type 2 diabetes. It was found to be approximately 50% lower in individuals whose physical activity and dietary habits indicated low risk and approximately 80% lower in those whose physical activity, dietary habits, alcohol use and smoking habits all indicated low risk [24,29]. In addition, having relatives with type 2 diabetes substantially increases the chance of developing type 2 diabetes. The Insulin Receptor Substrate-1 (IRS-1) gene has been associated with type 2 diabetes, insulin resistance and hyperinsulinemia in a large scale study that screened 14,000 people all around the world [30].

Signs and symptoms

Type 2 diabetes often develops slowly from a condition of pre-diabetes, and symptoms may not be apparent for years [7]. The characteristic symptoms of type 2 diabetes including; dehydration, blurred vision, excessive thirst, polydipsia (increased fluid intake) and polyuria (excessive urine production); can develop because of hyperglycaemia. In diabetes, insulin producing β -cells are either partially or completely unable to use glucose as a fuel and therefore switch to using fat, carbohydrates and protein metabolism as a fuel source instead. This process requires more energy and leads to polyphagia (excessive eating), weight loss and lethargy [4,5,31]. Additionally, hyperglycaemia can lead to skin infection as a result of open and slow healing sores because it is more difficult for the body to heal itself [32].

Serious long-term complications of type 2 diabetes include nerve dysfunction, cardiovascular disease, microvascular damage, renal failure, blindness, impotence and poor wound healing and are a result of prolonged hyperglycaemia [4,5,33]. These complications may also occur if the disease is controlled inappropriately. Hypoglycemia is caused by inaccurately administered insulin. A shortage of insulin causes the body to switch to metabolising fatty acids and as a result produces ketone bodies. This response results in ketoacidosis and causes dehydration in addition to many of the symptoms and complications already described [34]. Another metabolic complication is known as hyperglycaemia hyperosmolar state and is the end result of sustained osmotic diuresis. It is characterised by severe hyperglycaemia, hyperosmolarity, and dehydration, but without the significant ketoacidosis hyperosmolar hyperglycemic state [34,35].

Diagnosis

Diabetes is diagnosed by recurrent or persistent hyperglycaemia. This can be demonstrated by any of the following criteria: a fasting plasma glucose level of 7.0 mM; a single plasma glucose reading in excess of 11.1 mM; and an Oral Glucose Tolerance Test (OGTT) administered two hours after 75 g oral glucose with fasting plasma glucose concentrations in excess of 11.1 mM [4,5].

Glycosylation of haemoglobin (HbA1c) is primarily used as a treatment-tracking test and reflects average glucose levels over 8-12 weeks [36,37]. Measurements can be performed at any time and there is no need for fasting. It is recommended that HbA1c be used to measure blood glucose control in both pre-diabetics and patients with diabetes. A reading of 6.5% HbA1c or above is used to diagnose diabetes [37]. OGTT or intravenous glucose tolerance tests are used

to determine insulin response of the pancreas and degree of insulin resistance. However, it was noted that glucose administered orally promoted a significantly greater insulin response than glucose administered intravenously (Figure 2), although plasma glucose levels were the same [38-40]. Further, cross reactivity with partially degraded proinsulin and insulin may occur and as a result insulin measurement may be problematic. It is especially problematic in patients who have developed anti-insulin antibodies through administering animal insulin. As a result, C-peptide concentration has been used as a semi quantitative measure of β -cell secretory activity instead of insulin itself. C-peptide has a half-life 2.5 times longer than insulin and therefore higher concentrations exist in the peripheral circulation and levels fluctuate less [41].

Approaches and goals for the treatment of type 2 diabetes

More intensive glucose control, mainly determined by HbA1c levels, can delay or prevent the development and progression of serious complications in type 2 diabetics [33]. Initial treatment of type 2 diabetes generally begins with non-pharmacological interventions such as diet, lifestyle and exercise. These interventions combined with antihyperglycaemic agents (such as metformin) are expected to improve blood glucose control. If an HbA1c level greater than 7% is not achieved within 2-3 months, then the recommended second stage is the addition of hypoglycaemic agents (such as sulfonylureas) or insulin injections to the treatment. Hypoglycaemic agents reduce plasma glucose levels by increasing insulin secretion, reducing insulin resistance and/ or delaying glucose absorption in the gut [42-44].

In many cases treatment with either antihyperglycaemic or hypoglycaemic agents is not usually enough to achieve adequate blood glucose control and therefore insulin therapy is intensified [42,45,46]. However, insulin therapy has a number of risks associated with it including, hypoglycaemia, weight gain, and increased risk of colorectal cancer [47]. These risk factors together with the route of administration (usually subcutaneous injection); contribute too many patients being reluctant to maintain intensive insulin therapy [48].

Consequently, these classic treatments are often unsatisfactory and there is an imperative need for new classes of glucose lowering agents. Recently, incretin-based therapies have been used in the treatment of type 2 diabetes; namely Exenatide and Liraglutide. These drugs have the ability to preserve normal physiological responses to food intake and improve glycaemic control.

GLP-1 Treatment for Type 2 Diabetes

Incretin hormones

Incretins are gastrointestinal hormones that contribute to postprandial insulin release [40,49]. GLP-1 and Glucose-dependent Insulinotropic Polypeptide (GIP) are two major incretins and are thought to be responsible for up to 70% of insulin secreted from the β -cells of the pancreas following food intake. This increase in insulin is called the 'incretin effect' and maintains glucose concentrations at low levels irrespective of the amount of glucose ingested. This is achieved by increasing the sensitivity of β -cells to glucose [50]. The 'incretin effect' has been shown to be either reduced or absent in type 2 diabetics due to the loss of insulinotropic activity of GLP-1 and GIP. However, more recently it's been suggested that the secretion of GIP and GLP-1 is normal in type 2 diabetic patients [51]. This strongly suggests a role for incretin hormones or their actions in the treatment of type 2 diabetes [39,52-55].

The GIP gene is mainly expressed in K-cells and enterochromaffin

cells of the proximal small intestine. GIP secretion is stimulated by enteral glucose, lipids and products of meal digestion in a concentration dependent manner [56]. In patients with type 2 diabetes, GIP concentrations after food intake are either normal or slightly elevated. GIP infusion does not reduce plasma glucose concentrations in patients with type 2 diabetes. As a result GIP has not been thought of as a suitable candidate for therapeutic development [57,58]. In contrast, patients with type 2 diabetes have decreased GLP-1 activity [53,55,59]. It is currently unknown whether reduced GLP-1 activity is a cause or consequence of diabetes. First degree relatives of patients with type 2 diabetes have normal GLP-1 activity in response to glucose, this suggests that a reduction in GLP-1 activity seen in type 2 diabetic patients is more likely acquired [60,61]. Additionally, GLP-1 is able to stimulate glucose-dependent insulin secretion in type 2 diabetic patients under hyperglycaemic conditions [54,62,63]. Furthermore, administration of exogenous GLP-1 to type 2 diabetic patients leads to normalisation of hyperglycaemic conditions [54,64,65]. As a result, GLP-1 based strategies appear an interesting and more suitable target for the treatment of type 2 diabetes [66].

Synthesis and secretion of GLP-1

GLP-1 is 42 amino acids in length and is synthesised from the posttranslational modification of proglucagon, by Prohormone Convertase 1 (PC1) within the intestinal L-cells. PC1 is specific to GLP-1 production in the L-cells [67,68]. The proglucagon gene (Figure 3A) is expressed in both the pancreatic α -cells and in the intestinal L-cells, but posttranslational processing differs in these two tissues [69-71]. In the pancreatic α -cells (Figure 3B), proglucagon is processed to glucagon, Intervening Peptide-1 (IP1), Major Proglucagon Fragment (MPGF), and Glucantin-related Pancreatic C-peptide (GRPP) by Prohormone Convertase 2 (PC2) [68,72]. In the intestinal L-cells (Figure 3C), proglucagon is cleaved to GLP-1, Glucagon like Peptide-2 (GLP-2), Intervening Peptide-2 (IP2), oxyntomodulin and glucantin by PC1 [73-75]. However, emerging evidence suggests that pancreatic α -cells can also adapt to produce GLP-1 with recombinant expression of PC1 [76-78].

In secretory vesicles, the first six amino acids of GLP-1 are cleaved from the N-terminus forming the bioactive peptides. Approximately 80% of truncated GLP-1 forms the predominantly secreted GLP-1 (7-36)-NH₂ and the remaining 20% is released as GLP-1 (7-37) (Figure 3D) [79]. Both GLP-1 (7-36)-NH₂ and GLP-1 (7-37) bind to the GLP-1R with similar affinity and shows similar potency [80]. GLP-1 is produced in response to food intake, in particular glucose

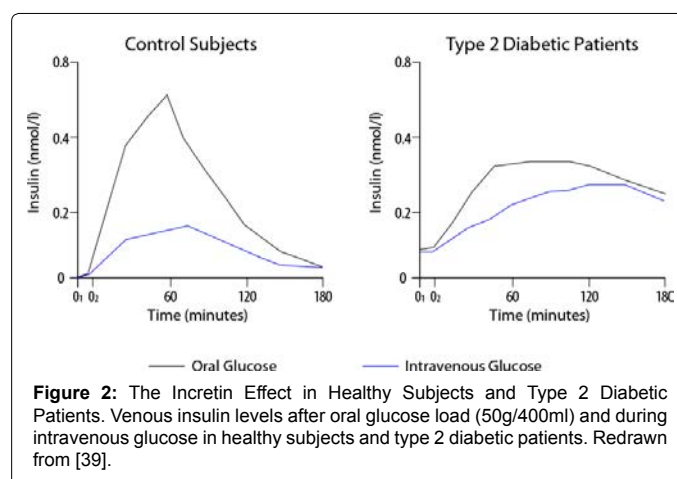
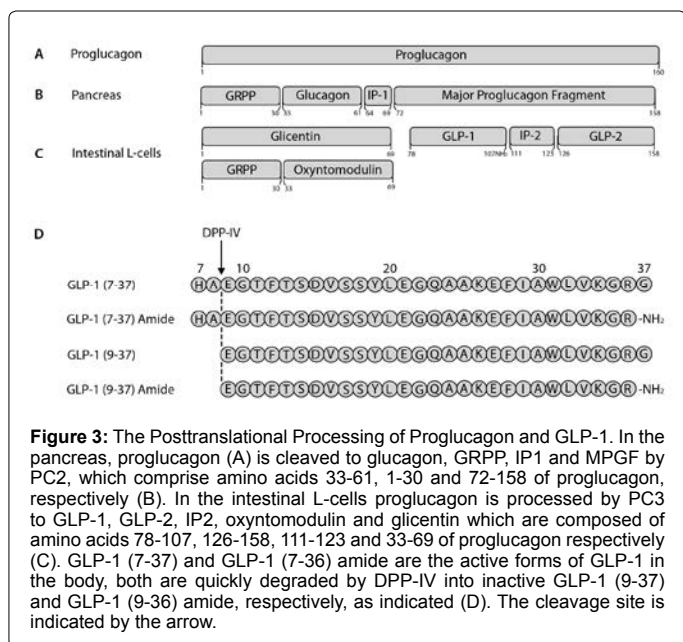


Figure 2: The Incretin Effect in Healthy Subjects and Type 2 Diabetic Patients. Venous insulin levels after oral glucose load (50g/400ml) and during intravenous glucose in healthy subjects and type 2 diabetic patients. Redrawn from [39].



and triacylglycerols, and lowers blood glucose levels [49,81]. In times of fasting, GLP-1 plasma concentrations are very low and can be lowered even further with administration of somatostatin in humans, suggesting there are some basal rates of secretion [69]. Typically, 'total' GLP-1 concentrations are about 5-15 pmol/l in basal state, rising to about 20-60 pmol/l after food intake [49]. The secretion of GLP-1 from L-cells increases in about 10 minutes after food intake, which is later than the 'cephalic phase' stimulation of insulin secretion. This suggests that neuronal signals generating insulin release does not influence GLP-1 secretion. Evidence suggests that the presence of nutrients in the gut and the interaction with the microvilli of L-cells are responsible for GLP-1 secretion [69].

In vivo, GLP-1 has a very short half-life of 1.5 minutes due to the rapid proteolytic degradation by enzyme Dipeptidyl Peptidase-IV (DPP-IV) [82-85]. This enzyme cleaves the active GLP-1 (7-36) to its inactive GLP-1 (9-36) form by removing two amino acids at the N-terminus of the peptide [86-88]. GLP-1 (9-36) and GLP-1 (9-37) (Figure 3D) have both been identified as products of GLP-1 cleavage by DPP-IV action *in vitro* and *in vivo* [84]. The degradation occurs so quickly that less than 25% of active GLP-1 that is secreted enters the portal vein prior to reaching the liver [89]. As a result, it is estimated that approximately 85% of circulating postprandial GLP-1 is either GLP-1 (9-36) or GLP-1 (9-37) [90].

Biological activities of GLP-1

GLP-1 has several actions in various tissues and exerts its effects through its cell surface receptor, the GLP-1R (Figure 4). Structurally related members of the glucagon family of peptides such as GLP-2, glucagon and GIP do not bind the GLP-1R at physiologically relevant concentrations [69]. The human GLP-1R gene is transcribed in pancreatic islet, brain, heart, intestine, kidney, liver, lung and stomach. However, the actions of GLP-1 in fat and muscle most likely occur through indirect mechanisms and do not occur in many species [91-94]. The expression of the GLP-1R is consistent with the roles of GLP-1 in glucose homeostasis, β -cell proliferation, heart rate, food intake and appetite and even learning [91].

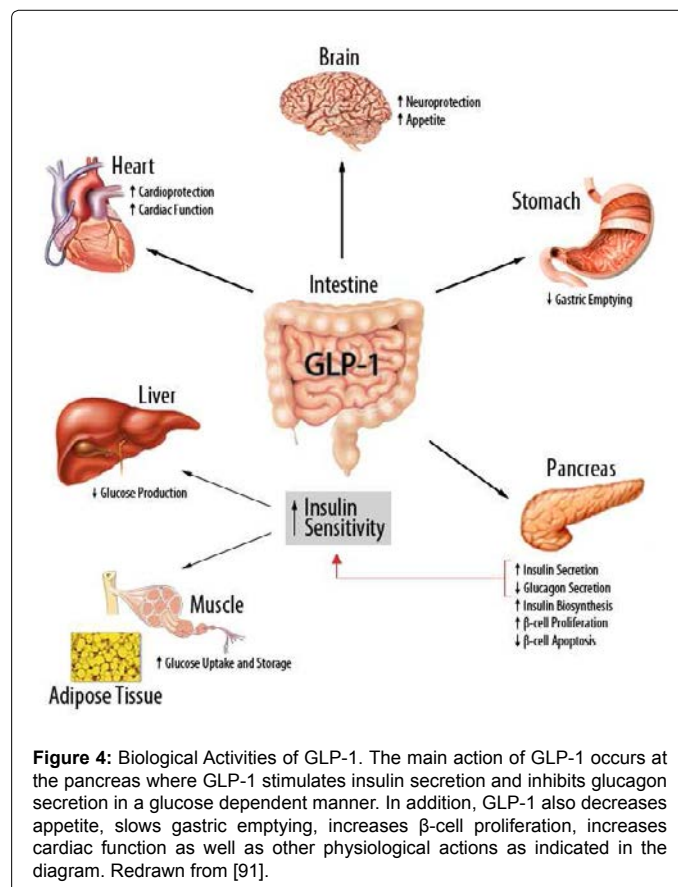
In the pancreas, GLP-1 increases insulin secretion from islet

β -cells and suppresses glucagon secretion for islet α -cells, in a glucose dependent manner [95,96]. Additionally, GLP-1 has been shown to promote β -cell proliferation and neogenesis, while preventing apoptosis [97-99]. In the gastro intestinal tract, GLP-1 delays gastric emptying and acts as a postprandial satiety signal to the brain to suppress appetite and food intake [56,100]. Furthermore, GLP-1 plays an important role in the enteric and central nervous system. The release of GLP-1 is tightly regulated and involves the gut-to-brain and the brain-to-periphery axis [101-103]. Pharmacological applications of GLP-1 have demonstrated a number of positive affects in the cardiovascular system, suggesting GLP-1 may play an important role in that system [104]. Additionally, evidence suggests GLP-1 and its receptor may modulate components of the insulin signalling pathway and decrease hepatic steatosis *in vitro* [94].

Interestingly, evidence is emerging to suggest GLP-1 (9-36) and GLP-1 (9-37), the inactive forms of GLP-1, have insulin-like actions on heart, liver and vasculature and strongly reduce activity on the GLP-1R of β -cells. It has therefore been proposed that they may act through a novel signalling pathway by binding to a different cell surface receptor [105].

GLP-1 based therapies

The binding of GLP-1 to its receptor results in insulin secretion from pancreatic β -cells, making them an important target in the treatment of type 2 diabetes. The biological and pharmacological activities of GLP-1 have been the basis for two type 2 diabetic therapies. The first therapy is based on the use of DPP-IV inhibitors to prevent the breakdown of GLP-1 from its active to inactive form [66]. The second therapy is based on the use of DPP-IV resistant GLP-1 mimetics that replicate the



physiological actions of the native GLP-1 peptide but with a longer half-life. DPP-IV, also named adenosine deaminase complexin G-protein or CD26 (cluster of differentiation 26), is an antigenic enzyme. It is associated with signal transduction, immune regulation and apoptosis and therefore is expressed on the surface of most cell types. DPP-IV is highly specific and cleaves between X-proline and X-alanine dipeptides (where X is any amino acid) at the N-terminus, but is unable to cleave peptides with a third proline (for example glycine-proline-proline) [84]. DPP-IV inhibitors increase GLP-1 levels by 2-3 fold over 24 hours by inhibiting 90% of plasma DPP-IV activity *in vivo*. They also have an additional advantage of oral administration [106]. There are currently three DPP-IV inhibitors, saxagliptin, sitagliptin and vildagliptin, used in the treatment of type 2 diabetes in Europe [107]. These inhibitors significantly decrease postprandial glucose levels and HbA1c by 0.5-1.0% [66,108]. Sitagliptin and vildagliptin have been shown to improve β -cell function and reduce systolic blood pressure [109]. However, the long-term inhibition of DPP-IV may have adverse effects because this enzyme is expressed in many types of tissues and has many functions [110]. Experimental evidence has demonstrated an increase in infection and some tumours, supporting adverse immunological and oncological effects after prolonged use of DPP-IV inhibitors [111].

The main limitation of GLP-1 is a very short half-life (1.5 minutes) due to the rapid proteolytic degradation of GLP-1 by DPP-IV, cleaving the active GLP-1 (7-36) to the inactive GLP-1 (9-36) form [82,83,85]. DPP-IV cleaves GLP-1 between alanine and glutamic acid at positions 8 and 9. A substitution at position 8 from alanine to valine (Ala⁸Val) stabilises the peptide without affecting its activity and prevents peptide degradation. However, the half-life of the modified peptide is still too short (4-5 minutes) to be used as a drug [112]. As a result therapeutic strategies that activate the GLP-1R and improve GLP-1 actions have been extensively studied and developed. This has led to the development of two DPP-IV resistant GLP-1R agonists, Liraglutide and Exenatide. Liraglutide is a long-acting GLP-1 analogue with 97% sequence homology to human GLP-1 [113]. It is chemically similar to human GLP-1 but with structural modifications resulting in resistance to GLP-1 inactivation by DPP-IV and prolonged duration of action [114]. Liraglutide has a half-life of approximately 11-13 hours and it is administered once a day irrespective of meal times [115]. Exenatide is a peptide found within the salivary glands of the Gila monster lizard and has 52% sequence homology to GLP-1 [116]. It is also not enzymatically degraded by DPP-IV and therefore has a prolonged *in vivo* half-life of 3.4-4 hours compared with GLP-1. As a result it is administered twice daily within 60 minutes of a meal [117]. Both GLP-1R agonists are currently in use as drugs for the treatment of type 2 diabetes, as they are effective insulinotropic agents regulating blood glucose levels by increasing insulin secretion and suppressing glucagon secretion in a glucose dependent manner [118,119]. Liraglutide and Exenatide significantly reduce both fasting and postprandial glucose levels and HbA1c levels by 0.8-1.5% [113]. The most common side effects of GLP-1 strategies are dyspepsia or nausea, which may lead to delayed gastric emptying. However, the effects seem to subside with continuous administration [66,120]. Acute pancreatitis has been reported in a few rare cases but their clinical significance remains unclear [121]. These side effects associated with the long-term administration of these peptides have necessitated the search for orally active small molecule agonists of the GLP-1R [122].

A series of eleven-amino acid peptide, agonists of the GLP-1R, have been reported to have excellent potency and *in vivo* activity in ob/ob mouse models of diabetes [123,124]. These peptides are closely related structurally to nine C-terminal residues of GLP-1 but are

substituted with several unnatural amino acids at position 11, such as homohomophenylalanine. This gives rise to the opportunity to increase stability against proteolytic degradation by DPP-IV. However, the activity of these peptides can be blocked with inactive exendin (9-39) (exendin antagonist) [124].

The GLP-1R in Type 2 Diabetes

Characterisation of the GLP-1R

Regulation of GLP-1R expression and function is clinically important because of its role in GLP-1 based therapy for type 2 diabetes. The GLP-1R belongs to a group of receptors known as GPCRs (also named seven transmembrane receptors). They are the largest family of cell surface receptors and are the most common target for medical therapeutics due to their involvement in many physiological and pathological processes. Over 50% of drugs available on the market act on GPCRs [125]. All GPCRs are made up of a single polypeptide chain of up to 1100 amino acid residues, which pass through the plasma membrane seven times. This membrane topology results in an extracellular N-terminal domain, seven transmembrane α -helices joined by three Extracellular Loops (ECL) and three Intracellular Loops (ICL) followed by an intracellular C-terminal domain that interacts with G proteins (Figure 5). GPCRs are classically divided into three classes: A, B and C based on their sequence homology and functional similarities [126].

The GLP-1R belongs to the family B GPCRs, also known as the secretin receptor family and is made up of only 15 members [126,127]. This family is distinguishable from the other two families by the large N-terminal extracellular domain that is 100-160 amino acids in length and has an important role in agonist binding (Figure 5B). Additionally, this family contains several conserved disulphide bonds in the N-terminus of the receptor, which stabilises the large N-terminal structure [127]. The gene encoding the GLP-1R is located on the short arm of chromosome 6 (6p21) and encodes a 463 amino acid length protein (Figure 6) [128-130]. The GLP-1R is predicted to contain a large hydrophilic N-terminal extracellular domain with a putative signal peptide, seven hydrophobic transmembrane domains (TM1-TM7) joined by three hydrophilic ICL (ICL1, ICL2, ICL3) and three ECL (ECL1, ECL2, ECL3); ending in an intracellular C-terminal domain (Table 2) [131].

Following GLP-1 binding to the GLP-1R, the receptor undergoes a conformational change and transmits extracellular signals through heterotrimeric G-proteins (Figure 7). Heterotrimeric G-proteins are so called because they interact with Guanosine Diphosphate (GDP) and Guanosine Triphosphate (GTP) [132]. Heterotrimeric G-proteins are made up of α , β , and γ subunits and can activate or inhibit a number of effectors. The α subunit ($G\alpha$) consists of an α -helical domain that binds guanine nucleotides, and a GTPase domain that binds and hydrolyses GTP. The $G\alpha$ subunit has been categorised into four families based on similarities within their primary sequence: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, $G_{\alpha 12/13}$. The β and γ subunits are bound in a complex ($G\beta\gamma$) through an N-terminal coil on the $G\gamma$ subunit to the base of the $G\beta$ subunit. The $G\beta\gamma$ subunit binds to hydrophobic pocket in the $G\alpha$ subunit in the inactive state [132]. Agonist-occupied GPCRs activate $G\alpha$, which dissociates from $G\beta\gamma$. The GLP-1R has been shown to activate members of the $G_{\alpha s}$, $G_{\alpha i/o}$ and $G_{\alpha q/11}$ [133,134]. Members of the $G_{\alpha s}$ family activate AC, increasing cAMP levels and in turn activate both Exchange Protein Activated by cAMP (EPAC) and Protein Kinase A (PKA) [135]. Activating members of the $G_{\alpha i/o}$ family inhibit AC activity and regulate inward rectifier potassium channels [136]. $G_{\alpha q/11}$ family

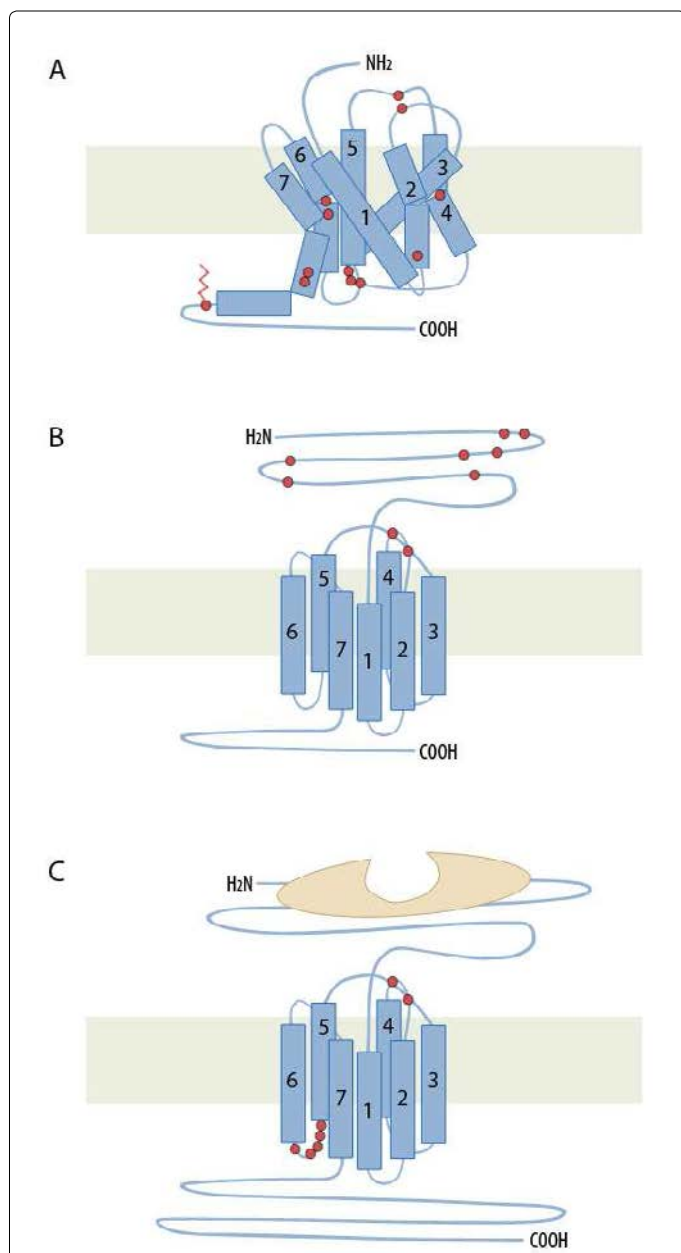


Figure 5: Structure of GPCRs. All GPCRs share a common membrane topology consisting of an NH₂-terminal extracellular domain, seven transmembrane α helices joined by three ECL and ICL and an intracellular COOH-terminal domain. Red circles denote conserved residues. (A) The Family A GPCRs contain a disulphide bridge that connects ECL1 and ECL2 causing the receptor to 'kink' and 'tilt'. The C-terminal domain contains a conserved palmitoylated cysteine residue. (B) The Family B GPCRs are characterized by a long N-terminal tail consisting of many conserved disulphide bonds. (C) The Family C GPCRs have very large N and C- terminal domains with an agonist binding domain described as a 'venus fly trap' located at the N- terminus. Additionally, a conserved disulphide bridge connects ECL1 and ECL2 and a short and conserved ICL3 also define family C GPCRs. Redrawn from [246].

members activates Phospholipase C (PLC) which in turn hydrolyses phosphatidylinositol-4,5-bisphosphate to Inositol-1,4,5-triphosphate (Ins(1,4,5)P₃; IP₃) and Diacylglycerol (DAG). DAG activates Protein Kinase C (PKC) and IP₃ induces intracellular calcium release from the ER [137]. G α 12/13 family members regulate intracellular actin through Rho GTPase activity [138]. The G $\beta\gamma$ complex can also

activate a number of intracellular signalling molecules and pathways including phospholipases and phosphatidylinositol 3-kinase, Ras, Raf, Extracellular signal-regulated Kinase (ERK) and ion channels, but its role in GLP-1R activation is not fully known [136,139].

After stimulation with agonist, most GPCRs internalise from the cell surface to dampen the biological response, resensitisation of the system or propagation of the signal through novel transduction pathways. Agonist induced GPCR internalisation typically occurs in a clathrin-dependent fashion via GRKs (GPCR kinases), β -arrestins and ADP-ribosylation Factor (ARF) proteins (Figure 8) [140,141]. Currently, there is some confusion by which pathway the GLP-1R is internalised. It has been reported that clathrin-coated vesicles mediate GLP-1R internalisation and three PKC phosphorylation sites within the C-terminal tail are important for this to occur (Figure 6) [142]. However, more recently it has been shown that the GLP-1R is internalised by caveolae-mediated endocytosis upon agonist stimulation (Figure 9).

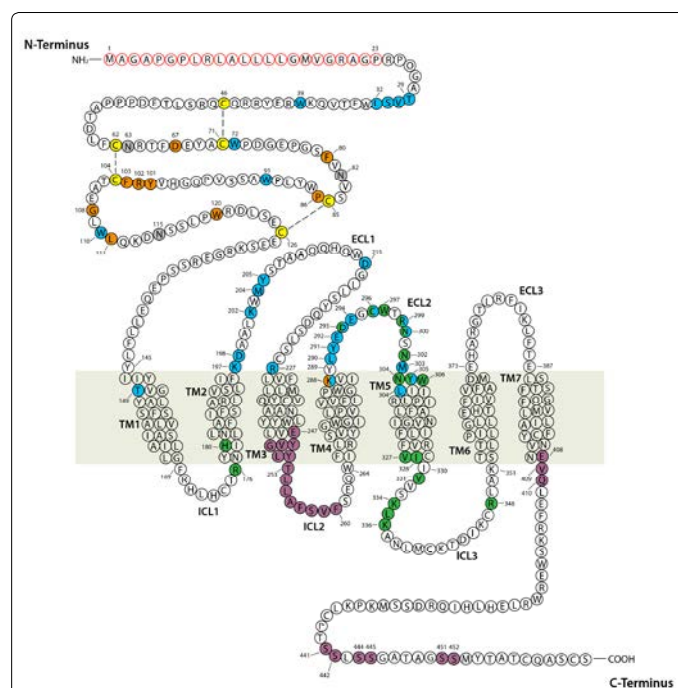
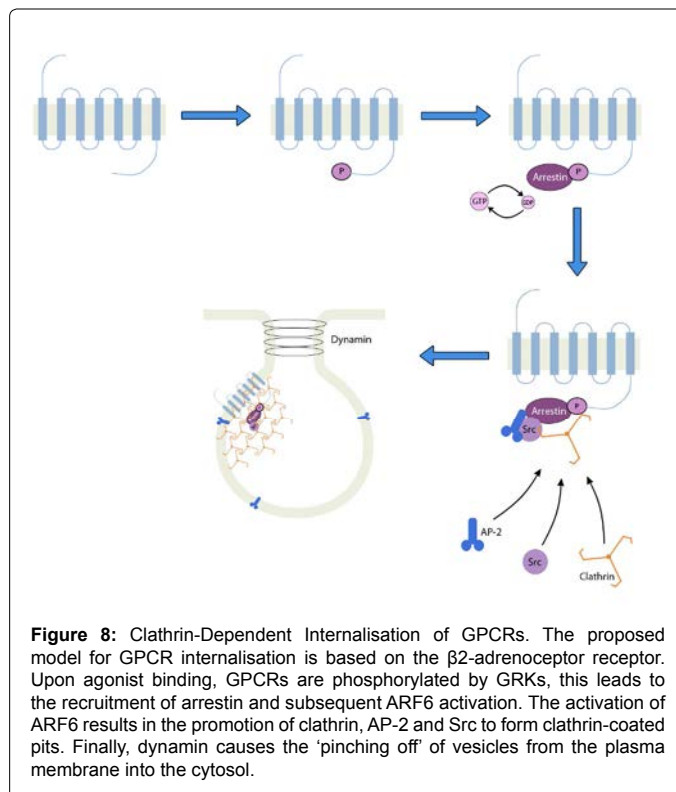
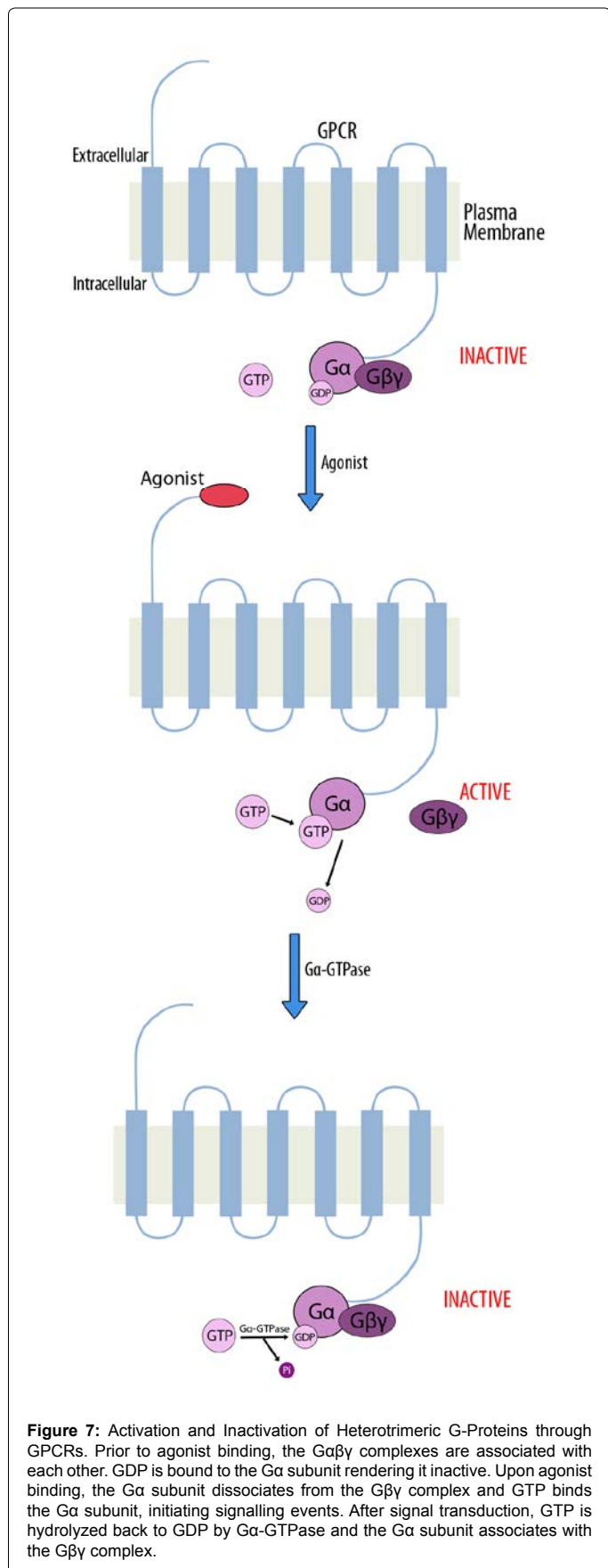


Figure 6: Amino acid Sequence of the Human GLP-1R. The signal peptide is highlighted in red circles (1-23). Residues in yellow highlight conserved cysteine residues that form disulphide bonds. Residues in blue show amino acid important in agonist binding. Amino acids that have a structural role are highlighted in orange. Glycosylation sites are shown in grey. Residues important in receptor internalization are shown in purple and for activation and functions are in green. Redrawn and adapted from [1].

Amino Acids Length (from-to)	Description	Amino Acids Length (from-to)	Description
23 (1-23)	Putative SP	122 (24-145)	NT
23 (146-168)	TM1	8 (169-176)	ICL1
20 (177-196)	TM2	31 (197-227)	ECL1
25 (228-252)	TM3	12 (253-264)	ICL2
24 (265-288)	TM4	15 (289-303)	ECL2
26 (304-329)	TM5	22 (330-351)	ICL3
21 (352-372)	TM6	15 (373-387)	ECL3
21 (388-408)	TM7	55 (409-463)	CT

SP= Signal peptide; NT= N-terminal domain; CT= C-terminal domain

Table 2: The amino acid sequence of the GLP-1R domains.

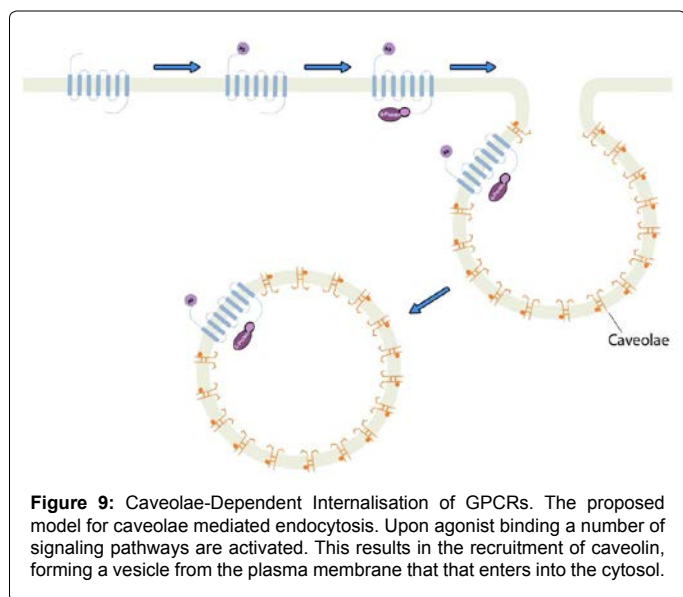


A feature of GPCRs that are endocytosed via caveolae is their ability to bind caveolin-1, a protein weighing approximately 21-24 kDa. Caveolin-1 is the principle component of caveolae and can interact with a number of signalling molecules including receptor tyrosine kinases, G proteins and GPCRs. This occurs via a common caveolin-binding motif, $\Phi X\Phi X X X X \Phi$ and $\Phi X X X X \Phi X X \Phi$, where Φ is an aromatic residue and X is any amino acid [143,144]. Endocytosis in this manner can lead to fission of caveolae enriched vesicles and then fusion with caveosomes, large intermediate intracellular organelles [145]. The GLP-1R was reported to contain a classical caveolin-1 binding motif, ²⁴⁷EGVLYLTLA²⁶⁰FSVF²⁶⁰, in ICL2 (Figure 6) [146].

Recently, there has been increasing interest in the stoichiometry of GPCRs and how this impacts receptor function [147,148]. For family B GPCRs, homodimerisation has been shown to occur with the calcitonin receptor [149], secretin receptor [150] and parathyroid receptor [151]. There is also interest in the development of allosteric agonists and whether they interact with a single receptor (in cis) or across dimers (in trans). Currently, most drug development is dependent on an in cis conformation and mechanism of action [152,153]. The GLP-1R has been shown to form a homodimer through an interface along TM4 and is required for receptor signalling. Alanine substitutions to Leu²⁵⁶, Val²⁵⁹ or Gly²⁵², Leu²⁵⁶, Val²⁵⁹ abolished GLP-1 binding, reduced cAMP and ERK signalling and abolished calcium signalling. Dimerisation of the GLP-1R was important for signal bias and discriminated between peptide and non-peptide activation. Additionally, dimerisation was not required for allosteric modulation by compound 2 demonstrating that this small molecule agonist acted in cis [152].

Allosteric modulation of the GLP-1R

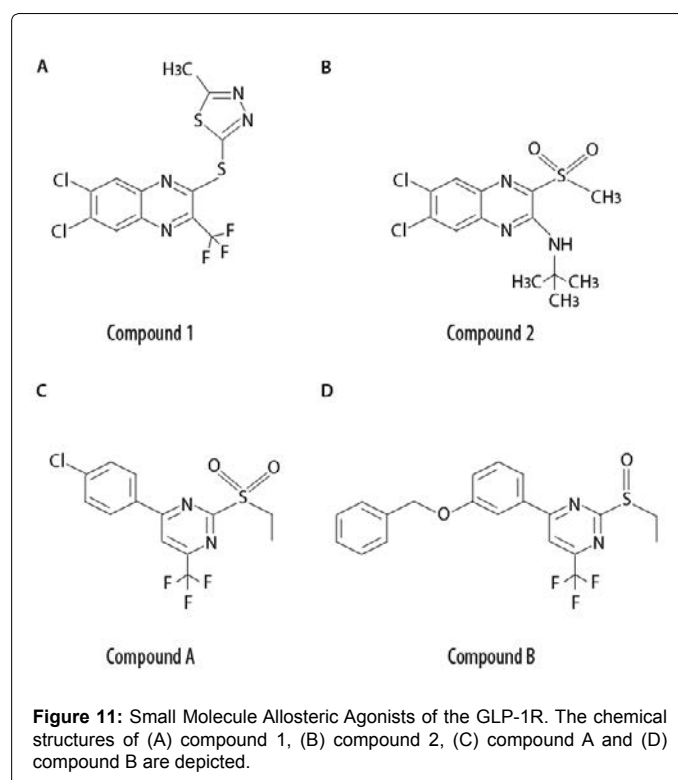
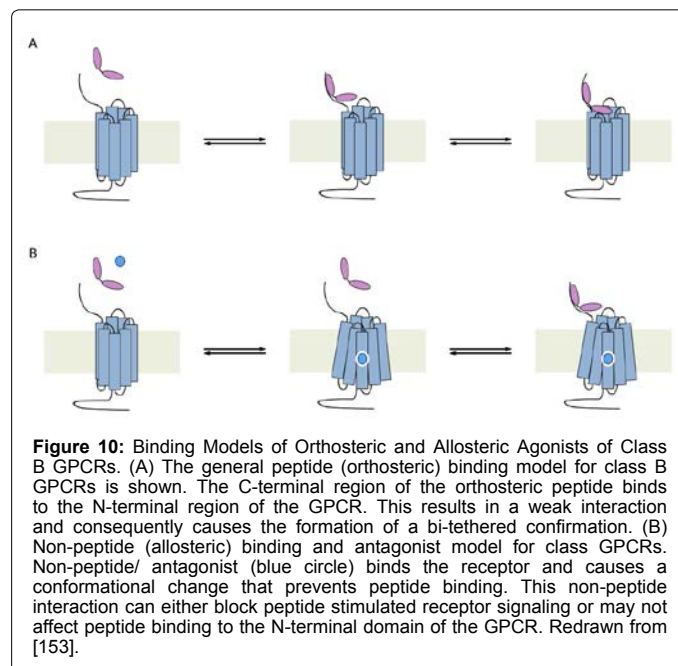
Many GPCRs have been shown to have allosteric binding sites that are spatially and often functionally distinct to the primary agonist (orthosteric) binding site (Figure 10) [154,155]. Small molecule



allosteric agonists can either increase or decrease the binding efficiency of an orthosteric agonist. Such agonists are generally termed positive allosteric modulators or negative allosteric modulators depending on what effects they have on the receptor [156]. Allosteric sites may provide novel therapeutic targets and a number of advantages compared to classical orthosteric agonists. This is advantageous where selective orthosteric therapy has been difficult, for example, where the orthosteric site is highly conserved. Targeting the allosteric sites allows for greater selectivity to be obtained [157,158]. Additionally, allosteric agonists may provide a second advantage in that they can be selectively regulated by endogenous agonists [158]. Finally, low molecular weight agonists that have the potential for oral administration can be used to target allosteric binding sites [154]. Some small molecule agonists, named ago-allosteric agonists, can bind to the receptor and can act as both agonists and allosteric modulators in the absence of orthosteric agonists. It is unknown how these agonists affect the binding or efficiency of compounds acting at the orthosteric site. Compounds with allosteric or ago-allosteric properties increase the potential for receptor subtype selectivity. This allows for more improved, targeted and novel therapeutics [159]. Receptor internalisation and signalling mediated by ago-allosteric agonism may provide further information into the activation and regulation of this receptor.

A small molecule GLP-1R agonist, compound 1 (2-(2-methylthiadiazolylsulfanyl)-3-trifluoromethyl-6,7-dichloroquinoxaline) (Figure 11A), has demonstrated low-affinity, low potency allosteric agonism to the GLP-1R. In an effort to produce a more potent agonist, compound 2 was developed (6,7-dichloro-2-methylsulfanyl-3-N-tert-butylaminoquinoxaline) (Figure 11B). Compound 2 is an ago-allosteric agonist that not only increases the affinity of GLP-1 for its receptor, but also acts as an agonist. Additionally, exendin (9-39) antagonist did not inhibit compound 2 binding, showing a second binding site on the GLP-1R distinct from the orthosteric binding site [160]. The effectiveness of compound 2 to stimulate insulin secretion has also been assessed *in vivo*. Although, compound 2 was able to stimulate insulin secretion it was unable to do so as effectively as GLP-1, Liraglutide or Exenatide. Further, combining compound 2 with either Liraglutide or Exenatide did not show a substantial improvement in insulin secretion response in mice [161]. Two additional small molecule agonists of the GLP-1R, compound A (4-(3,4-dichlorophenyl)-2-(ethanesulfonyl)-

6-(trifluoromethyl)pyrimidine) and compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfonyl)-6-(trifluoromethyl)), have also demonstrated ago-allosteric properties (Figure 11C and 11D). Like compound 2, these compounds induced cAMP signalling and increased insulin secretion in rat islets and animal studies. Further studies showed treatment with compound B to near-normalise insulin secretion with human islets isolated from a donor with type 2 diabetes [162]. These small molecule agonists indicate a useful starting point for the identification and design of orally active allosteric GLP-1R compounds.



An alternative model for agonist induced activation

An alternative model for agonist induced activation of family B GPCRs has been proposed. It has been suggested that upon binding of an orthosteric agonist to the receptor, the N-terminal domain of the receptor undergoes a conformational change and interacts with another region of the receptor, which results in GPCR activation (agonism) [163]. This hypothesis originally arose from observations with the Corticotropin-releasing Hormone Receptor (CRHR; also known as corticotropin-releasing factor receptor), another family B GPCR. Nuclear Magnetic Resonance (NMR) analysis of the CRHR showed agonist induced conformational changes where the C-terminal region of the agonist binds the N-terminal domain of the receptor, which in turn causes the N-terminus to dock with the transmembrane bundle [164]. Additionally, similar conformational changes were noticed with the secretin receptor where secretin peptides with minor modifications to the N-terminus were no longer able to interact with the receptor, but still resulted in full agonism [165]. These findings could not be explained by current agonist binding models of family B GPCRs. Further, it was shown that the synthetic peptide corresponding to a conserved sequence in the N-terminal region of the secretin receptor, Trp⁴⁸-Asp⁴⁹-Asn⁵⁰ (WDN), acts as a full agonist and docks where the top of TM6 continued onto the ICL3 in the secretin receptor [166]. This suggests that the N-terminal domain of the secretin receptor folds to allow a 'built in agonist' to interact with the transmembrane bundle [167]. More recently, a synthetic peptide encoding an N-terminal sequence of the GLP-1R, Asn⁶³-Arg⁶⁴-Thr⁶⁵-Phe⁶⁶-Asp⁶⁷ (NRTFD), was shown to have full agonist activity. Further, this peptide was also able to activate the secretin and vasoactive intestinal peptide type 1 receptors because it was able to form an intradomain salt bridge between side chains of arginine and aspartate in ECL3 above TM6, similar to the WDN peptide. Moreover, GLP-1 (9-37) antagonist failed to block the NRTFD action, confirming that the site of action of NRTFD peptide is different from that of endogenous agonist GLP-1 [168].

The GLP-1R N-terminal domain and its signal peptide

Approximately 15% of GPCRs show evidence of a signal peptide sequence that is often critical for synthesis and processing of the receptor [169]. This signal peptide sequence is usually located in the N-terminal domain of the protein. It is about 20 amino acids in length and contains a run of hydrophobic residues. The first stage of protein targeting is insertion into the ER by binding to the Signal Recognition Particle (SRP). This is usually mediated by a signal peptide sequence within the N-terminal domain of the protein [170]. Two types of signal peptide sequences can be observed. One group contains a signal peptide sequence that is cleaved by a signal peptidase and is required for ER targeting and insertion. The second group possesses a non-cleavable anchor sequence within the first transmembrane domain for this process. Interestingly, the ER targeting and insertion of GPCRs can occur in either manner but the majority have a non-cleavable anchor sequence. Subsequently, the mature receptor is subjected to further post-translational modifications in the Golgi prior to translocation and insertion into the plasma membrane [171].

Cleavage of the signal peptide sequence is not essential for all GPCRs that contain them. Deleting the signal peptide sequence of the thyrotropin receptor abolished functionality [172,173]. However, the corticotropin-releasing factor receptor type 2a signal peptide although present, was found to be incapable of mediating ER targeting [174,175]. The GLP-1R has been shown to contain a cleavable N-terminal signal peptide that is essential for receptor processing and trafficking to the cell surface (Figure 6). A mutation to the signal peptide cleavage site

(Ala²¹Arg) still allowed receptor synthesis but prevented cleavage and resulted in receptor retention within the ER [176]. It is unclear why some GPCRs require a cleavable signal sequence and other do not. Statistical analysis suggests that the length of the N-terminal domain and the number of positively charged residues it contains denotes the presence of a cleavable signal peptide sequence [171].

The GLP-1R has six highly conserved cysteine residues at the N-terminal domain, highlighting their structural importance. These cysteine residues form disulphide bonds between Cys⁴⁶ and Cys⁷¹, Cys⁶² and Cys¹⁰⁴, and between Cys⁸⁵ and Cys¹²⁶ [177] (Figure 6). Additionally, Asp⁶⁷, Trp⁷², Pro⁸⁶, Arg¹⁰², Gly¹⁰⁸, and Trp¹¹⁰ are six other residues that are highly conserved across family B GPCRs, of which Trp⁷² and Trp¹¹⁰ have been shown to be important in GLP-1R agonist binding [1,178,179]. The crystal structure of the GLP-1R extracellular domain has shown these conserved residues to be positioned centrally. For example, Asp⁶⁷ is centrally located and forms intermolecular interactions directly with Trp⁷² and Arg¹²¹ and indirectly interacts with Arg¹⁰² via a water molecule. Asp⁶⁷ interacts with Tyr⁶⁹ and Ala⁷⁰. Arg¹⁰² is sandwiched between the side chains of Trp⁷² and Trp¹¹⁰. These interactions, and Gly¹⁰⁸, stabilise the receptors N-terminal domain. Pro⁸⁶ plays a critical role in forming the agonist binding site (Figure 6) [180]. In addition to the two highly conserved tryptophan residues, Trp⁷² and Trp¹¹⁰, already mentioned. Substitution of Trp³⁹, Trp⁷², Trp⁹¹, Trp¹¹⁰, or Trp¹²⁰ by alanine in the full-length rat GLP-1R abolished GLP-1 binding, whereas substitution of Trp⁸⁷ had no effect on agonist binding [178]. The role of Trp³³ still remains unclear. Trp¹²⁰ has no role in agonist binding but instead plays a structural role by forming a hydrophobic cluster with Phe⁸⁰, Tyr¹⁰¹, Phe¹⁰³ and Leu¹¹¹ (Figure 6) [180].

Residues Thr²⁹-Val³⁰-Ser³¹-Lys³² also located within the N-terminal domain have been shown to confer peptide specificity. A mutation to this region of the GLP-1R resulted in a 7-fold decrease in GLP-1 affinity showing its importance in agonist binding (Figure 6) [181].

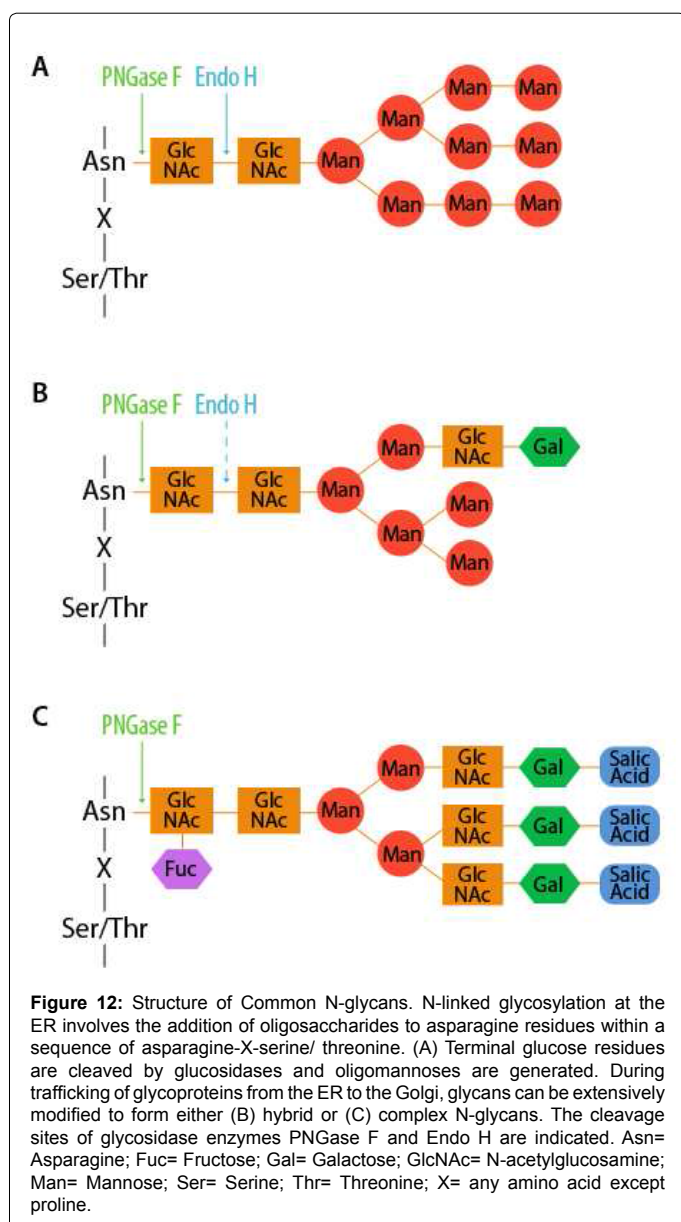
N-linked glycosylation of the GLP-1R

The GLP-1R has been shown to undergo N-linked glycosylation [182,183]. GPCRs are synthesised in the ER and require translocation to the Golgi. In this trafficking process, GPCRs undergo post- or co-translational modifications including glycosylation, methylation, phosphorylation, sulfation and lipid addition. It is likely that glycosylation may play an important role in cell surface trafficking and receptor maturation [184,185].

N-linked glycosylation usually occurs in the ER, which adds a glycan core unit (Glucose3-Mannose9-N-acetylglucosamine2) to an asparagine residue within a sequence of asparagine-X-serine/ threonine, where X can be any amino acid but proline [186-188]. Terminal glucose residues are cleaved by glucosidases and oligomannoses are formed (Figure 12A) [189]. During trafficking of glycoproteins from the ER to the Golgi, glycans can be extensively modified to form either complex or hybrid N-glycans (Figure 12B and C) [188,190]. Hybrid N-glycans are formed in the medial Golgi and are due to the incomplete actions of α -mannosidase II. Hybrid N-glycans are unable to be processed to complex N-glycans [190]. O-linked glycosylation that occurs within the Golgi is not very well understood. The process involves the addition of N-acetyl-galactosamine to serine or threonine residue and may occur at any residue with no sequence protein [191,192]. Glycans can be cleaved with the use of enzymes. PNGase F cleaves between asparagine and N-acetylglucosamine residues on oligomannoses and both hybrid and complex N-glycans. Endo H cleaves between N-acetylglucosamine residues on oligomannoses and some hybrid glycans (Figure 12).

O-linked glycosylation has been shown to occur in the V2 vasopressin receptor [193] and δ -opioid receptor [194]. However, most GPCRs undergo N-linked glycosylation but the role of this varies between receptors. The rat GLP-1R has previously been demonstrated to undergo N-linked glycosylation [195,196]. Further, the N-terminal domain of the human GLP-1R contains three N-linked glycosylation sites at positions Asn⁶³, Asn⁸² and Asn¹¹⁵ [182,183]. Tunicamycin, an inhibitor of N-linked glycosylation, interfered with GLP-1R biosynthesis and trafficking and abolished agonist binding. Further, mutations to Asn⁶³, Asn⁸² and Asn¹¹⁵ with leucine were made individually and in combination. Individual mutations did not affect receptor cell surface expression or agonist binding. However, mutations of two or three residues resulted in complete loss of GLP-1 binding. Immunofluorescence staining of the mutant receptors transfected cells demonstrated that the mutant receptors were still synthesised but were localised to the ER or Golgi [182,183].

ICLs, ECLs and TM domains of the GLP-1R



The ICLs of GPCRs are known to interact with G-proteins and play a role in receptor activation [197]. For the GLP-1R, ICL3 has been shown to mediate signalling via G-proteins. However, ICL1 and ICL2 have demonstrated an importance in discriminating between different types of G-proteins. ICL1 and ICL3 specifically mediate Gas whereas ICL2 activates Gas, Gai/o and Gaq/11 [134]. An alanine substitution at Arg¹⁷⁶ within ICL1, caused a reduction in GLP-1 mediated stimulation of cAMP but had no effect on receptor expression or internalisation (Figure 6) [198]. Additionally, different domains of ICL3 have been shown to be responsible for the Gas and Gai/o activation. The entire ICL3 (amino acids 329-351) has been shown to prefer Gas over Gai/o. However, the C-terminal end of ICL3 (amino acids 329-341) stimulates both Gas over Gai/o subtypes. Further, the N-terminal end of ICL3 (amino acids 341-351) also stimulates both subtypes, but favours Gas over Gai/o [133]. Residues important in coupling to G-proteins are mainly located in ICL3 and where TM5 meets ICL3 [199]. Alanine substitutions to Val³²⁷, Ile³²⁸ or Val³³¹, where TM5 meets ICL3, caused significantly lowered cAMP production but had no effect on receptor expression (Figure 6). These residues and Lys³³⁴ form a hydrophobic face that interacts directly with the G-protein [198]. A single block deletion of Lys³³⁴-Leu³³⁵-Lys³³⁶ within the N-terminal half of ICL3 caused a significant decrease in cAMP production in response to GLP-1, of which Lys³³⁴ showed most significance with no effect on receptor expression (Figure 6). This indicated that the region was required to couple Gas and stimulate AC [199]. A glycine substitution to Arg³⁴⁸, near the C-terminal end of ICL3, nearly abolished cAMP production and decreased receptor affinity in response to GLP-1 (Figure 6) [200].

The ECLs of GPCRs have been shown to be important in agonist binding and receptor trafficking. A disulphide bridge between ECL1 and ECL2 is conserved across all GPCRs and has been suggested to be involved in stabilising the receptor during agonist binding [160]. Residues within TM2 and ECL1 appear to be more important in GLP-1 binding than exendin-4 binding [87,201]. Mutations within ECL1 of the GLP-1R (Lys¹⁹⁷, Asp¹⁹⁸, Lys²⁰², Met²⁰⁴, Tyr²⁰⁵, Asp²¹⁵ or Arg²²⁷) have been shown to decrease agonist binding affinity (Figure 6) [87,179,201]. The GLP-1R has a number of conserved amino acids within ECL2 including Lys²⁸⁸, Asp²⁹³, Cys²⁹⁶, Trp²⁹⁷ and Trp³⁰⁶. These residues were demonstrated to be essential for GLP-1R function because alanine mutations resulted in a significant loss of GLP-1 binding and attenuation of receptor signalling [202,203]. Mutations within ECL2 have been shown to affect GLP-1 binding and efficiency, indicating an important role in GLP-1R activation. Interestingly, some mutations resulted in distinct changes in pathway responses. For example alanine substitutions to Cys²⁹⁶, Trp²⁹⁷, Arg²⁹⁹, Asn³⁰⁰, Asn³⁰², Tyr³⁰⁵ and Leu³⁰⁷ increased signal bias towards ERK activation. However, an alanine mutation at Trp³⁰⁶ abolished all biological activity (Figure 6). Scanning alanine substitutions were made on ECL2 of the GLP-1R and the effect of GLP-1, exendin-4 and oxyntomodulin was assessed (Figure 6). Mutations at positions Glu²⁹², Cys²⁹⁶ and Asn³⁰⁰ resulted in a greater potency of exendin-4 but reduced oxyntomodulin efficacy possibly because the receptor is unable to form an active ternary complex. Met³⁰³ appeared to play a role in cAMP signalling and was more important for exendin-4 and oxyntomodulin than GLP-1. When positions Lys²⁹⁰, Tyr²⁹¹ and Glu²⁹⁴ were mutated, a significant loss in GLP-1 calcium signalling was witnessed and no effect was seen when stimulated with oxyntomodulin. In cAMP formation, Arg²⁹⁹ and Lys³⁰⁷ mutations had a reduced potency for GLP-1 than exendin-4 suggesting exendin-4 cAMP signalling required the distal portion of ECL2. Exendin-4 mediated calcium responses were abolished in mutations at Asp²⁹³, Arg²⁹⁹, Tyr³⁰⁵ and Lys³⁰⁷ yet reduced but measurable responses were observed with GLP-1 suggesting subtle difference in calcium signalling mechanisms.

Cys²⁹⁶, Arg²⁹⁹ and Tyr³⁰⁵ mutants demonstrated no detectable calcium signalling and increased ERK signalling. Collectively, these mutations have suggested that GLP-1, exendin-4 and oxyntomodulin activate the receptor using different mechanisms [203]. The ECL3 of the GLP-1R has originally been hypothesised to act as an endogenous agonist [166]. However, this hypothesis was disproven when it was recognised that ECL3 could not establish necessary spatial approximation with the agonist binding region of the GLP-1R [204]. The GLP-1R has recently been shown to bind an agonist peptide (NRTFD), corresponding to the sequence of the GLP-1R, Asn⁶³-Asp⁶⁷, at the N-terminal region of ECL3 [168,205]. Furthermore, ECL3 has been shown to be important for endogenous agonist action of several members of family B GPCRs, suggesting that this region is likely to be important for drug binding [206-208].

Residues of TM1 through to TM3 are also important for agonist binding and receptor function. For example, a missense mutation of Thr¹⁴⁹ in TM1 reduced agonist binding [209]. Additionally, substitution of His¹⁸⁰ by arginine in TM2 resulted in a reduction in both the potency of cAMP production and affinity of the receptor for GLP-1 (Figure 6) [200]. A positively charged Lys²⁸⁸ in TM4 is highly conserved in all family B GPCRs and has been demonstrated to be important for the interaction of GLP-1 to its receptor (Figure 6). Further, Lys²⁸⁸ has

been hypothesised to be important in stabilising the top of TM4 [202]. Substitution of Lys²⁸⁸ by neutral leucine or alanine also reduced the affinity of GLP-1 for its receptor. However, substitution with a positively charged arginine had very little effect, demonstrating a positive charge was essential at this particular location [210]. Additionally, mutating at Lys²⁸⁸ resulted in a reduced binding affinity of GLP-1 than exendin-4 [203,210]. For some GPCRs such as the GLP-1R, serine and threonine rich amino acid sequences in TM3 or the cytoplasmic domain are required for receptor internalisation [142,211,212].

The C-terminal tail in agonist induced internalisation

The C-terminal domain of GPCRs is known to interact with intracellular proteins involved in receptor internalisation. There are three regions that are involved including: a region just downstream of TM7; the very end of the C-terminus; and the region in between (Figure 13) [213]. The C-terminal domain plays a critical role in agonist induced internalisation, desensitisation, down regulation and arrestin signalling [214].

The first region is called the helix-8 and is an α -helix that terminates with palmitoylated cysteine residues. It is located just downstream of TM7 and has been shown to associate with a number of proteins (Figure 13) [213]. For example the dopamine receptor interacting

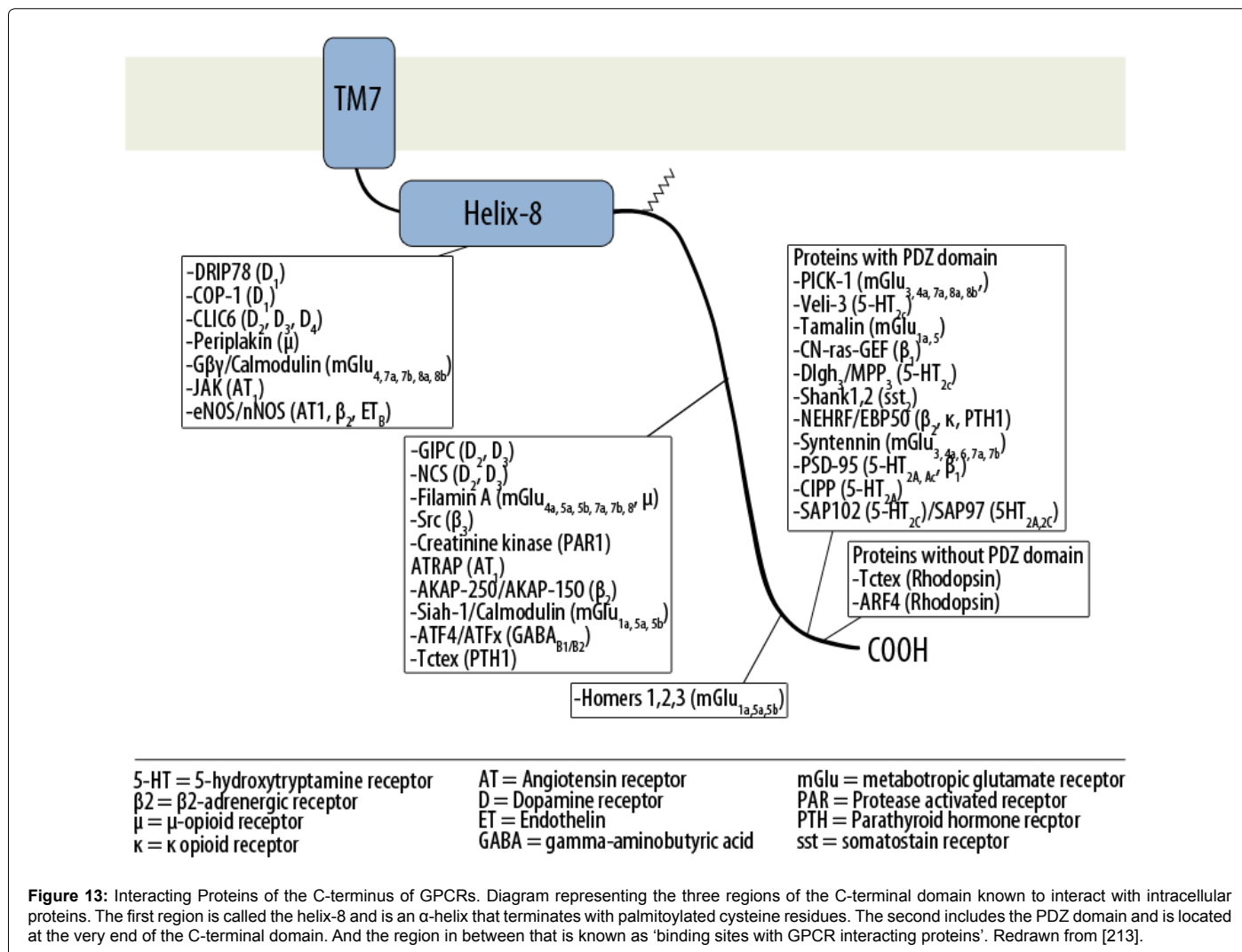


Figure 13: Interacting Proteins of the C-terminus of GPCRs. Diagram representing the three regions of the C-terminal domain known to interact with intracellular proteins. The first region is called the helix-8 and is an α -helix that terminates with palmitoylated cysteine residues. The second includes the PDZ domain and is located at the very end of the C-terminal domain. And the region in between that is known as 'binding sites with GPCR interacting proteins'. Redrawn from [213].

protein 78 binds to a conserved sequence located in the helix-8 domain of the dopamine D1 receptor and is responsible for receptor trafficking to the plasma membrane [215].

At the very end of the C-terminal domain, many GPCRs possess a PDZ binding domain that plays a role in receptor targeting, internalisation, recycling and signalling (Figure 13) [216]. The PDZ binding domains are grouped into three classes based on their amino acid sequences [217,218]. GPCRs without a PDZ binding domain have shown to interact with other proteins through the extreme C-terminus. For example the C-terminus of the rhodopsin receptor was also reported to interact with ARF4 [219]. The GLP-1R lacks this PDZ binding domain. The region between helix-8 and the very end of the C-terminus is referred to as 'binding sites with GPCR interacting proteins' (Figure 13) [213]. A PxxP motif within the C-terminus of the β 3-adrenergic receptor interacts with the Src Homology (SH) 3 domain of Src and results in the activation of ERK [220]. In addition, the extreme of TM7 close to the C-terminal domain is also known to interact with other proteins. An NPxxY motif within the serotonin 5-hydroxytryptamine receptor 2a interacts with ARF1 and couples to phospholipase D in a G-protein independent manner [221].

GPCRs including the GLP-1R regulate intracellular effector proteins such as PLC and AC via heterotrimeric G-proteins. Upon high or sustained levels of agonist stimulation, G-protein mediated responses typically desensitise [222]. Desensitisation occurs by either an agonist specific response (homologous desensitisation) or activation of a different receptor (heterologous desensitisation). Receptor phosphorylation and arrestins mediate receptor desensitisation and cause uncoupling from G-proteins [223]. Additionally, GPCRs are phosphorylated at regions of the C-terminal domain in response to agonist binding [224]. The C-terminal domain of GPCRs is also required for targeting to endosomes, Golgi and the cell surface. These motifs are four to six amino acids in length and contain a critical tyrosine residue and follow a general consensus of YXX Φ , where Y is a tyrosine residue, X denotes any amino acid and Φ is a hydrophobic residue [225-227].

The last 33 amino acids of the C-terminal domain of the GLP-1R containing serine 441/442, 444/445 and 451/452 phosphorylation sites were required for efficient receptor activation and therefore internalisation (Figure 6) [142,228]. Interestingly, receptor internalisation was quickened when amino acids ⁴⁰⁸EVQ⁴¹⁰ were substituted with alanine at the C-terminal domain of the GLP-1R [229].

GLP-1R signal transduction in pancreatic β -cells

In β -cells, the main action of GLP-1 through the GLP-1R is the formation of cAMP and its insulinotropic activity [69]. Upon agonist binding, the Gas subunit dissociates from the receptor, couples to AC and generates cAMP [122,230]. When blood glucose levels rise, it enters the β -cell through GLUT1 and GLUT2 transporters. Glucose is phosphorylated by glucokinase to glucose-6-phosphate, and results in the ATP/ADP ratio in the cytosol increasing and the plasma membrane depolarising by closing KATP channels. The closure of KATP channels, in turn opens calcium channels, releasing intracellular stores of calcium. The increase of cytosolic calcium causes secretory granules containing insulin to fuse to the plasma membrane and insulin is exocytosed [231,232]. It is also likely that human glucokinase activity is more important in glucose-induced insulin secretion than the rate at which glucose enters the β -cell [233].

GLP-1 has been shown to increase the quantity of insulin secreted per cell and cause more β -cells to become more sensitive to increased

glucose levels by GLP-1 modulated KATP channels [234,235]. Activation of GLP-1 can also increase calcium concentration by partial activation of L-type voltage dependent calcium channel and/or increase calcium-induced calcium release from intracellular stores and is mediated by PKA phosphorylation in an ADP-dependent manner [69]. The release of intracellular stores of calcium is achieved by one of two ways: either due to PKA activation or EPAC activation [236,237]. It has been suggested that PKA activation is achieved through the IP3 receptor (PKA dependent) and EPAC activation is achieved through ryanodine receptors (PKA independent) [238,239].

The increase in calcium levels causes an exocytotic response and is potentiated by elevated cAMP levels due to an increase in the amount of vesicles available for release [58]. In pancreatic β -cells, there are three different pools of insulin secretory vesicles. A reserve pool is situated in the cytoplasm; a readily release pool and immediately release pool are situated close to the membrane. GLP-1 increases the amount of insulin secretory vesicles in the readily release pool. GLP-1 depolarises the cell membrane closing KATP channels and therefore the current is inactivated before the cell can begin repolarising. Consequently, the cell does not reach its resting membrane potential and starts to depolarise before it has recovered from inactivation [240].

Additionally, a sustained increase in cAMP induced nuclear translocation leads to the activation of cAMP Response Element Binding-protein (CREB) and cell proliferation. The phosphorylation of PKA is said to activate CREB, interact with Transducer of Regulated CREB activity (TORC2), increase insulin receptor substrate-1 expression and cause activation of a serine-threonine protein kinase, Akt [241]. Akt has been described to link GLP-1 signalling to β -cell growth and survival [242]. Furthermore, the activation of Ribosomal protein S6 (rbs6) in animal models has been reported as a key regulator of glucose homeostasis and β -cell mass [243].

Two mutations within the GLP-1R have been shown to alter insulin secretion. In a Japanese study, one patient diagnosed with type 2 diabetes had a missense mutation that resulted in the substitution of Thr⁴⁴⁹ with methionine [244]. The patient exhibited impaired glucose tolerance, insulin secretion and sensitivity. The mutated receptor had reduced affinity *in vitro* for GLP-1 and exendin-4 [209]. Further, GLP-1R mutants lacking Lys³³⁴-Leu³³⁵-Lys³³⁶ of ICL3 in the HIT-T15 insulinoma cell line showed an absence of GLP-1 induced cAMP production, calcium channel activation and insulin secretion [245].

Conclusions and Future Prospects

The ability of GLP-1 to lower postprandial hyperglycemia by increasing insulin secretion and inhibiting glucose secretion makes this peptide an ideal candidate for the treatment of type 2 diabetes. Additionally, as GLP-1 is able to retain its glucose lowering activity in patients with type 2 diabetes it is also of significant clinical relevance. The main limitation of GLP-1 is a very short half-life and as a result therapeutic strategies that activate the GLP-1R and improve GLP-1 actions have been extensively studied and developed.

GLP-1R activation by GLP-1 has many beneficial effects, most likely due to the activation of a number of signalling pathways upon agonist binding. But the precise signalling pathway that is activated and is critical for GLP-1 to exert its effects on the β -cell is still unknown. Therefore, agonists that act through the GLP-1R would be the perfect treatment in type 2 diabetes. However, only Liraglutide and Exenatide are currently available and have a number of severe side effects. As a result, there is a need for small molecule agonists that have a longer half-life and are orally active. It is also important to note that receptor-

agonist interactions are more complex than was previously believed. Some GPCRs do not function as monomers and can be regulated by more than one agonist and can also 'self activate'. This knowledge is important for further agonist development of GPCRs. Overall a lot still remains to be determined in GLP-1R pharmacology and drug development in the treatment of type 2 diabetes.

Acknowledgements

The work in VK's laboratory was funded by BBSRC, UK and MRC, UK. AT holds a BBSRC, UK PhD studentship. We thank members of the VK laboratory.

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This article was originally published in a special issue, **GPCR Signalling & Diabetic Complications** handled by Editor(s). Dr. Wenbo Zhang, The University of Texas Medical Branch, USA