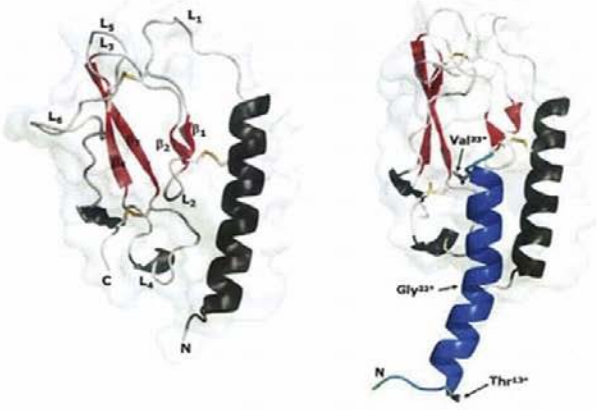
Incretins and Insulin Secretion

Edited by
Gerald Litwack



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VOLUME EIGHTY-FOUR

VITAMINS AND HORMONES

Incretins and Insulin Secretion

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VOLUME EIGHTY-FOUR

VITAMINS AND HORMONES

Incretins and Insulin Secretion

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PREFACE

Incretins Secretion

Incretins, so far, are the active agents, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide (GLP-1), which are secreted from specific cells in the intestine following a meal. These peptides have an array of activities but are important for their abilities, through specific receptors in the plasma membranes of beta-cells of the pancreas, to affect the secretion of insulin. Consequently, these peptides and their synthetic chemical mimics recently have become useful treatments for type 2 diabetes. These and related topics are reviewed in this volume.

The volume opens with a treatise on 1. "Evolution of Genes for Incretin Hormones and their Receptors" by Irwin. 2. McIntosh *et al.* review "Pleiotropic Actions of the Incretin Hormones." 3. "Dietary Effects on Incretin Hormone Secretion" by Wu *et al.* follows. Then, 4. Cho and Kieffer report on "K-cells and Glucose-Dependent Insulinotropic Polypeptide in Health and Disease." A discussion of 5. "The Emerging Role of Promiscuous 7TM Receptors as Chemosensors for Food Intake" is presented by Wellendorph *et al.* 6. Yavropoulou and Yovos review "Central Regulation of Glucose-Dependent Insulinotropic Polypeptide Secretion." Following this, 7. Ahren *et al.* offer "Incretin Hormone Secretion over the Day." 8. "Using the Lymph Fistula Rat Model to Study Incretin Secretion" is the subject of Yoder *et al.*

A consideration of structure is encompassed by the contribution of 9. Underwood *et al.* entitled "Structural Basis for Ligand Recognition of Incretin Receptors." This manuscript also provides the figures included on the cover of this volume. 10. "Epac2-Dependent Rap1 Activation and the Control of Islet Insulin Secretion by Glucagon-Like Peptide-1" is the subject of Leech *et al.* 11. Velasquez *et al.* discuss "Central GLP-1 Actions on Energy metabolism". 12. Hellstrom reports on "Glucagon-Like Peptide-1: Gastrointestinal Regulatory Role in Metabolism and Motility." 13. Holscher reviews "The Role of GLP-1 in Neuronal Activity and Neurodegeneration." 14. "Wnt and Incretin Connections" is contributed by Garcia-Jimenez.

Therapeutic aspects are covered in the last two offerings: 15. "Incretin-Based Therapy and Type 2 Diabetes" by Hare and Knop and finally 16. "GPR119 Agonists for the Potential Treatment of Type 2 Diabetes and Related Metabolic disorders" by Shah and Kowalski.

The two figures on the cover derive from the manuscript by Reedtz-Runge and collaborators, Chapter 9 in this volume entitled "Structural Basis for Ligand Recognition of Incretin Receptors." The left-hand figure shows a ribbon presentation of the structure of the GLP-1 receptor extracellular domain (α-helix in black; β-strands in red and loops in gray). Disulfide bridges appear as orange sticks and the loop regions L1–L6 are indicated. The figure on the right shows GLP-1 (blue) bound to the extracellular domain.

Narmada Thangavelu and Sujatha Thirugnanasambandam direct the final processes at the Press and Lisa Tickner oversees the Serial. They earn my thanks.

Gerald Litwack
Scranton, PA

CHAPTER ONE

Evolution of Genes for Incretin Hormones and their Receptors

David M. Irwin

Contents

Abstract

The incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are essential components in the regulation of blood glucose levels in mammals. These two incretins are produced by evolutionarily related genes and these hormones show similarity in sequence as both are glucagon-like sequences. Genes for these hormones have been identified in a number of diverse vertebrate species indicating that they originated prior to the earliest divergences of vertebrate species. However, analysis of functional and sequence data suggest that each of these hormones acquired incretin activity independently, and only since the divergence of tetrapods from fish. Not only are the hormones related, but so are their receptors. Like the hormones, the incretin action of the receptors is not a product of a shared common ancestral history, as the receptors for GLP-1 and GIP are not most closely related. Further study of the physiological functions of GLP-1 and GIP in additional vertebrates is required to better understand the origin of incretin action.

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I. Introduction

Blood glucose levels are maintained within a narrow range in mammals by a complex system that is primarily regulated by two hormones, insulin and glucagon, and failure to properly regulate glucose levels has serious health consequences (Gerich, 1993; Jiang and Zhang, 2003; Kahn, 2003). As blood glucose levels increase, insulin is secreted by pancreatic islet beta-cells thus signaling other tissues, including liver, muscle, and adipose, to increase their rate of glucose uptake and to store excess sugar for future use (Gerich, 1993; Kahn, 2003). In contrast, when blood glucose levels are low, pancreatic islet alpha-cells secrete glucagon which leads to the release of glucose from energy stores into the blood (Gerich, 1993; Jiang and Zhang, 2003). The ingestion and digestion of food, the source of glucose, can result in a rapid, and large, increase in blood glucose levels and this sudden increase can be a challenge for pancreatic islet beta-cells and their ability to secrete insulin as a means of regulating blood glucose levels (Holst, 2007; Nauck, 2009). Mammals, though, have evolved mechanisms that helps pancreatic islet beta-cells to prepare for an anticipated increase in blood sugar levels caused by the ingestion of food — the incretin hormones (Asmar and Holst, 2010; Baggio and Drucker, 2007; Holst, 2007; Nauck, 2009; Parker *et al.*, 2010).

Incretin hormones are hormones that are secreted by intestinal cells into the blood in response to the presence of food in the intestine and these hormones act on pancreatic islet beta-cells, priming them for the release of insulin (Asmar and Holst, 2010; Baggio and Drucker, 2007; Holst, 2007; Nauck, 2009; Parker *et al.*, 2010; Wideman and Kieffer, 2009). Incretin hormones, by themselves, do not cause the release of insulin from islet beta-cells, but in the presence of both an incretin hormone and high blood glucose levels a greater amount of insulin is secreted which then leads to a more rapid uptake of glucose at other sites of the body and the blunting of the departure of blood glucose levels from the preferred range. The intestine has been found to secrete two different, but related, incretin hormones: glucagon-like peptide-1 (GLP-1) produced by intestinal L-cells and glucose-dependent insulinotropic polypeptide (GIP) synthesized by intestinal K-cells (Holst, 2007; Nauck, 2009). These two hormones have distinct but overlapping physiological actions and both act on pancreatic islet beta-cells to prime the release of insulin (Asmar and Holst, 2010; Baggio and Drucker, 2007; Holst, 2007; Nauck, 2009; Parker *et al.*, 2010; Wideman and Kieffer, 2009). In addition to its function as an incretin, GLP-1 stimulates an increase in the expression of the insulin gene, promotes the differentiation of pancreatic beta-cells from precursors, inhibit gastric emptying (thus delaying the production of glucose by the intestine), and regulate appetite (Baggio and Drucker, 2007; Holst, 2007; Nauck, 2009; Wideman and Kieffer, 2009). GIP, in addition to being an incretin, also functions in

lipid metabolism by adipose tissue and in bone remodeling (Asmar and Holst, 2010; McIntosh *et al.*, 2009). The secretion of GLP-1 and GIP by intestinal cells is regulated both by direct nutrient interactions with the cells secreting these hormones as well as by neuronal inputs (Baggio and Drucker, 2007; Vahl *et al.*, 2010; Wideman and Kieffer, 2009).

GLP-1 is a product of the proglucagon gene, and is one of three glucagon-like sequences encoded by the human proglucagon (*GCG*) gene (Irwin, 2001a; Kieffer and Habener, 1999; Sinclair and Drucker, 2005). The proglucagon gene is expressed not only in intestinal L-cells, but also in pancreatic alpha-cells and by some neurons of the brain stem and hypothalamus (Drucker, 2003; Jin, 2008; Kieffer and Habener, 1999). In some species (e.g., dog, chicken, and amphibians), the proglucagon gene is also expressed in additional tissues such as the stomach (Dugay and Mommsen, 1994; Irwin, 2001b; Irwin and Wong, 1995; Irwin *et al.*, 1997). In all mammals examined to date, only a single mRNA transcript is generated by the proglucagon gene that encodes an identical proglucagon protein precursor at all sites where the proglucagon gene is expressed (Drucker, 2003; Irwin, 2001a; Jin, 2008; Kieffer and Habener, 1999; see Fig. 1.1). In contrast, in other classes of vertebrates such as reptiles, birds, amphibians, and fish, several proglucagon mRNA transcripts have been identified that are generated by alternative mRNA splicing and lead to the production of different precursor proteins (Irwin, 2001a; Fig. 1.1). Most studies examining the expression of the proglucagon gene have focused on mammalian species where it has been demonstrated that different tissues generate different hormones despite the synthesis of an identical proglucagon precursor at all sites of gene expression (Baggio and Drucker, 2007; Kieffer and Habener, 1999). The production of the differing tissue-specific hormone complements is due to the proglucagon precursor being posttranslationally processed by the tissue-specific action of prohormone convertase enzymes to release different hormone products (Bataille, 2007). In addition to GLP-1, intestinal L-cells produce glucagon-like peptide-2 (GLP-2) which is cosecreted with GLP-1 (Bataille, 2007; Drucker, 2002). Pancreatic alpha-cells, in contrast, do not generate GLP-1 or GLP-2, but rather secrete glucagon (Bataille, 2007; Kieffer and Habener, 1999; Sinclair and Drucker, 2005). It appears that the neuronal cells that express the proglucagon gene process the proglucagon precursor in a pattern similar to that seen in intestinal cells liberating GLP-1 and GLP-2 (Larsen *et al.*, 1996). However, in some non-mammalian species some differences in the processing of proglucagon have been observed, where GLP-1 (often identified as glucagon-like peptide or GLP) along with glucagon have been identified as the products of the processing of proglucagon in pancreatic cells (Dugay and Mommsen, 1994; Nelson and Sheridan, 2006; Youson *et al.*, 2006).

GIP is the sole described hormone product of the *GIP* gene which is primarily expressed by intestinal K-cells (McIntosh *et al.*, 2009). Until recently, GIP had only been identified in mammals, but genes similar to *GIP* have now

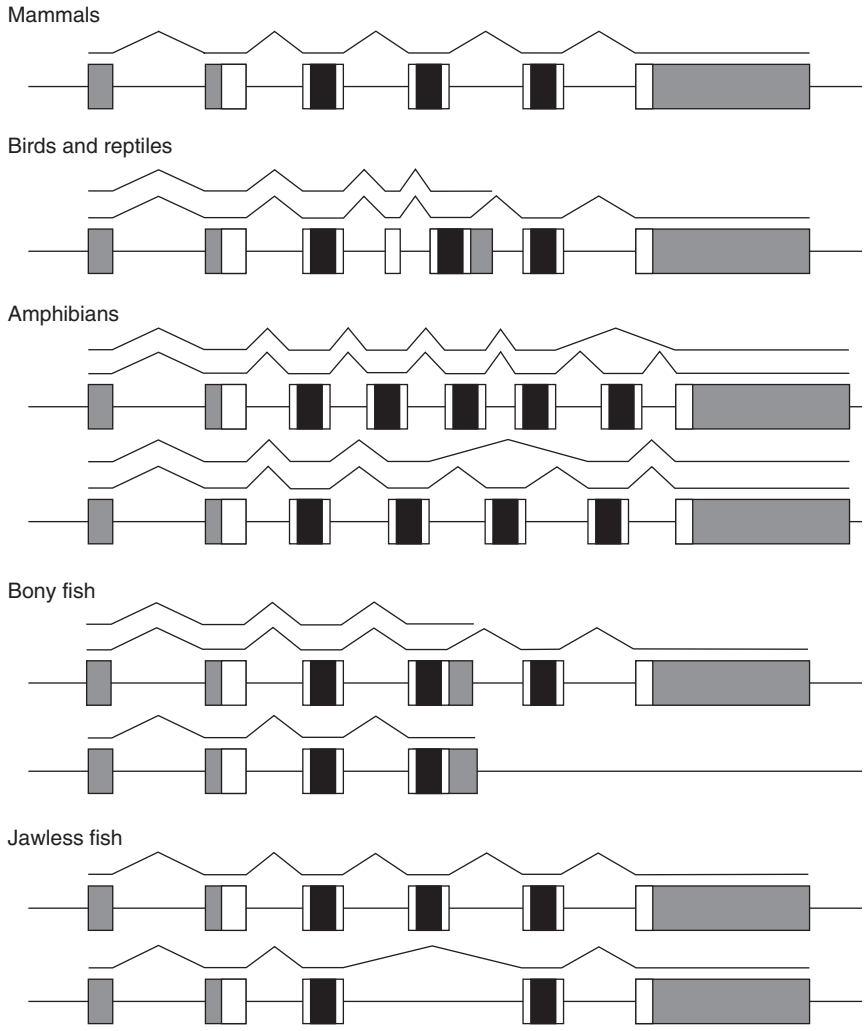

Figure 1.1 Structure and splicing of proglucagon genes in vertebrates. Summary of the exon–intron gene structure and splicing patterns for proglucagon genes identified in different classes of vertebrates. Exons are boxes and introns and flanking sequence shown as lines. Untranslated exon sequences are indicated in gray while sequences coding for glucagon-like sequences (glucagon, GLP-1, and GLP-1) are in black, and the remaining coding sequences are in white. Characterized splicing patterns are shown above each gene. In mammals only a single gene structure and mRNA splicing pattern has been observed. In birds and reptiles, the proglucagon gene encodes an extra exon and two different coding transcripts have been described. In chicken to different untranslated exon 1 (and promoter) sequences have been characterized (not shown). In Amphibians proglucagon genes containing five (*Xenopus laevis* and *Xenopus tropicalis*) or four (*Rana pipiens* and *Rana tigrina rugulosa*) have been identified and each have at least two different mRNAs (see text for details). Bony fish species (e.g., zebrafish) have duplicated proglucagon genes, one of which has two transcripts. Jawless fish (i.e., lamprey) also have two genes, but only a single transcript has been characterized for each gene.

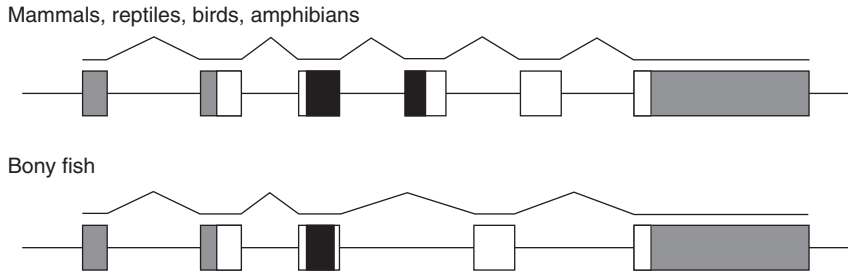

Figure 1.2 Structure of GIP genes in vertebrates. Summary of the exon–intron gene structure and splicing pattern for *GIP* genes identified in different classes of vertebrates. Exons are boxes and introns and flaking sequence shown as lines. Untranslated exon sequences are indicated in gray while sequences coding for GIP are in black, and the remaining coding sequences are in white. Splicing patterns are shown above for each gene. In most vertebrates, only a single gene structure and mRNA splicing pattern has been observed. In bony fish (i.e., zebrafish), the GIP gene has only five exons and a shorter GIP hormone sequence.

been identified in the genomic sequences of several diverse nonmammalian vertebrates (Irwin and Zhang, 2006; McIntosh *et al.*, 2009; Roch *et al.*, 2009; Fig. 1.2). While a GIP hormone has not yet been isolated and characterized from a nonmammalian vertebrate, cDNA sequences have been determined from intestinal mRNA from the chicken (*Gallus gallus*) and a frog (*Xenopus tropicalis*, also called *Silurana tropicalis*, Western clawed frog) indicating that this gene is expressed at least some intestinal cells (Irwin and Zhang, 2006). The first full-length zebrafish (*Danio rerio*) GIP-encoding cDNA clone was isolated from whole adult cDNA library (Irwin and Zhang, 2006); however, a more recent study has shown that the zebrafish *GIP* gene is expressed in the pancreas rather than the intestine (Musson *et al.*, 2009). Intriguingly, genomic sequences similar to *GIP* have not yet been found in most of the available near-complete fish genomic sequences including those of *Takifugu rubripes* (Japanese pufferfish), *Tetraodon nigroviridis* (Green-spotted pufferfish), *Gasterosteus aculeatus* (Three-spined stickleback), or *Oryzias lapites* (Japanese medaka), although *GIP*-like genes have been identified in the genomes of *D. rerio* (zebrafish) and *Oncorhynchus mykiss* (Rainbow trout) (Irwin and Wong, 2005; Musson *et al.*, 2009; Roch *et al.*, 2009).

II. Evolution on Incretin Hormone Genes

A. The glucagon-like gene family

The incretin hormones GLP-1 and GIP are both glucagon-like sequences and share similarity in sequence not only with each other but also with a small number of other hormones of the secretin gene family (Hoyle, 1998;

Roch *et al.*, 2009; Sherwood *et al.*, 2000). In mammals, six genes, including *GCG* and *GIP*, have been described that encode glucagon-like sequences (Hoyle, 1999; Roch *et al.*, 2009; Sherwood *et al.*, 2000). As described above, the proglucagon gene (*GCG*) encodes two other glucagon-like sequences (glucagon and GLP-2) in addition to GLP-1 (Irwin, 2001a, 2009; Kieffer and Habener, 1999), while the *GIP* gene encodes only a single glucagon-like sequence (McIntosh *et al.*, 2009). The mammalian adenylate cyclase-activating polypeptide (*ADCYAP*) and vasoactive intestinal peptide (*VIP*) genes both encode two glucagon-like sequences (Hoyle, 1999; Roch *et al.*, 2009; Sherwood *et al.*, 2000). The *ADCYAP* gene encodes the glucagon-like sequences PACAP and a PACAP-related peptide (PRP, also called growth hormone releasing hormone-like peptide, GHRH-LP), while *VIP* encodes VIP and a peptide called either peptide histidine methionine (PHM) or peptide histidine isoleucine (PHI) depending on the identity of the C-terminal amino acid of the hormone (methionine or isoleucine; Hoyle, 1999; Roch *et al.*, 2009; Sherwood *et al.*, 2000). The other two genes, growth hormone releasing hormone (*GHRH*) and secretin (*SCT*), each encode a single glucagon-like sequence and these genes are named after their encoded peptides (Hoyle, 1999; Roch *et al.*, 2009; Sherwood *et al.*, 2000). It had previously been proposed that the *ADCYAP* and *GHRH* genes were generated by a gene duplication that occurred on the mammalian lineage (Sherwood *et al.*, 1993) as at that time only a single *ADCYAP*-like sequence had been found in nonmammalian vertebrate species and this gene that appeared to encode both PACAP and GHRH; however, more recent studies have shown that both of these genes can be found in the genomes of diverse vertebrates and thus they must be the product of a more ancient gene duplication event (Tam *et al.*, 2007). A *SCT* gene has still not been identified in the genomes of any fish species (Roch *et al.*, 2009) and its relationships with the other genes of the secretin family remain unresolved (Hoyle, 1999; Sherwood *et al.*, 2000). *GCG*, *GIP*, *VIP*, *GHRH*, and *ADCYAP* genes have been describe in both fish and mammalian genomes indicating that they are the products of gene duplications that occurred very early in vertebrate evolution (Hoyle, 1999; Roch *et al.*, 2009; Sherwood *et al.*, 2000). Indeed, evidence suggests that the proglucagon and *GIP* were generated during one of the two genome duplications that occurred in the early vertebrate as both genes reside in related genomic neighborhoods, that is, the genes that flank the proglucagon gene are related to genes near the *GIP* gene (Irwin, 2002). Whether the genes that encode the remaining glucagon-like sequences were generated during genomic duplications, or were generated by other gene duplication events is unclear as they are not found in regions that have synteny with the proglucagon or *GIP* genes, or each other (Irwin, 2002).

B. Evolution of proglucagon

Proglucagon genes have been identified in a wide variety of vertebrates, and these genes potentially produce glucagon, GLP-1, and GLP-2 as hormones (Irwin, 2001a; Fig. 1.1). Glucagon, GLP-1, and GLP-2 are similar sequences and have descended from a common ancestor (Irwin, 2001a, 2005; Irwin *et al*., 1999; Lopez *et al*., 1984) but now have distinct biological functions (Baggio and Drucker, 2007; Kieffer and Habener, 1999; Sinclair and Drucker, 2005) thus these functions must have evolved as these peptides diverged from each other in sequence. Glucagon and GLP-1 hormones have been isolated from wide diversity of vertebrates; however, GLP-2 peptides have only been purified from mammalian species (Baggio and Drucker, 2007; Dugay and Mommsen, 1994; Kieffer and Habener, 1999; Sinclair and Drucker, 2005). The conservation of the GLP-2 sequence, though, does suggest that this peptide is produced and functions in most vertebrates; however, its site of production and function has not been fully characterized, potentially due to low expression or difficulty in detection. GLP-2 is likely produced in the intestine, rather than the pancreas, of most vertebrates as only intestinal proglucagon transcripts predict this peptide in fish and amphibians (Irwin and Wong, 1995; Irwin *et al*., 1997; Yeung and Chow, 2001). The function of GLP-2 is known for some mammals, where it has been described to have important roles in the homeostasis of rodent and human intestinal cells (Baggio and Drucker, 2007). Glucagon has been characterized from a wide variety of vertebrate species, and essentially has the same function in all these species, that of inducing the production of glucose by the liver when blood glucose levels are low (Dugay and Mommsen, 1994). In contrast, while the function of GLP-1 has been identified in mammals and fish, GLP-1 function is not conserved. In mammals, GLP-1 is an incretin hormone (Holst, 2007; Nauck, 2009), however, in fish GLP-1 (where it is often called glucagon-like peptide, GLP) has a physiological role similar to that of glucagon inducing glucose production by the liver (Dugay and Mommsen, 1994; Moon, 2004).

In addition to a change in function in the glucagon-like sequences, there have been changes in the structure of the vertebrate proglucagon gene (see Fig. 1.1). While mammalian proglucagon genes all encode the three glucagon-like sequences, glucagon, GLP-1, and GLP-2 (Irwin, 2009), some proglucagon genes encode a different number of glucagon-like sequences that range from two to five (Irwin, 2001a, 2005; Fig. 1.1). Within amphibians, proglucagon genes from *Xenopus lavevis* (African clawed frog; Irwin *et al*., 1997) and *X. tropicalis* (unpublished results) encode five glucagon-like sequences, three of which are most similar to GLP-1. In other amphibians, including *Rana pipiens* (Leopard frog) and *Rana tigrina rugulosa* (*Hoplobatrachus rugulosus*, Chinese edible frog), the proglucagon gene has been shown to encode at least four glucagon-like sequence, two of which are GLP-1-like

peptides (Irwin and Sivarajah, 2000; Yeung and Chow, 2001). Peptide sequence data from other amphibian species (e.g., *Bufo marinus*, cane toad) also suggest the presence of multiple GLP-1-like sequences (Conlon *et al.*, 1998). Some fish proglucagon genes encode only two glucagon-like sequences. Analysis of draft genome sequences from bony fish (Class Osteichthyes) indicate that these species (including zebrafish and pufferfish) have two proglucagon genes, one of which encodes all three glucagon-like sequences and a second gene that only encodes glucagon and GLP-1 (Zhou and Irwin, 2004). The lamprey (*Petromyzon marinus*), a jawless fish (Class Agnatha), also has two proglucagon genes, one of which encodes three glucagon-like sequences while the second only encodes glucagon and GLP-2 (Irwin *et al.*, 1999; Fig. 1.1). While all characterized fish genomes contain duplicated proglucagon genes with differing coding potential, all species retain the potential to encode all of the three different glucagon-like sequences (Irwin, 2001a). To date, no known vertebrate species has been identified that does not have the potential to produce all three of the proglucagon-derived glucagon-like sequences (glucagon, GLP-1, and GLP-2), although some species have the potential to produce more than one variant sequence of some, or all, of these peptides (Irwin, 2001a, 2005).

Associated with the variation in the structure of the proglucagon gene are variations in the structure of the transcripts that these genes produce (Irwin, 2001a, 2005; see Fig. 1.1). While the mammalian proglucagon gene is transcribed as a single mRNA, which is translated into a single precursor that is processed in a tissue-specific manner to release different hormones (Drucker, 2003; Jin, 2008; Kieffer and Habener, 1999), multiple proglucagon mRNA exist in many nonmammalian vertebrates (Irwin, 2001a, 2005). Several different patterns of alternative mRNA splicing have been described. While a transcript that encodes all of the glucagon-like sequences has been described for every species examined, in bony fish, chicken (*G. gallus*, a bird), and the Gila monster (*Heloderma suspectum*, a reptile) an alternate mRNA is also generated due to the failure of splicing after the GLP-1 encoding exon (in genes that encode GLP-2) and terminate transcription within the intron that separates the GLP-1 and GLP-2 encoding exons (Irwin, 2001a, 2005; Fig. 1.1). The conserved 5′ splice donor site for the intron that separates the GLP-1 and GLP-2 encoding exons also codes for the stop codon that terminates the proglucagon precursor that is generated by the alternative transcript, preventing it from encoding GLP-2. The very first proglucagon cDNAs characterized were isolated from the Anglerfish (*Lophius americanus*) and were found to only encode a GLP in addition to glucagon (Lund *et al.*, 1982, 1983) and these two cDNAs represented the two different proglucagon genes found in fish (Zhou and Irwin, 2004). However, these two sequences failed to identify the full diversity of proglucagon-encoded peptides from Anglerfish as the longest proglucagon mRNA, which likely encodes GLP-2, was not characterized (Lund *et al.*,

1981, 1982, 1983). In addition to splicing that alters the coding potential, two alternative first exons (and promoters) have been identified for the chicken proglucagon gene, although these alternative exons do not change the coding potential they may have a role in regulating expression (Richards and McMurtry, 2008; Yue and Irwin, 2005).

Within amphibians two additional forms of alternative mRNA slicing have been described (Fig. 1.1). In *Xenopus laevis*, two different mRNAs were characterized for one of the two expressed genes and the difference in the translated content of these mRNAs was the presence or absence of GLP-2, however, the mechanism of alternative splicing that leads to the absence of GLP-2 differed from that described in fish, chicken, and Gila monster (Irwin *et al.*, 1997). In *X. laevis*, the GLP-2 lacking transcript does not terminate prior to GLP-2, but rather skips the GLP-2 encoding exon. That is, one the proglucagon transcripts does not contain the GLP-2 exon, but does contain sequences derived from exons that are both 5′ and 3′ to the GLP-2 encoding exon (Irwin *et al.*, 1997). A similar exon skipping mechanism has been described in a different amphibian species, *Rana tigrina rugulosa*, but in this case the alternative splicing skips one of the two GLP-1-encoding exons (Yeung and Chow, 2001).

In addition to changes in proglucagon gene number, structure, coding potential, and splicing, change has also occurred in the coding sequences (Irwin, 2001a, 2009). Conservation of coding sequence is correlated with conservation of function. Sequences that show greater levels of divergence are more likely to have diverging function, especially those that have differing rates of sequence evolution compared to other species. The consequences of changes in proglucagon gene sequence upon function can be illustrated with the evolution of glucagon in Hystricognath rodents (i.e., guinea pig (*Cavia porcellus*) and relatives). In Hystricognath rodents, a large number of amino acid substitutions have occurred in the glucagon sequence, resulting in a glucagon hormone that is not very effective in stimulating glucose release from the liver, changes that appear to be driven as a compensation for changes in the function, and sequence, of insulin (Irwin, 2001a; Seino *et al.*, 1988). When a diverse set of mammalian proglucagon sequences were examined it was observed that the GLP-1 portion of the sequence is extremely well conserved (Irwin, 2001a, 2009). If the highly variable platypus (*Ornithorhynchus anatinus*) sequence is excluded, only one mammalian sequence shows any variation, the squirrel (*Spermophilus tridecemlineatus*) that differs at one amino acid position (and this difference may simply represent a sequencing error as this peptide sequence is predicted from a draft genome sequence; Irwin, 2009). The GLP-1 sequence from the platypus, a representative of the earliest diverging mammalian linage, differs at about half of the positions within its sequence raising the possibility that this peptide differs in, or lost, function (Irwin, 2009).

In other vertebrates though, GLP-1 shows greater levels of sequence variability, especially within amphibians and fish (Irwin, 2001a). In mammals, GLP-1 is an incretin hormone. In the chicken, GLP-1 has been described to have anorexic effects in the brain that are similar to those seen in mammals, however, it has not yet been demonstrated to be an incretin hormone affecting insulin secretion, although some observations are consistent with it being an incretin hormone (Richards and McMurty, 2009). Avian and reptilian GLP-1 sequences differ from the mammalian GLP-1 sequences at only a few positions, though it is not as well conserved as the glucagon sequence (Irwin, 2009), consistent with no large change in function. The functions of the multiple GLP-1-like peptides in amphibians are unknown. While GLP-1-like peptides from amphibians have been described to act in a incretin-like manner in mammalian cells (Irwin *et al.*, 1997) and to act in the expected glucagon-like manner in fish (Mommsen *et al.*, 2001), few experiments with proglucagon-derived peptides have been done in amphibians (Dugay and Mommsen, 1994). In fish, GLP-1 does not act as an incretin hormone, rather it acts similar to glucagon (Dugay and Mommsen, 1994; Moon, 2004), however this has likely been due to a change in GLP-1 activity on fish lineage (Irwin, 2005; Irwin and Wong, 2005). The ancestral function of GLP-1 on the fish lineage is unknown. It has been suggested that the redundancy of GLP-1 due to presence of multiple peptides, encoded by the single genes in amphibians or by the duplicate genes in fish, may have reduced the stringency of selection on these peptides allowing them to show greater sequence diversity (Irwin, 2001a).

The glucagon and GLP-2 sequences encoded by the proglucagon gene also tend to be well conserved among vertebrates (Irwin, 2001a). Within placental mammals (i.e., mammals excluding platypus and opossum) GLP-1 is the most conserved of the proglucagon-derived sequences, with glucagon and GLP-2 showing greater levels of sequence variability (Irwin, 2009). Among vertebrates, though, the glucagon sequence is better conserved, with GLP-2 being least conserved (Irwin, 2001a, 2009). Like GLP-1, the function of GLP-2 in nonmammalian vertebrates is poorly defined; in fact the peptide has not been identified in a nonmammalian vertebrate (Dugay and Mommsen, 1994; Irwin, 2001a; Richards and McMurty, 2009). Phylogenetic analysis of the peptide has suggested that the GLP-2 sequence underwent rapid evolution on the early mammalian lineage and may have acquired a new function, potentially the intestinal function, at this time (Irwin, 2001a, 2009).

C. Evolution of GIP

Until recently, *GIP* genes were only known, both in sequence and function, from mammals (McIntosh *et al.*, 2009). With the recent public release of a number of draft genome sequences (e.g., see Ensembl, www.

ensembl.org) as well as expressed sequence tag (EST) cDNA databases (e.g., see NCBI, www.ncbi.nlm.nih.gov) from diverse vertebrate species it was discovered that sequences similar to *GIP* could be identified in some nonmammalian vertebrate genomes (Irwin and Zhang, 2006; Musson *et al.*, 2009; Roch *et al.*, 2009; Fig. 1.2). As described above, not only were these genes similar in genomic sequences, but some were also expressed in intestinal cells and the encoded mRNAs that predict protein sequences that potentially could be processed to produce a GIP-like peptide (Irwin and Zhang, 2006). Mammalian GIP is 42 amino acids in length, about 10 residues longer in length than the related proglucagon-derived glucagon-like sequences (Irwin, 2009; Irwin and Zhang, 2006). Predicted reptilian, avian, and amphibian GIP peptide sequences are also about 42 amino acids in length, however the predicted zebrafish GIP sequence is only 31 residues in length, a length similar to that of the proglucagon-derived glucagon-like sequences (Irwin and Zhang, 2006; Musson *et al.*, 2009). This observation suggested that the change in length of the GIP peptide occurred on the tetrapod lineage (Irwin and Zhang, 2006). The function of GIP in nonmammalian vertebrates has not been studies extensively. Intriguingly, *GIP* is expressed in the pancreas, rather than the intestine, of the zebrafish and the expression of this gene is increased during food deprivation, suggesting that in zebrafish GIP does not have an incretin-like function (Musson *et al.*, 2009). Studies with an anti-GIP antibody suggested that GIP is produced in the pancreas rather than the intestine of a snake, the Burmese python, and that its plasma levels were depressed after food intake (Secor *et al.*, 2001). These observations would indicate that GIP function, like that of the other incretin GLP-1, varies among vertebrate species. An analysis of GIP peptide sequences indicate that the GIP hormone sequence underwent rapid sequence evolution on the early mammalian lineage (Irwin, 2009; Irwin and Zhang, 2006), but whether it was changes due to rapid sequence evolution or its increase in length during early tetrapod evolution (or some other event) that are responsible for GIP acquiring an incretin function is not known.

D. Exendin

In addition to GLP-1 and GIP some other peptides have been described that have incretin-like activity (Conlon *et al.*, 2006), the best characterized of these are the exendin-3 and exendin-4 peptides which have been isolated from the venom of two species of lizards, the Beaded lizard (*Heloderma horridum*) and Gila monster (*H. suspectum*), respectively (Eng *et al.*, 1990, 1992). Not only do these exendin peptides have incretin-like activity, but also have similarity to the GLP-1 sequence (Raufman, 1996; Raufman *et al.*, 1992). Peptides with incretin-like activity have also been isolated from some amphibians; however, these peptides do not have any similarity in sequence

with GLP-1 or GIP (Conlon *et al.*, 2006). Exendin-4 had promise as a pharmaceutical agent and is now used in therapy for Diabetes (Lovshin and Drucker, 2009). The isolation of cDNAs for exindin-4 and proglucagon from the Gila monster demonstrated that these two peptides are encoded by separate genes, and it appears that exendin is not encoded by any of the known member of the secretin gene family (Chen and Drucker, 1997; Chen *et al.*, 2006; Pohl and Wank, 1998). Searches for exendin-like genes in the chicken and mammalian genomes have failed to identify a closely related gene (Pohl and Wank, 1998), and a similar sequence could not be found in the genome sequence of the Anole lizard (*Anolis carolinensis*), the only reptile with a draft genome sequence (unpublished observations). In addition to exendin-4, Gila monster venom was found to contain additional peptides, helodermin (exendin-1) and helospectin (exendin-2), with sequence similarity to glucagon (Raufman, 1996). A cDNA for helodermin has also been characterized, and intriguingly when this protein sequence was compared to the exendin-4 precursor sequence it showed greater conservation of the sequences N-terminal to the glucagon-like venom sequences than of the glucagon-like sequences (Fry *et al.*, 2010; Pohl and Wank, 1998). In contrast, the glucagon-like sequences encoded by the proglucagon and *GIP* genes are better conserved than the flanking or intervening peptide sequences (Irwin, 2001a, 2009; Irwin and Zhang, 2006; McIntosh *et al.*, 2009). It has been suggested that the decreased similarity of the glucagon-like portion of the exendin and helodermin sequences in the Gila monster and Beaded lizard venom could be due to these sequences experiencing rapid adaptive sequence evolution due to positive selection to become toxins (Fry *et al.*, 2010), a process similar to that observed for other reptilian venom proteins (Aoki *et al.*, 2008; Gibbs and Rossiter, 2008).

III. Evolution of Incretin Hormone Receptor Genes

A. Receptors for glucagon-like sequences

Not only are the incretin hormones GLP-1 and GIP related in sequence, but so are their receptors (Harmar, 2001; Laburthe *et al.*, 1996). Both GLP-1 and GIP bind to target cells through specific G-protein-coupled receptors that are expressed in tissue-specific patterns (Mayo *et al.*, 2003). GLP-1 and GIP receptors are both members of Class B of G-protein coupled receptors, a class that not only includes the GLP-1 and GIP receptors but also receptors for the other proglucagon-derived peptides, glucagon, and GLP-2, and for other glucagon-like sequences including VIP, GHRH, PACAP, and secretin (Cardoso *et al.*, 2006; Fredriksson

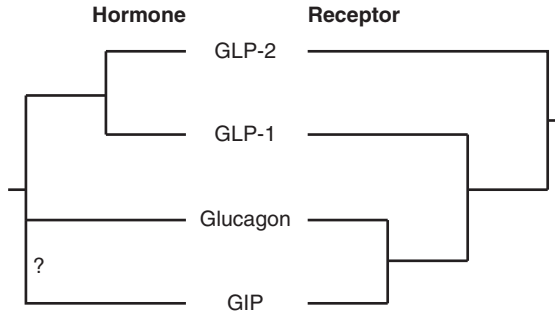

Figure 1.3 Contrasting evolutionary histories for glucagon-like hormone sequences and their receptors. The evolutionary relationships of proglucagon-derived peptides (Glucagon, GLP-1, and GLP-2) and GIP is shown on the left while the relationship of their receptors is shown on the right. The glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) are most closely related glucagon-like sequences, while the relationship to GIP to the proglucagon-derived peptides is unclear. The glucagon and GIP receptors are most closely related, while the GLP-2 receptor diverged from the others earliest.

et al., 2003; Harmar, 2001; Laburthe *et al.*, 1996). Within this group of receptors, the receptors for the proglucagon-derived peptides (glucagon, GLP-1, and GLP-2) and GIP were found to be most closely related (see Fig. 1.3; Cardoso *et al.*, 2006; Fredriksson *et al.*, 2003; Harmar, 2001; Irwin and Wong, 2005; Sivarajah *et al.*, 2001). It might have been expected that peptide hormones and their receptors coevolved, thus the relationships of the hormones should mirror that of the receptors. The GLPs, GLP-1 and GLP-2 are the most closely related to each other than to glucagon (Irwin *et al.*, 1999; Lopez *et al.*, 1984) however the relationship of GIP to the proglucagon-derived peptides remains unclear (Clynen *et al.*, 2004; Hoyle, 1998; Irwin, 2005; Sherwood *et al.*, 2000; see Fig. 1.3). If the hormone and receptor genes had coevolved, then we would have expected identical phylogenies for both the proglucagon-derived peptides and their receptors; however, the phylogeny of the receptors differs from that of the proglucagon-derived peptides (Irwin, 2005; Irwin and Wong, 2005). The GLP-1 and GLP-2 receptors are not most closely related, and instead, it was found that the GLP-1 receptor was more closely related to the Glucagon receptor than to the GLP-2 receptor (Cardoso *et al.*, 2006; Fredriksson *et al.*, 2003; Irwin and Wong, 2005; Sivarajah *et al.*, 2001). Intriguingly, the GIP receptor is most closely related to the glucagon receptor and is not closely related to the GLP-1 receptor (Cardoso *et al.*, 2006; Fredriksson *et al.*, 2003; Irwin and Wong, 2005; Sivarajah *et al.*, 2001). An implication of these observations is that coevolution was not a driving force in the duplication and divergence of the hormones and receptors, but rather that the selectivity of the hormone–receptor interaction evolved only after the duplication and

divergence of the hormones and the receptors (Irwin, 2005). Knowledge of the relationships of the receptors also allows it to be concluded that the incretin action of GLP-1 and GIP is not due to these hormones interacting with closely related receptors that had incretin action as an ancestral state, as these two receptors are not closely related (Cardoso *et al.*, 2006; Fredriksson *et al.*, 2003; Irwin and Wong, 2005; Sivarajah *et al.*, 2001) (Fig. 1.3). Incretin action of the GLP-1 and GIP receptors must have evolved independently and in parallel.

B. Receptors for incretin hormones

Most experimental studies on the characterization of incretin hormone receptors have focused on receptors from mammalian species (Ahrén, 2009; Mann *et al.*, 2007; Mayo *et al.*, 2003; McIntosh *et al.*, 2009). While the sequences for GLP-1 and GIP receptors have been predicted from a number of nonmammalian genomes (Cardoso *et al.*, 2005, 2006; Irwin and Wong, 2005; Ji *et al.*, 2009; Richards and McMurty, 2009; Roch *et al.*, 2009) only a few nonmammalian receptors have been functionally characterized. Glucagon receptors from a few species of fish and a frog have been isolated and partially characterized (Chow *et al.*, 2004; Ngan *et al.*, 1999; Sivarajah *et al.*, 2001) as well as receptors from two species of fish that can be bound and activated by GLP-1 (Mojsov, 2000; Yeung *et al.*, 2002). There is a single report on the functional characterization of a receptor from zebrafish that binds GIP (Musson *et al.*, 2009). Phylogenetic analyses show that all of the fish and amphibian glucagon receptors are closely related to the mammalian glucagon receptors, and that the fish GIP receptor is most closely related to mammalian GIP receptors, however, surprisingly, fish GLP-1 receptors were found to be more closely related to fish and mammalian glucagon receptors than to mammalian GLP-1 receptors (Chow *et al.*, 2004; Irwin, 2005; Irwin and Wong, 2005). The best characterized fish GLP-1 receptor is from the goldfish (*Carassius auratus*), and this receptor was found to bind both GLP-1 and glucagon (Chow *et al.*, 2004). While the function of glucagon has been conserved in vertebrates (Dugay and Mommsen, 1994; Moon, 2004), and it binds and activates orthologous receptors in diverse vertebrates (Irwin, 2005; Irwin and Wong, 2005), differing evolutionary mechanisms appear to lead to differing function of GLP-1 and GIP.

The zebrafish GIP receptor binds and is activated by zebrafish GIP and to a small extent by a product of the zebrafish proglucagon II gene (glucagon or GLP-1 as this gene does not encode GLP-2; Zhou and Irwin, 2004), suggesting that there has been conservation of the ligand hormone interaction, however the fish receptor may be more promiscuous as it was activated to a small extent by a proglucagon-derived peptide (Musson *et al.*, 2009). Zebrafish GIP though does not appear to be an incretin hormone as its site

(pancreas) and pattern (expression increases with food depravation) of expression are not consistent with an incretin function (Musson *et al.*, 2009). These observations suggest that changes in the expression of the GIP receptor gene, and downstream signaling from the receptor (and possibly site of receptor expression) cause the change in the function of GIP, although whether this change occurred on the fish or mammalian lineage are unknown. The characterization of *GIP* and GIP receptor gene expression and function in more vertebrate species is needed to resolve this question.

A different picture emerges for the change in the function of GLP-1 between fish and mammals. Genes for GLP-1 receptors cannot be found in fish genomic sequences and their absence likely explains the lack of GLP-1 incretin action in these species (Irwin, 2005; Irwin and Wong, 2005). The absence of a GLP-1 receptor would prevent GLP-1 from transmitting its signal. But the change of GLP-1 function is not just the loss of incretin action but also the gain of a glucagon-like function in fish, a function that it does not have in mammalian species (Dugay and Mommsen, 1994; Moon, 2004). The glucagon-like action of GLP-1 in fish appears to be mediated by the fact that GLP-1, of endogenous or exogenous origin (Dugay and Mommsen, 1994; Moon, 2004), binds to the fish GLP-1 receptor in fish, however this receptor is orthologous to mammalian glucagon receptors and therefore generates a downstream signal that is similar to that generated by an activated glucagon receptor (Irwin, 2005; Irwin and Wong, 2005). The change in function of GLP-1 in fish is therefore caused by the loss of incretin function but instead is due to loss of the receptor that generates incretin signaling. GLP-1 in fish now binds to a glucagon receptor (that has evolved a new binding activity), thus GLP-1 has gained a new function (Irwin, 2005; Irwin and Wong, 2005).

IV. Evolution of Incretins

Mammals have evolved tight control of blood glucose levels with incretin hormones being an essential components in this regulation of blood glucose levels (Asmar and Holst, 2010; Baggio and Drucker, 2007; Holst, 2007; Nauck, 2009; Wideman and Kieffer, 2009). However, to date, incretin function has only been clearly functionally described in mammals (Dugay and Mommsen, 1994; McIntosh *et al.*, 2009; Moon, 2004). GLP-1 and GIP are the principal incretin hormones that act through specific receptors, the GLP-1 receptor and GIP receptor, respectively, and these peptides have evolved incretin function independently. Unfortunately, it still remains unclear when in vertebrate evolution

GLP-1 or GIP evolved incretin activity. GLP-1 and GIP appear to have originated through gene duplication (GIP) and exon duplication (GLP-1) in an ancestor of all vertebrates (Hoyle, 1999; Irwin, 2005; Irwin *et al.*, 1999; Lopez *et al.*, 1984; Sherwood *et al.*, 2000). Likewise, receptors for these hormones also existed in the common ancestor of all vertebrates (Cardoso *et al.*, 2006; Fredriksson *et al.*, 2003; Harmar, 2001; Irwin and Wong, 2005; Sivarajah *et al.*, 2001). However, the presence of these genes in an ancestor does not mean they had the same function that is currently found in some of their modern descendents, it can only be concluded in the common ancestor of those species that have the incretin trait, mammals, that GLP-1 and GIP had an incretin function. Some evidence from chicken suggests a function for GLP-1 that is not inconsistent with it being an incretin (Richards and McMurtry, 2008), which suggests that incretin function possibly existed in the ancestor of birds and mammals. GLP-1 function in Amphibians has not been carefully studied (Dugay and Mommsen, 1994) however the GLP-1 peptide is produced in the pancreas (Conlon *et al.*, 1998) which is inconsistent with an incretin function. Fish GLP-1 does not act as an incretin (Dugay and Mommsen, 1994; Moon, 2004) however this is due to a change in the identity of its receptor (Irwin, 2005; Irwin and Wong, 2005). The current data would suggest that GLP-1 may have acquired incretin action on the common ancestral lineage of amniotes (birds, reptiles, and mammals) after the divergence of amphibians, but an earlier origin cannot be excluded.

Compared to GLP-1, even less is known about GIP function in non-mammalian vertebrates. The *GIP* gene of chicken and *Xenopus* is expressed in the intestine (Irwin and Zhang, 2006), consistent with it being an incretin, while that of the zebrafish is expressed in the pancreas (Musson *et al.*, 2009), a pattern inconsistent with it being an incretin. These observations might suggest that GIP acquired incretin action in the ancestor of tetrapods (amphibians, birds, and mammals), and thus potentially the earliest evolving incretin hormone however we cannot exclude the possibility of an even earlier origin with a change of activity on the fish lineage. It is clear that to achieve a better understanding of the origin and evolution of incretin function that further studies of the function of GLP-1 and GIP from diverse vertebrate species are required.

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CHAPTER TWO

Pleiotropic Actions of the Incretin Hormones

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Contents

Abstract

The insulin secretory response to a meal results largely from glucose stimulation of the pancreatic islets and both direct and indirect (autonomic) glucose-dependent stimulation by incretin hormones released from the gastrointestinal

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tract. Two incretins, Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), have so far been identified. Localization of the cognate G protein-coupled receptors for GIP and GLP-1 revealed that they are present in numerous tissues in addition to the endocrine pancreas, including the gastrointestinal, cardiovascular, central nervous and autonomic nervous systems (ANSs), adipose tissue, and bone. At these sites, the incretin hormones exert a range of pleiotropic effects, many of which contribute to the integration of processes involved in the regulation of food intake, and nutrient and mineral processing and storage. From detailed studies at the cellular and molecular level, it is also evident that both incretin hormones act via multiple signal transduction pathways that regulate both acute and long-term cell function. Here, we provide an overview of current knowledge relating to the physiological roles of GIP and GLP-1, with specific emphasis on their modes of action on islet hormone secretion, β-cell proliferation and survival, central and autonomic neuronal function, gastrointestinal motility, and glucose and lipid metabolism. However, it is emphasized that despite intensive research on the various body systems, in many cases there is uncertainty as to the pathways by which the incretins mediate their pleiotropic effects and only a rudimentary understanding of the underlying cellular mechanisms involved, and these are challenges for the future.

I. Introduction

Following the discovery of secretin (Bayliss and Starling, 1902), Moore *et al.* (1906) proposed that a similar factor might be present in the intestinal mucosa that increased the "internal secretion" of the pancreas. They administered mucosal extracts orally to type 1 diabetes (T1DM) patients and reported successful reductions in glucosuria in some individuals. However, as pointed out by Creutzfeldt (2005), the positive responses obtained were unlikely to have been due to peptide hormones in the extracts and were probably a result of spontaneous remission. Subsequently, the effects on blood glucose of various crude secretin preparations were examined by a number of groups, with variable results (Creutzfeldt, 2005). The Belgian physiologist, Jean La Barre, was among those who found that intravenous (i.v.) administration of upper intestinal extracts produced a hypoglycemic response, and he introduced the term incrétine to describe the factor(s) responsible (La Barre, 1932). A regulatory role for the gut in glucose homeostasis was unambiguously established by two groups which determined that insulin responses to glucose delivered via gastric (Elrick *et al.*, 1964) or jejunal (McIntyre *et al.*, 1964) intubation were greater than when the same amount of glucose was infused intravenously. It was estimated that 50–60% of the total insulin secreted during a meal was a result of gut factors (Perley and Kipnis, 1967). The term "enteroinsular axis" was

later introduced to describe the hormonal link between the gut and the endocrine pancreas (Unger and Eisentraut, 1969) and the term "incretin" was reintroduced (Creutzfeldt, 1979) to describe hormone(s) that are released from the intestine in response to glucose and stimulate insulin secretion in a glucose-dependent manner (Creutzfeldt, 1979). There is also an important neural component of the enteroinsular axis that includes both autonomic reflexes and direct enteropancreatic innervation (Kirchgessner and Gershon, 1990).

Several groups sought the factor(s) responsible for incretin activity in intestinal extracts, and the first to be identified was gastric inhibitory polypeptide (GIP), a 42 amino acid peptide originally isolated on the basis of its acid inhibitory (enterogastrone) activity in dogs (Brown, 1971; Brown and Dryburgh, 1971). It was subsequently shown to stimulate insulin secretion in a glucose-dependent manner, (Brown *et al.*, 1975) and the name "glucose-dependent insulinotropic polypeptide" was introduced as an alternative definition of the acronym GIP (Brown and Pederson, 1976), to reflect its incretin status. Identification of a second incretin originated in the discovery by Lund and Habener that the anglerfish proglucagon gene coded for an additional glucagon-like peptide (GLP; Lund *et al.*, 1982). Two mammalian glucagon-related sequences were subsequently identified in the mammalian proglucagon gene (Bell *et al.*, 1983). Intestinal processing of proglucagon results in the production of GLP-$1_{7–36\ amide}$ and GLP-$1_{7–37}$, both of which potentiate glucose-induced insulin secretion (Holst *et al.*, 1987; Lund, 2005; Mojsov *et al.*, 1987), as well as GLP-2. The major circulating incretin form is GLP-$1_{7–36\ amide}$ and, by convention, combined tissue or plasma GLP-$1_{7–36\ amide}$ and GLP-$1_{7–37}$ levels are generally referred to as GLP-1. Although other insulinotropic hormones are synthesized in the gut, GIP and GLP-1 are the only physiological incretins identified so far.

GIP and GLP-1 are synthesized by enteroendocrine cells of the gastrointestinal tract, classified as K- and L-cells, respectively (Holst, 2007; McIntosh *et al.*, 2009). However, subpopulations of enteroendocrine cells demonstrating colocalization of the two hormones (Mortensen *et al.*, 2003) have been identified. Both GIP and GLP-1 are additionally synthesized in brain neurons (Nyberg *et al.*, 2005; Tang-Christensen *et al.*, 2001) and GLP-1 is produced in islet α-cells and in taste buds. Both hormones are released in response to a meal and exhibit almost identical insulinotropic activity (Drucker, 2006, 2007; McIntosh *et al.*, 2009; Meier *et al.*, 2002b; Vahl and D'Alessio, 2003). Termination of the insulinotropic activity of both GIP and GLP-1 is performed by the prolyl endopeptidase, dipeptidyl peptidase-IV (Drucker and Nauck, 2006; McIntosh, 2008).

It is now recognized that, in addition to their incretin effects, both GIP and GLP-1 exert pleiotropic actions (Brubaker, 2006; McIntosh *et al.*, 2005; Fig. 2.1), many of which contribute to the integration of processes involved in the regulation of food intake, and nutrient and mineral processing and

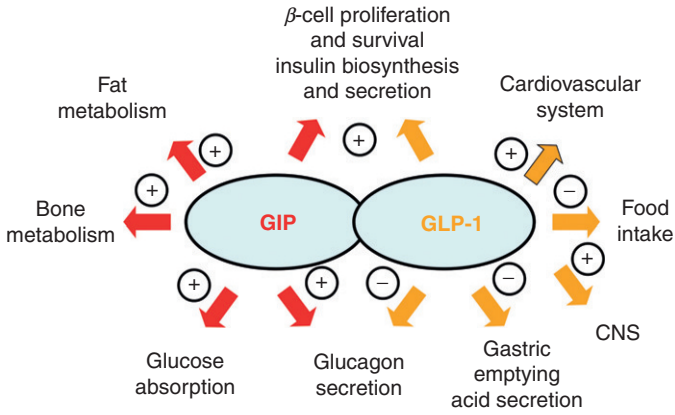


Figure 2.1 Major actions of GIP and GLP-1. In some cases, for example the cardiovascular system, both hormones have been shown to exert effects but, as indicated in the text, the hormone for which a physiological role has been most clearly demonstrated is indicated.

storage (Abu-Hamdah *et al.*, 2009; Drucker, 2007; McIntosh *et al.*, 2009). This includes the regulation of physiological events underlying food intake and satiety, passage of chyme through the gastrointestinal tract, nutrient digestion and absorption, as well as intravascular transport and storage of nutrients. This is an extremely complex set of processes, our understanding of which is still rudimentary. Additionally, both hormones play key regulatory roles in the proliferation and survival of pancreatic islets. Two classes of therapeutics that take advantage of the pleiotropic actions of the incretin hormones have recently been introduced for treatment of type 2 diabetes: DPP-IV resistant analogs of GLP-1 (incretin mimetics) and DPP-IV inhibitors (incretin enhancers; Drucker and Nauck, 2006; McIntosh, 2008). In this review, we have attempted to provide an up-to-date summary of current knowledge relating to the physiological roles of GIP and GLP-1 and their modes of action.

II. GIP and GLP-1 Actions: Hormonal and Neuronal Pathways

The pleiotropic actions of GIP and GLP-1 are mediated by interaction with their cognate G protein-coupled receptors (GIP-R and GLP-1R), both of which are present in the endocrine pancreas, gastrointestinal tract, brain, and immune and vascular systems (Baggio and Drucker, 2007; Drucker, 2007; Holst, 2007; McIntosh *et al.*, 2009). The GIP-R is also expressed in adipose tissue and bone, whereas the GLP-1R exhibits a much

broader distribution, including the ANS, lungs, heart, and kidneys (Baggio and Drucker, 2007; Drucker, 2007; Holst, 2007; McIntosh *et al.*, 2009). As discussed later, there is controversy over the identity of the GLP-1 responsive receptor in the liver and the muscle (Holst, 2007).

GIP secreted from the intestine is believed to act primarily in a classical endocrine fashion whereas, although GLP-1 acts on some tissues via the endocrine route, the ANS is thought to mediate many of its actions (Drucker, 2006; Holst, 2007; McIntosh, 2008). GLP-1 and GIP, as well as their receptors, are additionally synthesized in various regions of the brain (Nyberg *et al.*, 2005; Tang-Christensen *et al.*, 2001; Williams, 2009), where they act as neurotransmitters and are probably also involved in neuroprotection and the regulation of neuron proliferation (During *et al.*, 2003; Nyberg *et al.*, 2005). The GIP/GIPR system was also recently identified in neurons and glial cells of the peripheral nervous system (Buhren *et al.*, 2009), but the physiological significance of this localization is unclear.

A number of diverse studies have provided evidence for an indirect action of L-cell derived GLP-1, mediated via activation of autonomic nerves (Fig. 2.2; Holst, 2007; Holst and Deacon, 2005). Hansen *et al.* (1999) showed that endothelial cells in the vicinity of the enteroendocrine L-cells express DPP-IV, and the majority of secreted GLP-1 appears to be degraded within seconds of its release into the interstitial space. The close proximity of autonomic nerve terminals in the lamina propria to the mucosal L-cells suggested that locally released peptide activates afferent autonomic nerve fibers (Holst and Deacon, 2005). Additional sites of action on the ANS have also been identified (Fig. 2.2). GLP-1 receptors are present in the rat nodose ganglion (Nakagawa *et al.*, 2004; Vahl *et al.*, 2007), where cell bodies of afferent vagal neurons reside, as well as in nerves innervating the portal vein (Vahl *et al.*, 2007) and the liver. Intraportal infusion of GLP-1, but not GIP (Nishizawa *et al.*, 1996), increased the firing rate of both hepatic afferent nerves and efferent pancreatic branches of the vagus nerves (Nakabayashi *et al.*, 1996; Nishizawa *et al.*, 2000), showing that complete neural reflexes are activated by GLP-1. Ganglionic blockade (Balkan and Li, 2000) or sensory autonomic nerve ablation with capsaicin (Ahrén, 2004) reduced GLP-1-induced insulin secretion, demonstrating the physiological importance of such pathways. Administration of low concentrations of a GLP-1R antagonist into the rat portal vein, but not peripherally, resulted in intolerance to an enteral glucose load, providing further support for portal receptors regulating glucose homeostasis (Vahl *et al.*, 2007). GLP-1 is also involved in maintaining the competence of hepatoportal glucose sensors (Burcelin *et al.*, 2001). Vagal reflex circuits are now considered to play major roles in GLP-1-mediated stimulation of insulin secretion (Ahrén, 2004; Balkan and Li, 2000) and glucose homeostasis (Sandoval *et al.*, 2008), as well as in the inhibition of gastric emptying (Imeryuz *et al.*, 1997; Wettergren *et al.*, 1998; Fig. 2.2).

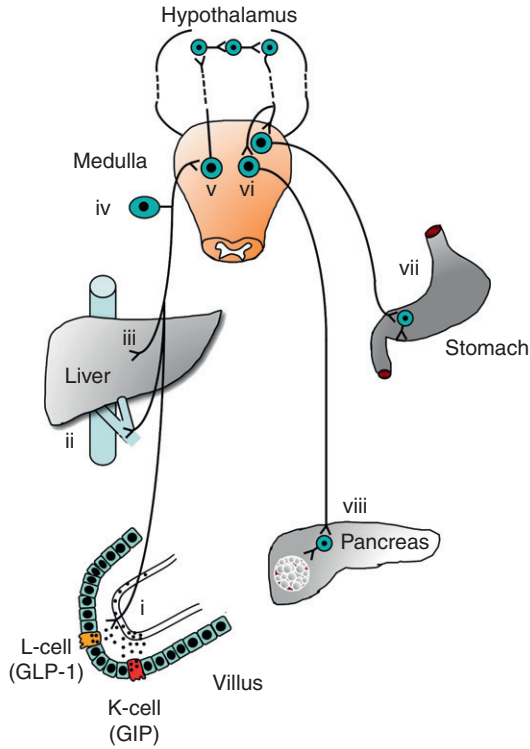


Figure 2.2 Proposed pathways by which GLP-1 and GIP exert their actions on the stomach and pancreas. In response to nutrients, GIP and GLP-1 are released mainly from K- and L-cells in the intestinal mucosa, respectively. Within the lamina propria of the intestinal villus (i), as well as within the hepatoportal system (ii) and the liver (iii), GLP-1 may activate afferent sensory neurons with cell bodies in the nodose ganglion (iv). Through activation of neuronal pathways involving the nucleus tractus solitarius (v), the hypothalamus and the dorsal vagal motor nucleus (vi) efferent vagal nerves are believed to be activated, resulting in inhibition of gastric emptying (vii) and stimulation of insulin secretion (viii). The transmitters released from intrinsic pancreatic nerves have not been identified with certainty, but may include NPY, PACAP, and VIP. Both GIP and GLP-1 can stimulate insulin secretion via the bloodstream. (Based on Figure 5 in McIntosh, 2008.)

The rat brainstem contains cell bodies of GLP-1 expressing neurons, localized in the nucleus tractus solitarius (NTS) and reticular nucleus of the medulla (Huo *et al.*, 2009; Jin *et al.*, 1988), as well as neurons that express GLP-1 receptors. The brainstem GLP-1 producing neurons innervate numerous sites, including the paraventricular nucleus (PVN), periventricular nucleus, and thalamic nucleus, and the brainstem itself. GLP-1 receptors are located throughout the rostrocaudal hypothalamus, including the arcuate, paraventricular and supraoptic nuclei, as well as in the anterior thalamic

nucleus and amygdala (Kanse *et al.*, 1988; Navarro *et al.*, 1996; Turton *et al.*, 1996). Intracerebroventricular (i.c.v.) GLP-1 administration was shown to markedly increase *c-fos* expression in the supraoptic nucleus and PVN of the hypothalamus, and central nucleus of the amygdala, with lesser increases in the arcuate nucleus (Tang-Christensen *et al.*, 2001; Turton *et al.*, 1996). There is therefore a complex intrinsic neuronal circuitry mediating responses to NTS-derived GLP-1, many components of which are associated with the regulation of feeding. However, circulating GLP-1 may also enter the brain from the circulation via diffusion across the blood–brain barrier (Kastin *et al.*, 2002), as well as act on circumventricular organs in regions such as the subfornical organ and area postrema (Kanse *et al.*, 1988). GLP-1 responsive neurons have been identified by electrophysiological recording in the latter region (Yamamoto *et al.*, 2003). Additionally, intraperitoneal (i.p.) or peripheral administration of the GLP-1 receptor agonist exendin-4 (ex-4) to mice increased c-fos expression in the area postrema, periventricular nucleus and the NTS, as well as decreasing food intake (Baggio *et al.*, 2004a). There is therefore abundant evidence for GLP-1 playing important roles in autonomic and central integrating systems and, during a meal, interaction between these and the GIP and GLP-1 endocrine responses are central to the regulation of glucose homeostasis. Equivalent neural pathways in humans remain to be established.

III. Effects of GIP and GLP-1 on Early Events During Feeding

The majority of GIP and GLP-1 actions are initiated following digestion and absorption of the major nutrients. However, subpopulations of GLP-1 containing cells were recently identified in oral circumvallate papillae (Feng *et al.*, 2008; Shin *et al.*, 2008). One of the GLP-1 producing cell types also expressed α-gustducin and the TIR3 sweet taste receptor subunit, while local terminals of afferent ANS nerves expressed GLP-1 receptors, suggesting the presence of a GLP-1 regulated taste perception system within the taste bud (Shin *et al.*, 2008). Altered taste perception was also identified in GLP-1 receptor knockout (GLP-1R$^{-/-}$) mice (Shin *et al.*, 2008) and it has been suggested that GLP-1 may impact on orosensory feedback systems involved in the control of rodent licking (Asarian *et al.*, 1998). GIP may also play a role in the regulation of salivary function, since there have been reports of GIP expression in the rat submandibular gland (Tseng *et al.*, 1995) and effects of GIP on Na^+ transport in the rabbit mandibular gland (Denniss and Young, 1978). Human fasting salivary levels of GIP were found to be higher than in plasma and to increase following swallowing (Messenger

et al., 2003), suggestive of a local secretion. Of interest is the identification of DPP-IV in exosome-like vesicles in human saliva (Ogawa *et al.*, 2008).

IV. Effects of Incretins on Functions of the Endocrine Pancreas

A. Effects of GIP and GLP-1 on β-cell secretion

It is generally accepted that GIP and GLP-1 are the major, if not the sole, incretin hormones in mammals (Drucker, 2006; Holst, 2007; McIntosh *et al.*, 2009). GIP acts directly on pancreatic β-cells and a direct mode of action for GLP-1 is supported by the presence of GLP-1Rs on β-cells (Moens *et al.*, 1996; Thorens *et al.*, 1993), *in vitro* islet responsiveness to GLP-1R agonists (Gromada *et al.*, 1998; Moens *et al.*, 1996), and the preserved GLP-1 stimulation of insulin secretion observed following orthotopic pancreas transplantation (Kissler *et al.*, 1999). As discussed in Section II, GLP-1 stimulates autonomic reflex pathways impacting on pancreatic endocrine function, including hepatopancreatic reflexes (Ahrén, 2004; Balkan and Li, 2000; Nakabayashi *et al.*, 1996; Nishizawa *et al.*, 1996, 2000). Pancreas-projecting neurons in the dorsal motor vagal nucleus that respond to GLP-1 have also been identified, but it is unclear whether they respond to both centrally and systemically derived peptide (Wan *et al.*, 2007). In the majority of studies on the *in vivo* effects of GLP-1 on pancreatic endocrine secretion, no attempt has been made to distinguish between direct effects on islet cells and indirect autonomic actions.

In humans, both GIP and GLP-1 stimulate insulin secretion at physiological concentrations (Andersen *et al.*, 1978; Elahi *et al.*, 1979; Kreymann *et al.*, 1987) and in a glucose-dependent manner (Vilsbøll *et al.*, 2003). In the rat, a glucose threshold of ~4.5 mM was established in the perfused rat pancreas, with maximum potentiation at 16.7 mM glucose (Jia *et al.*, 1995; Pederson and Brown, 1976). GIP and GLP-1 exhibited similar potencies in this model (Jia *et al.*, 1995). Circulating levels of GIP following a meal or glucose load are up to six-fold greater than those of GLP-1 (Holst, 2007). However in glucose clamp studies, it has been estimated that the two peptides contribute approximately equally to the incretin effect (Vilsbøll *et al.*, 2003). This conclusion is supported by studies in rodents, in which administration of antibodies against the GIP receptor (Lewis *et al.*, 2000) or GIP receptor antagonists (Gault *et al.*, 2002; Tseng *et al.*, 1996, 1999) reduced the incretin effect by 50–70%, with similar estimates for the contribution of GLP-1 obtained using the receptor antagonist exendin (9–39) (ex(9–39)) in rats (Kolligs *et al.*, 1995) and humans (Schirra *et al.*, 2006). Studies in GIP-R$^{-/-}$, GLP-1R$^{-/-}$, and double incretin receptor knockout (DIRKO) mice have confirmed roles for both GIP and GLP-1 in

glucose homeostasis (Hansotia *et al.*, 2004; Miyawaki *et al.*, 1999; Preitner *et al.*, 2004; Scrocchi *et al.*, 1996). However, GIP-R$^{-/-}$ and GLP-1R$^{-/-}$ mice exhibit only mild glucose intolerance and slightly reduced insulin responses to oral glucose (Miyawaki *et al.*, 1999; Scrocchi *et al.*, 1996), probably due to adaptation, and disturbances in glucose homeostasis were markedly greater in the DIRKO mice (Hansotia *et al.*, 2004; Preitner *et al.*, 2004).

B. Effects of GIP and GLP-1 on the secretion of other islet hormones

GLP-1 inhibits glucagon secretion in all species studied (Drucker, 2007; Dunning *et al.*, 2005; Fridolf *et al.*, 1991; Ørskov *et al.*, 1988), an action that contributes significantly to the antidiabetic effects of both incretin mimetics and incretin enhancers (Drucker and Nauck, 2006; Dunning and Gerich, 2007; Dunning *et al.*, 2005). However, there is considerable controversy over the mechanisms involved. Although GLP-1 receptors were initially found to be localized on rodent islet α-cells using immunocytochemistry (ICC; Heller *et al.*, 1997), GLP-1R mRNA transcripts have not been detected in FACS sorted α-cells (Franklin *et al.*, 2005; Moens *et al.*, 1996). Additionally, with more recent ICC determinations, GLP-1 receptors were found to be restricted to β-cells in islets from rats and humans (Tornehave *et al.*, 2008) and effects of GLP-1 on glucagon secretion observed in isolated islets (Ding *et al.*, 1997) could not later be replicated with purified α-cells (Franklin *et al.*, 2005). There is substantial evidence that the inhibitory effect of GLP-1 observed *in vivo* involves β-cell secretory products, including insulin and zinc (Dunning *et al.*, 2005; Franklin *et al.*, 2005). However, the retention of such effects under conditions of minimal β-cell secretion, including low glucose and in type 1 diabetes, indicates that non-β-cell-mediated actions also contribute (Gromada *et al.*, 2007). Studies on the perfused rat pancreas have led to conflicting conclusions, with evidence both for (de Heer *et al.*, 2008) and against (Silvestre *et al.*, 2003) a role for intraislet somatostatin (SS) in GLP-1R-mediated inhibition of glucagon secretion. Interestingly, studies on SS knockout mice revealed an essential role for the peptide in glucose-induced inhibition of α-cell secretion; unfortunately, responses to GLP-1 were not reported (Hauge-Evans *et al.*, 2009). In view of the receptor localization studies, there is a need to establish whether there are species differences in the inhibitory effects of GLP-1 on glucagon secretion or if there are multiple pathways involved, including intrinsic pancreatic neural pathways (Gromada *et al.*, 2007).

In contrast to GLP-1, GIP has been shown to stimulate pancreatic glucagon secretion from perfused rat (Pederson and Brown, 1978) and dog (Adrian *et al.*, 1978) pancreata with maximal effects in the presence of low glucose. In rat islets, the stimulatory effect of GIP involved activation of protein kinase A (PKA; Ding *et al.*, 1997). GIP-induced glucagon release

may play a role in maintaining normoglycemia during high protein meals in these species. Most studies in humans have examined the effect of GIP on glucagon secretion at elevated glucose concentrations and found no effect in normal individuals (Elahi *et al.*, 1979; Nauck *et al.*, 1993). However, GIP exerted glucagonotropic actions in perfused pancreata from human cadavers (Brunicardi *et al.*, 1990) and increased glucagon levels under fasting conditions (Meier *et al.*, 2002a). GIP may therefore play a physiological role in the regulation of α-cell secretion in humans. Similar to GLP-1 (de Heer *et al.*, 2008; Silvestre *et al.*, 2003), GIP also stimulates secretion of pancreatic SS (Ipp *et al.*, 1977; Szecowka *et al.*, 1982) and both incretin hormones may stimulate pancreatic polypeptide secretion (Adrian *et al.*, 1978; Fehmann *et al.*, 1995) in rodents, although the significance of these effects for humans is unclear.

C. Mechanisms of GIP and GLP-1 action on insulin secretion

There is an extensive literature on the molecular characteristics of the class B GIP and GLP-1 G protein-coupled receptors and the signal-transduction pathways involved in regulating insulin secretion, including a number of recent reviews (Doyle and Egan, 2007; Drucker, 2006; Holz *et al.*, 2006; Kim and Egan, 2008; McIntosh *et al.*, 2009). In this section, we focus mainly on the mechanisms by which GIP and GLP-1 potentiate glucose-stimulated insulin secretion. β-cell uptake and metabolism of glucose results in increases in the intracellular ATP/ADP ratio and closure of ATP-sensitive K^+ (K_{ATP}) channels (Fig. 2.3). The resultant membrane depolarization activates voltage-dependent Ca^{2+} channels (Ca_v), thus increasing the intracellular (i) [Ca^{2+}] and triggering of insulin granule exocytosis (Ashcroft and Rorsman, 2004; Hinke *et al.*, 2004; Hiriart and Aguilar-Bryan, 2008; Fig. 2.3). Membrane repolarization is mediated by voltage-dependent K^+ (K_v; MacDonald and Wheeler, 2003) and Ca^{2+}-sensitive K^+ (K_{Ca}) channels. The incretin hormones act at multiple levels within this complex series of events.

GLP-1 and GIP share a common mechanism in stimulating adenylyl cyclase (AC) through a Gs-coupled process resulting in localized increases in cyclic AMP (cAMP; Seino and Shibasaki, 2005; Figs. 2.3 and 2.4). This has been demonstrated in pancreatic tumor cell lines, isolated pancreatic islets, FACS-purified β-cells, and cell lines transfected with GIP or GLP-1 receptor cDNA (reviewed in Baggio and Drucker, 2007; Doyle and Egan, 2007; Hinke *et al.*, 2004; Holz *et al.*, 2008; McIntosh *et al.*, 2009). Multiple members of the AC family are expressed in β-cells (Doyle and Egan, 2007) and it is unclear as to which subtypes couple to the incretin pathway. However, type VIII AC is dually activated by Ca^{2+}-calmodulin and Gsα, and it has been suggested to act as a "coincidence detector" (Delmeire *et al.*, 2004; Hinke *et al.*, 2004) of signals from glucose (Ca^{2+}) and the incretins

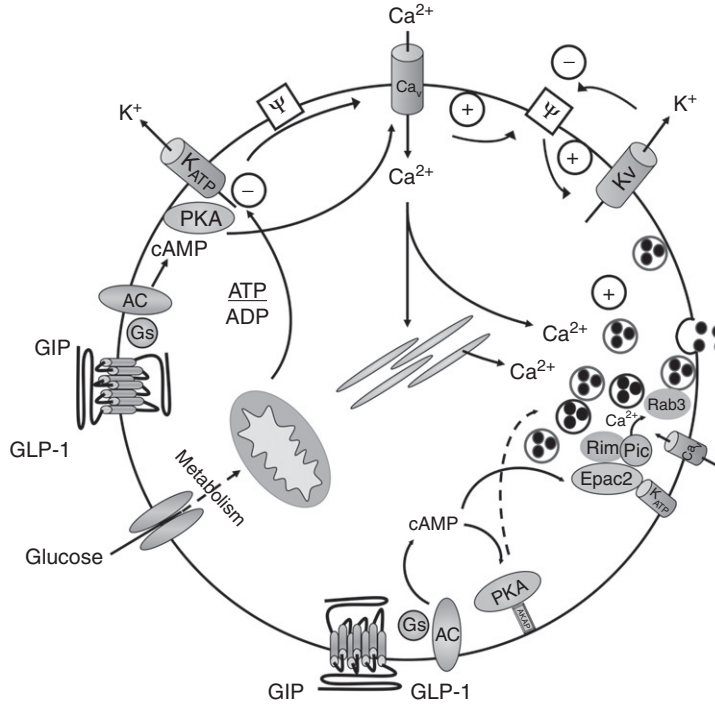


Figure 2.3 Representation of β-cell events resulting in stimulation of insulin secretion through interaction between glucose and GIP and GLP-1 signaling via activation of adenylyl cyclase. See text for details. Gs, Stimulatory G protein; cAMP, Cyclic AMP; PKA, Protein kinase A; AKAP, A-Kinase Anchoring Protein; Epac2, exchange protein directly activated by cAMP 2/cAMP-specific guanine nucleotide exchange factor (GEF) II; Pic, Piccolo; Rim2, Regulating synaptic membrane exocytosis 2; Rab2 (Member RAS oncogene family); K_{ATP}, ATP-dependent K^+ channel; Ca_v, Voltage-dependent Ca^{2+} Channel; K_v, Voltage-dependent K^+ channel.

(cAMP). The importance of dual signaling from Ca^{2+} and cAMP is evident from studies on the "glucose competence" of β-cells. Pipeleers (1987) first suggested that purified β-cells exhibited poor glucose-responsiveness due to the necessity for synergistic interaction between cAMP, resulting from glucagon stimulation, and a nutrient-induced messenger. In electrophysiological studies, it was later shown that treatment of isolated β-cells with GLP-1 instilled competence in cells previously unresponsive to glucose (Holz *et al.*, 1993). Glucagon also induces glucose competence in the intact islet (Huypens *et al.*, 2000), and the combined effects of GIP, GLP-1, and glucagon probably contribute to overall β-cell glucose responsiveness.

Synchronous, in-phase, oscillations in intracellular levels of cAMP and Ca^{2+} have recently been identified using real-time measurements

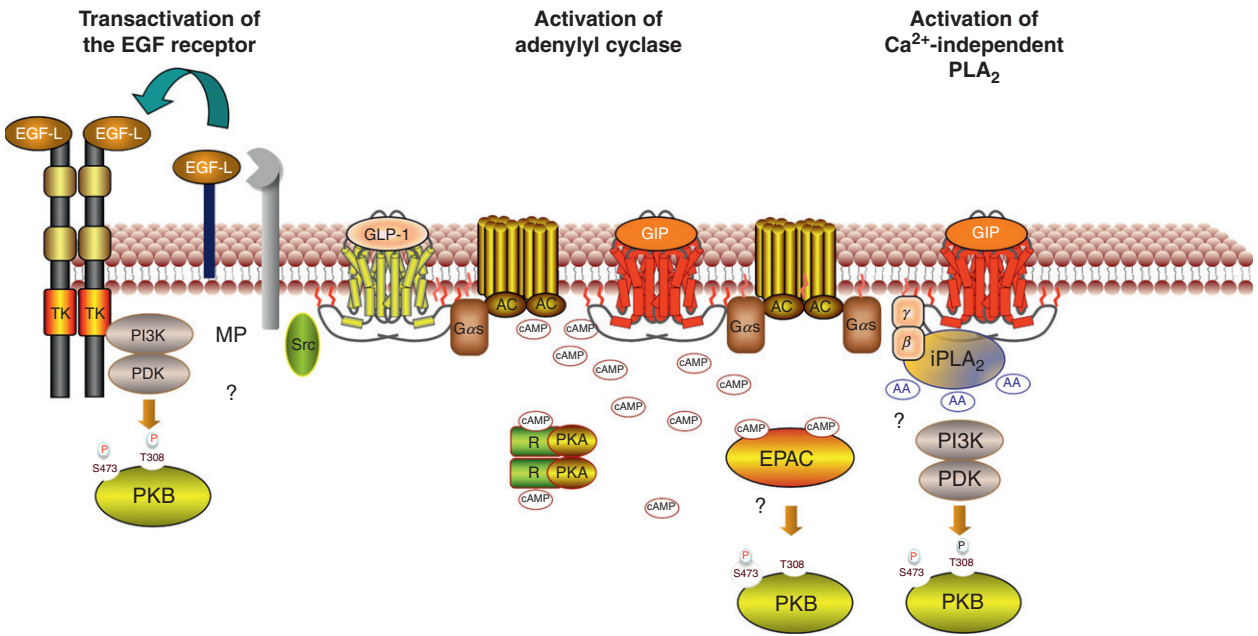


Figure 2.4 Alternative pathways by which GIP and GLP-1 may act In the β-cell. There is strong evidence that both GIP and GLP-1 act via stimulation of adenylyl cyclase and activation of EPAC and PKA. GIP also activates Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) through a mechanism that appears to involve Gβγ. GIP and GLP-1 both stimulate PKB (Akt). GIP can increase PKB enzyme activity without increasing phosphorylation of serine (S) 473 and threonine (T) 308, probably via EPAC2 activation. Chronic stimulation of the β-cell by GIP results in phosphorylation of both S473 and T308 through a Phosphoinositide 3 Kinase (PI3K)/PDK (phosphoinositide-dependent protein kinase-1)-mediated pathway. GLP-1 activates membrane-bound metalloproteinase(s) (MP), through a c-Src-mediated pathway, resulting in release of an activating ligand for the EGF receptor, leading to activation of PI3K and phosphorylation of S473 and T308 in PKB. ?, Uncertainties. Other MAP Kinase pathways are also activated, as discussed in the text.

(Fridlyand *et al.*, 2007). Ca^{2+}-calmodulin also modulates the activity of phosphodiesterase (PDE) 1C, and it has been suggested that synergistic interaction between GLP-1/Gsα and Ca^{2+}-calmodulin account for the in-phase oscillations (Fridlyand *et al.*, 2007; Holz *et al.*, 2008). There is also evidence for a role for PDE 3B in GLP-1 action (Doyle and Egan, 2007). Incretin-induced increases in β-cell cAMP result in the activation of both PKA (Ding and Gromada, 1997) and cAMP-specific guanine nucleotide exchange factor (GEF)/exchange protein directly activated by cAMP (Epac; Holz, 2004; Holz *et al.*, 2006; Seino and Shibasaki, 2005; Figs. 2.2 and 2.3). It is not known whether GIP and GLP-1 receptors are localized in distinct microdomains of the β-cell, but β-arrestin-1 (Sonoda *et al.*, 2008) and scaffolding proteins, such as AKAP18, AKAP95, and AKAP79/150 (Faruque *et al.*, 2009; Fraser *et al.*, 1998; Lester *et al.*, 2001), are likely shared regulators of signaling pathways. Multiple PKA isoforms have also been identified in islets and β-cell lines, and phosphorylation of a number of targets is involved in the effects of incretins on both proximal and distal events involved in insulin granule exocytosis.

In rodents, the high K_m glucose transporter GLUT2 rapidly equilibrates glucose across the β-cell plasma membrane, after which it is phosphorylated by glucokinase. Surprisingly, GLP-1 was found to phosphorylate GLUT2 in the C-terminus, thus reducing its rate of glucose transport (Thorens *et al.*, 1996). Although the significance of this effect has not been established, it is possible that phosphorylated GLUT2 interacts with, and regulates, intracellular proteins involved in insulin secretion (Thorens *et al.*, 1996). Alternatively, phosphorylation may modulate receptor-related functions of GLUT2 (Leturque *et al.*, 2009) or its ability to direct glucose toward nonmetabolic functions such as glucose-stimulated gene expression. Similar studies have not been performed in human islets. K_{ATP} channels couple glucose metabolism to insulin secretion. Patch-clamp studies have shown that GLP-1 inhibits β-cell K_{ATP} channel activity in a glucose-dependent manner (Gromada *et al.*, 1998, 2004) and both PKA and Epac are now thought to be involved. PKA-mediated phosphorylation of Ser_{1448} of the β-cell SUR1 subunit was shown to result in ADP-dependent K_{ATP} channel closure (Light *et al.*, 2002). Epac-2 interacts with the nucleotide-binding fold-1 of SUR1 (Holz *et al.*, 2006; Kang *et al.*, 2006), and in a series of studies using selective Epac agonists and a dominant negative form of Epac1, Holz and coworkers showed that Epac sensitizes the K_{ATP} channel to inhibition by ATP (Kang *et al.*, 2006, 2008). It is currently unknown whether Epac-1 is also involved in GLP-1 action, since both forms of Epac are present in the β-cell, whether GIP exerts identical effects to GLP-1, or whether Epac and PKA actions occur over different concentration ranges of glucose. There is also uncertainty over the effects of incretins on ATP levels. In studies on MIN6 cells, GLP-1 was shown to stimulate β-cell mitochondrial ATP production through a mechanism involving PKA- and Epac-2-mediated increases in

endoplasmic reticulum (ER) Ca^{2+} release (Rutter *et al.*, 2006; Tsuboi *et al.*, 2003). This would be expected to result in greater inhibition of K_{ATP} channel activity and potentiation of insulin release. However, no major effects of GLP-1 receptor agonists on intermediary metabolism or ATP production were observed in a study on rodent islets (Peyot *et al.*, 2009). Clearly, this discrepancy needs to be resolved.

Incretin modulation of Ca^{2+} fluxes in β-cells involves the effects on cell uptake and release from intracellular stores (Doyle and Egan, 2007; Gromada *et al.*, 1998). Both GLP-1 (Gromada *et al.*, 1998) and GIP (Lu *et al.*, 1993; Wheeler *et al.*, 1995) increase influx of Ca^{2+} through L-type voltage-dependent channels (Ca_ν). Signaling by both incretins is coupled to $Ca_v1.3$ through K_{ATP} channel-dependent effects and pathways involving PKA, PKC, and Erk1/2 (Jacobo *et al.*, 2009). The incretins also act on nonselective ion channels (Gromada *et al.*, 1998; Lu *et al.*, 1993; Wheeler *et al.*, 1995), as well as stimulating calcium release from intracellular stores (Gromada *et al.*, 1998; Lu *et al.*, 1993; Wheeler *et al.*, 1995) via release channels (ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors; Gromada *et al.*, 1998; Holz *et al.*, 2006). Although it has been suggested that IP_3 derived from GLP-1 activation of phospholipase C (Suzuki *et al.*, 2006) is involved in this process, there is no direct evidence for such an effect (Kang *et al.*, 2003), and it is more likely that Epac- and PKA-mediated activation of ryanodine and IP_3 receptors, respectively, are involved (Holz *et al.*, 2006; Kang *et al.*, 2005; Tsuboi *et al.*, 2003).

Repolarization of the β-cell occurs in response to voltage-dependent K^+ currents mediated by K_ν and K_{Ca} channels (Hiriart and Aguilar-Bryan, 2008; MacDonald *et al.*, 2002a). Biophysical and pharmacological characterization has resulted in the identification of two major classes of K_ν currents: delayed rectifier and A-type. Numerous subtypes of K_ν channels are expressed in β-cells, and studies to establish their specific roles in regulating insulin secretion are still ongoing (MacDonald and Wheeler, 2003; MacDonald *et al.*, 2002c). Both GIP and GLP-1 reduce K_ν channel currents, resulting in more prolonged β-cell action potentials and potentiation of the Ca^{2+} signal. GLP-1 receptor activation reduces K_ν channel currents via both PKA and PI3Kinase (PI3K)/Protein kinase Cζ (PKCζ)-mediated effects on delayed rectifier channels (MacDonald *et al.*, 2002b, 2003), and the $K_{\nu2.1}$ channel is the major delayed rectifier channel in rodent β-cells, playing a dominant role in GLP-1 action. GIP also inhibits delayed rectifier currents in INS-1 cells and human β-cells (Choi *et al.*, Unpublished observations), and it exerts a novel regulatory action on A-type currents by increasing the endocytosis of Kv1.4 channels through cAMP/PKA-dependent phosphorylation (Kim *et al.*, 2005a).

In addition to their actions on membrane depolarization, GLP-1 and GIP exert distal effects on exocytosis, also via both PKA- (Ding and Gromada, 1997) and Epac-dependent pathways (Holz *et al.*, 2006; Seino and Shibasaki, 2005). PKA phosphorylates proteins that are associated with

the exocytotic machinery (Seino and Shibasaki, 2005), including α-SNAP and Munc 13-1 (Yu and Jin, 2010). Two models have been presented for the Epac-2 regulated events (Eliasson *et al.*, 2003; Holz *et al.*, 2006; Seino and Shibasaki, 2005). A pool of Epac-2 is located close to the secretory granules, where it acts to increase the probability of the readily releasable pool of granules undergoing exocytosis (Holz *et al.*, 2006). In the model proposed by Eliasson *et al.* (2003), Epac-2 interacts with SUR1 associated with secretory granules and/or the plasma membrane. Localization at both sites may be necessary for activation of the secretory granule chloride channel ClC-3 (Holz *et al.*, 2006), resulting in granule acidification and priming via a v-type H^+-ATPase (Eliasson *et al.*, 2003). In the model of Seino and coworkers, a cAMP–Epac-2–Rim2 complex plays a central role (Holz, 2004; Holz *et al.*, 2006; Kashima *et al.*, 2001; Rucha and Verspohl, 2005). Under unstimulated conditions, Epac-2 is proposed to be associated with Sur1. Following AC activation, cAMP–Epac-2 dissociates from Sur1 and induces Ca^{2+}-dependent heterodimerization of the secretory granule associated proteins Rim2 and piccolo and interaction with the binding protein, Rab3A, a core component of the exocytotic system (Holz *et al.*, 2006; Kashima *et al.*, 2001; Seino and Shibasaki, 2005).

Other signaling pathways contribute to both GIP and GLP-1 stimulation of insulin secretion. GIP receptors couple functionally to a Group VIA islet Ca^{2+}-independent phospholipase A_2 ($iPLA_2$; Ehses *et al.*, 2001; Fig. 2.4). It has been proposed that activation of $iPLA_2$ increases arachidonic acid production (Ehses *et al.*, 2001; McIntosh *et al.*, 2009), resulting in the release of Ca^{2+} from intracellular stores, coupling signaling to insulin secretion. Arachidonic acid has recently been shown to increase the rate of inactivation of $K_{v2.1}$ channels (Jacobson *et al.*, 2007), raising the possibility that GIP-activation of $iPLA_2$ is also linked to Kv channel regulation by this pathway. A physiological role for $iPLA_2$ is also supported by the fact that inhibition (Song *et al.*, 2005), or siRNA suppression (Bao *et al.*, 2006a), of $iPLA_2$ in INS-1 β-cells reduced insulin secretion, and $iPLA_2$ knockout mice exhibited greater impairments in glucose intolerance than wild-type mice on a high fat diet (Bao *et al.*, 2006b). Activation of PI3K/PKCζ is involved in the regulation of Kv delayed rectifier currents (MacDonald *et al.*, 2003), whereas activation of PKCα and PKCε has been linked to GLP-1-induced increases in iCa^{2+} in INS-1 cells (Suzuki *et al.*, 2006), although further studies are needed to establish whether this amplifies insulin secretion. GLP-1 was recently shown to activate ADP ribosyl cyclase/CD38, as well as an unidentified cyclase, resulting in increased production of the Ca^{2+}-mobilizing molecules nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (Kim *et al.*, 2008a). Finally, GLP-1 also increased phosphorylation of the polypyrimidine tract binding protein (PTB1) in islets and INS-1 cells (Knoch *et al.*, 2006). PTB1 undergoes glucose-induced nucleocytoplasmic translocation, resulting in reduced degradation

of mRNA and increased expression of specific secretory granule proteins (Knoch *et al.*, 2006), demonstrating that the incretins can exert long-term effects on the secretory machinery.

D. Effects of GIP and GLP-1 on insulin biosynthesis

Both GLP-1 and GIP stimulate proinsulin biosynthesis (Drucker, 2007; Drucker *et al.*, 1987; Fehmann and Göke, 1995; Schäfer and Schatz, 1979). GLP-1 was shown to increase insulin gene transcription, stabilize preproinsulin mRNA, and increase translation (Chepurny *et al.*, 2002; Doyle and Egan, 2007). Insulin gene expression is regulated by promoter sequences ~400 bp upstream from the start site (Hay *et al.*, 2005) and, although the regulatory sequences in the rat insulin I and human genes are similar, there are differences in their regulation (Ban *et al.*, 2000; Chepurny *et al.*, 2002; Hay *et al.*, 2005; Skoglund *et al.*, 2000). In β-cell lines, ex-4 (Chepurny *et al.*, 2002; Skoglund *et al.*, 2000) or GLP-1 (Lawrence *et al.*, 2002) stimulated rat insulin I gene promoter activity, and two transcription activation systems have been implicated: interaction of an uncharacterized bZIP protein with the single identified CRE site (Chepurny *et al.*, 2002; Skoglund *et al.*, 2000) and interaction of nuclear factor of activated T-cells (NFAT) with NFAT elements (Lawrence *et al.*, 2002). In contrast to the rat, the human insulin gene promoter contains 3 active CREs, of which one was found to be activated by the bZIP protein ATF-2, but inhibited by cAMP response element-binding protein (CREB; Ban *et al.*, 2000). Since NFAT and bZIP proteins can interact, they may constitute a common pathway. It is currently unclear as to the upstream pathways by which GLP-1 receptor agonists increased insulin gene expression, with Ca^{2+}/calmodulin-dependent phosphatase 2B (calcineurin) and cAMP/PKA implicated in the NFAT responses (Lawrence *et al.*, 2002) and a cAMP-independent pathway for bZIP/Cre-mediated activation (Chepurny *et al.*, 2002). GLP-1 also increased expression and Pdx-1 binding to the A1 element of the rat insulin II promoter (Wang *et al.*, 1999). The majority of studies on the regulation of insulin gene expression have been performed on immortalized β-cell lines. Importantly, in primary β-cells, ex-4 was found to be without effect on preproinsulin mRNA levels and its major action was to enhance translational control via a cAMP-mediated pathway (Alarcon *et al.*, 2006). Further studies on the mechanisms by which GLP-1 increases proinsulin levels are needed, as are similar studies with GIP.

E. Effects of GIP and GLP-1 on pancreatic islet mass

Since it is now recognized that loss of β-cell mass is a key event in both T1DM and T2DM, several laboratories are involved in studying the mechanisms by which a relatively stable number of β-cells is normally

maintained. Studies on GIP and GLP-1 receptor knockout (GIP-R$^{-/-}$, GLP-1R$^{-/-}$) and DIRKO mice showed no major changes in β-cell volume or islet cell number (Ling *et al.*, 2001; Pamir *et al.*, 2003; Preitner *et al.*, 2004), although cell topography was altered. However, there is strong evidence (Baggio and Drucker, 2007; Doyle and Egan, 2007; Drucker, 2007; McIntosh *et al.*, 2009) that both GLP-1 and GIP exert major effects on the maintenance of β-cell mass and promote survival of islets in animal models of diabetes. A number of elegant studies have also been performed on the potential developmental role of GLP-1 (Stoffers, 2004) and the differentiation of stem or exocrine pancreatic progenitor cells toward endocrine phenotypes (List and Habener, 2004), but these will not be discussed further.

1. Effects of GIP and GLP-1 on β-cell proliferation

Both incretins have been shown to increase proliferation *in vitro* with rodent β-cell lines (Brubaker and Drucker, 2004; Buteau *et al.*, 1999, 2001, 2003; Ehses *et al.*, 2003; Trümper *et al.*, 2001) and primary rat (Friedrichsen *et al.*, 2006) and human (Movassat *et al.*, 2002) β-cells. However, although *in vivo* effects of GLP-1 receptor agonists on β-cell proliferation have been extensively studied (Baggio and Drucker, 2006; Doyle and Egan, 2007; Drucker, 2007; Li *et al.*, 2005; Vahl and D'Alessio, 2003; Wajchenberg, 2007), little is known about the relative importance of GIP.

Numerous hormones increase β-cell mass when administered to rodents (List and Habener, 2004). Among these, both GLP-1 and ex-4 treatment for periods of 2 days to several weeks, increased β-cell mass 1.5−6-fold, in a number of rodent models (Li *et al.*, 2003; Park *et al.*, 2006; Perfetti *et al.*, 2000; Stoffers *et al.*, 2000; Tourrel *et al.*, 2001; Wang and Brubaker, 2002). There is controversy as to the relative contributions of β-cell neogenesis and proliferation to increases in β-cell mass and there is evidence for the effects of GLP-1 on both events (Brubaker and Drucker, 2004; Xu *et al.*, 1999).

Glucose and GLP-1 act in a synergistic fashion to increase the expression of a number of immediate-early response genes coding for transcription factors involved in the regulation of cell proliferation and differentiation, including *c-fos*, *c-jun*, and *junB* (Susini *et al.*, 1998). Activation of the GLP-1 receptor also increased expression of the key transcription factor Pdx-1, both *in vitro* (Movassat *et al.*, 2002; Wang *et al.*, 1999) and *in vivo* (Stoffers *et al.*, 2000), through a pathway involving activation of PI3K and PKCζ translocation into the nucleus (Buteau *et al.*, 1999; Wang *et al.*, 2004). Pdx-1 nuclear translocation and its DNA-binding activity are also increased by GLP-1 treatment (Buteau *et al.*, 1999; Doyle and Egan, 2007). The importance of these effects on GLP-1R activation was exemplified in β-cell$^{Pdx\text{-}1-/-}$ mice, in which β-cell proliferative responses to ex-4 were ablated (Li *et al.*, 2005). Both GIP (Kim *et al.*, 2005b) and GLP-1 (Buteau *et al.*, 2006) also regulate gene transcription by phosphorylation of the transcription factor Foxo1 via

activation of PI3K/Protein kinase B/Akt (PKB; see Section IV.E.2), resulting in its nuclear exclusion (Kim *et al.*, 2005b). In transgenic mice expressing a form of Foxo1 that constitutively resides in the nucleus, ex-4 was incapable of increasing β-cell proliferation (Buteau *et al.*, 2006). Since Foxo1 represses expression of both *Pdx1* and *Foxa2*, inhibition of its nuclear translocation results in potentiation of proliferative responses (Buteau, 2008). Both GIP and GLP-1 also increased the expression of cyclin D1 (Friedrichsen *et al.*, 2006; Kim *et al.*, 2006), an important component of the cell cycle regulatory system, via CREB-mediated transactivation (Kim *et al.*, 2006).

The cellular events involved in regulating cell proliferation are complex and exemplify the pleiotropic nature of both GLP-1 and GIP action. Since both incretins activate multiple signal transduction modules, it is likely that they demonstrate promiscuous receptor/G protein interactions (Liu and Habener, 2008). Inhibition of PKA reduced mitogenic responses to both GIP and GLP-1 (Friedrichsen *et al.*, 2006). PKB is also central to the mitogenic actions of incretins (Buteau *et al.*, 1999; Friedrichsen *et al.*, 2006; Trümper *et al.*, 2005; Wang *et al.*, 2004). Phosphatidylinositol-3 kinase may be activated by GIP and GLP-1 through direct Gβγ interaction, epidermal growth factor (EGF) transactivation, or through IRS2 binding, resulting in phosphorylation of PKB at Ser_{308} and Ser_{473}, sites that have been considered critical for activation. However, a noncanonical pathway was recently identified, by which GIP increases PKB activity without elevating Ser_{308} phosphorylation levels (Widenmaier *et al.*, 2009b). This pathway induces rapid PKB activation and it may be mediated via EPAC, thus constituting a system for cross talk with cAMP-mediated events (Widenmaier *et al.*, 2009b).

The cAMP-PKA and Epac2 modules activated in response to incretin receptor-binding regulate a number of downstream signaling pathways, including p44/42 mitogen-activated protein kinase (MAPK)/Extracellular signal-Regulated Kinase 1/2 (Erk1/2; Buteau *et al.*, 2001; Ehses *et al.*, 2002; Trümper *et al.*, 2001, 2002, 2005) and MKK3/6-p38 MAPK (Buteau *et al.*, 2001; Ehses *et al.*, 2003). Influx of Ca^{2+} and activation of calcium/calmodulin-dependent protein kinase II (CaMKII) may also be involved (Gomez *et al.*, 2002). GIP-activated Mek1/2 and Erk1/2 (Ehses *et al.*, 2002; Trümper *et al.*, 2001) were shown to result in Rap-1-mediated phosphorylation of Raf-B in INS-1 β-cell lines (Ehses *et al.*, 2002), and a similar GLP-1-activated pathway was subsequently identified in human islets (Trümper *et al.*, 2005). In response to GIP treatment, INS-1 β-cells demonstrate increased phosphorylation of a number of proteins that are substrates of ERK1/2 (Elk-1; p90 RSK; Ehses *et al.*, 2002), p38 MAPK (ATF-2; Ehses *et al.*, 2002), and PKB (p70S6K, Foxo1, glycogen synthase kinase (GSK) 3β, Kim *et al.*, 2005b; Trümper *et al.*, 2001). The ERK1/2 pathway acts on proliferation additively with cAMP/PKA-activated CREB in β-cells (Klinger *et al.*, 2008). It has recently been demonstrated that

physiological negative regulators of these pathways are present in β-cells, removal of which potentiates GLP-1-induced proliferation (Klinger *et al.*, 2008; Schuit and Drucker, 2008). Finally, a number of Wnt signaling target genes, including cyclin D1 and c-Myc, are involved in stimulating β-cell proliferation, and the β-catenin-dependent Wnt pathway was shown to be a target of GLP-1 signaling. Exendin-4 increased phosphorylation of β-catenin Ser_{675}, through activation of PKA and/or PKB, thus increasing its stability, resulting in enhanced T-cell factor 7-like 2 (TCF7L2)-activated gene transcription (Liu and Habener, 2008). Interestingly, common variants of the *TCF7L2* gene are associated with T2DM, and levels of TCF7L2 in β-cells are strongly linked to GIP and GLP-1 receptor gene expression (Shu *et al.*, 2009)

There appears to be an extensive cross talk between GLP-1 signaling and tyrosine kinase receptors in relation to β-cell growth and replication. In studies on INS-1 β-cell lines, GLP-1 increased β-cell proliferation via transactivation of the EGF receptor (Buteau, 2008; Buteau *et al.*, 2001, 2003). GLP-1 activates membrane-bound metalloproteinase(s), through a c-Src-mediated pathway, resulting in release of an activating ligand for the EGF receptor (Fig. 2.4), such as betacellulin (Buteau *et al.*, 2003). The pathway by which GLP-1 activation of the EGF receptor leads to PI3K activation of PKB has been proposed to be mediated through a complex of the EGF-R with Growth factor receptor-bound protein 2 (Grb2) and Grb2-associated-binding protein 1 (Gab1; Holz and Chepurny, 2005; Mattoon *et al.*, 2004; Trümper *et al.*, 2000). GLP-1 mediated increases in proliferative responses also involve interaction, at several levels, with the insulin-like growth factor (IGF) signaling cascade (Cornu *et al.*, 2009, 2010; Holz and Chepurny, 2005; Park *et al.*, 2006). GLP-1 was found to increase β-cell IGF-1 receptor expression over the long term via a process involving translational control (Cornu *et al.*, 2009, 2010). Since studies with IBMX suggested that higher concentrations of cAMP were necessary to increase IGF-1 receptor gene transcription, additional stimuli, including GIP, may be necessary for maximal responses. Acutely, GLP-1 stimulated IGF-2 secretion from β-cells and IGF-2 acts in an autocrine manner, resulting in PKB phosphorylation and activation (Cornu *et al.*, 2009). It has also been shown that GLP-1 increases insulin receptor substrate 2 (IRS2) expression through activation of CREB (Jhala *et al.*, 2003), but it is unclear how this relates to PKB responses (Cornu *et al.*, 2009).

2. Effects of GIP and GLP-1 on β-cell apoptosis

Pancreatic islets normally exhibit low rates of apoptosis, but increased levels are central to the development of diabetes. In T1DM, the main contributing factor is the autoimmunity, whereas chronic hyperglycemia and hyperlipidemia, elevated cytokines, amyloid deposits, ER stress and insulin resistance all contribute to T2DM (Rhodes, 2005). Elevated levels of

β-cell apoptosis were found in GLP-1R$^{-/-}$ mice, indicating that incretins normally play a role in maintaining islet integrity (Li *et al.*, 2003). Both *in vivo* and *in vitro* studies show that GIP and GLP-1 exert powerful prosurvival effects on β-cells (Baggio and Drucker, 2006, 2007; Drucker, 2006; McIntosh *et al.*, 2009). GIP-R or GLP-1R agonists have been shown to exert antiapoptotic effects in a number of rodent models, including *db/db* mice (Wang and Brubaker, 2002), ZDF (Farilla *et al.*, 2002; Widenmaier *et al.*, 2010) and VDF (Kim *et al.*, 2005b; Widenmaier *et al.*, 2010) Zucker rats and streptozotocin-induced diabetic mice (Li *et al.*, 2003; Maida *et al.*, 2009; Widenmaier *et al.*, 2010).

In order to elucidate the mechanisms involved, studies have been performed on the effects of GIP, GLP-1, or ex-4 on isolated islets, primary β-cell lines, or β-cell tumor lines that have been subjected to apoptosis-inducing stressors that include high glucose ± fatty acids (glucolipotoxicity; Buteau *et al.*, 2004; Kim *et al.*, 2005b), serum depletion with a low-glucose environment (Ehses *et al.*, 2003), exposure to cytokines (Li *et al.*, 2003), or treatment with agents that induce oxidative (Hui *et al.*, 2003), genotoxic (Widenmaier *et al.*, 2009a), mitochondrial (Widenmaier *et al.*, 2009a), or ER (Widenmaier *et al.*, 2009a; Yusta *et al.*, 2006) stress. Multiple components of the cell's survival system are influenced by incretins. For example, GIP treatment of INS-1 β-cells reduced or ablated staurosporine-induced mitochondrial translocation of Bad and BimEL, activation of mitochondrial Bax, release of mitochondrial cytochrome C, and caspase 3 activation (Widenmaier *et al.*, 2009a; Fig. 2.5).

As with proliferative effects, both cAMP/PKA (Hui *et al.*, 2003; Jhala *et al.*, 2003; Yusta *et al.*, 2006) and PI3K/PKB-mediated (Wang *et al.*, 2004) pathways are involved in incretin-induced antiapoptotic effects. Involvement of the cAMP/PKA pathway in GLP-1 receptor-mediated inhibition of apoptosis was shown in MIN6 cells subjected to oxidative (Hui *et al.*, 2003) and ER (Yusta *et al.*, 2006) stress. *In vivo* administration of ex-4 reduced β-cell levels of ER stress-related factors, including spliced XBP-1 and Bip (Yusta *et al.*, 2006). Although ex-4, GLP-1, and GIP–all reduced apoptosis induced by the ER-stress-inducing agent thapsigargin (Widenmaier *et al.*, 2009a; Yusta *et al.*, 2006), GLP-1 increased translation of the transcription factor ATF-4 and induced expression of a number of proteins involved in the unfolded protein response (UPR), including CHOP and GADD34, and increased dephosphorylation of eIF2a. Such responses would appear to favor progression to apoptosis, but Yusta *et al.* (2006) suggested that accelerated recovery of global protein synthesis would be beneficial to the β-cell. However, in studies on GLP-1R-mediated protection against lipotoxic ER stress enhancement of defense mechanisms, the ER chaperone BiP, Bcl-2 and the early response gene JunB were found to be the predominant factors (Cunha *et al.*, 2009). Therefore, it appears that both the activating stimulus for ER stress and the timing at which responses

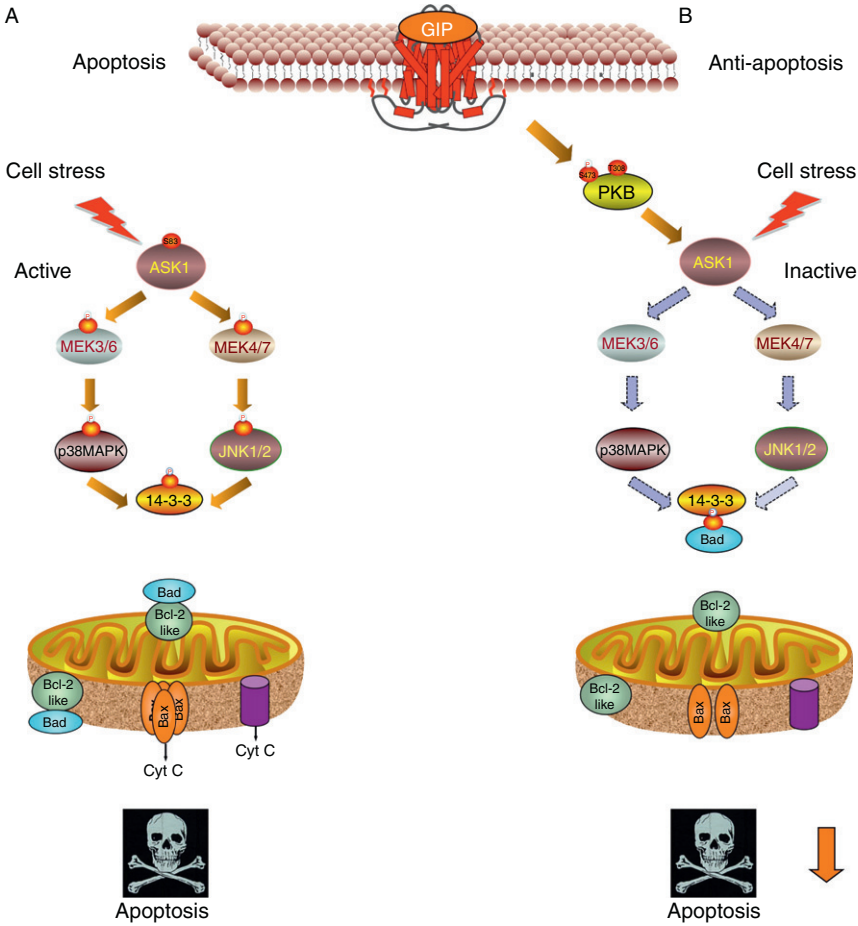
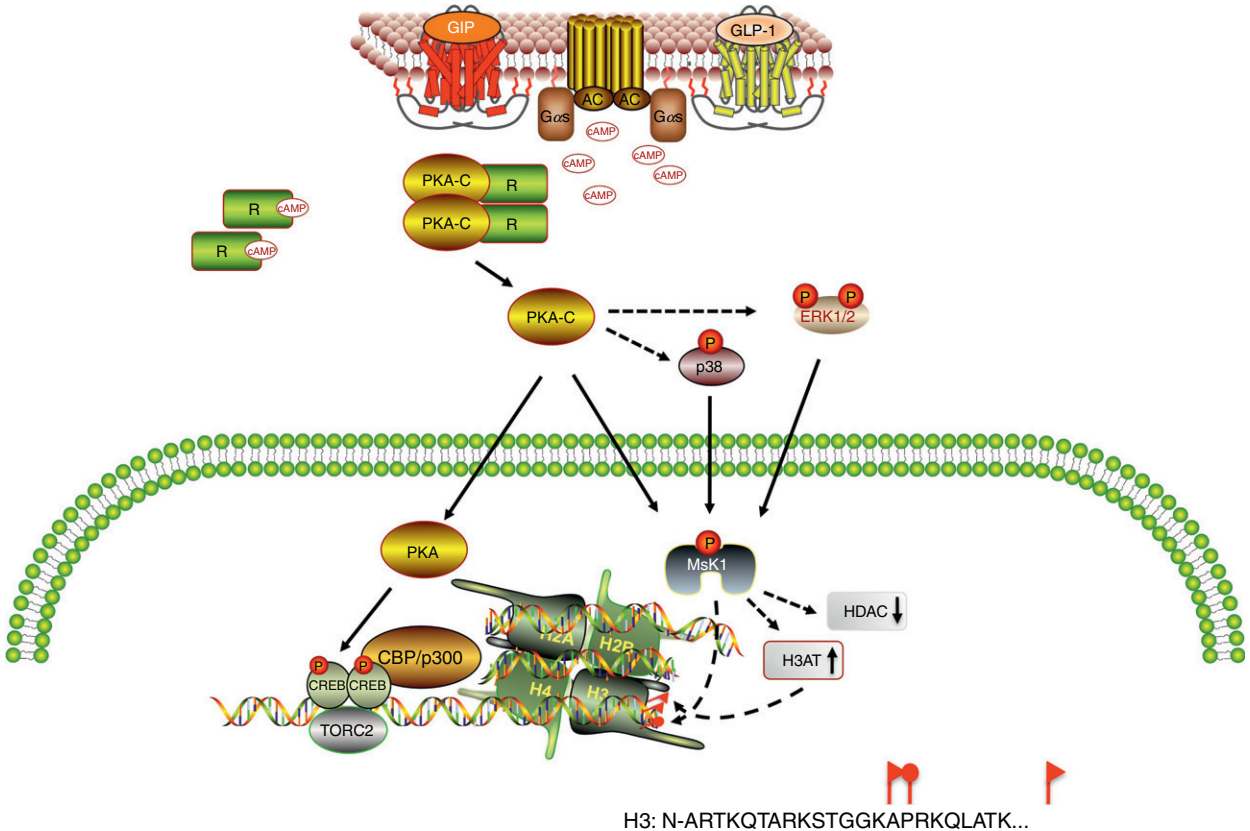


Figure 2.5 Suppression of apoptosis signal regulating kinase-1 (ASK1) activation by GIP is central to incretin-mediated inhibition of β-cell apoptosis. (A) Stress-activation of ASK1 results in the phosphorylation and activation of MAP Kinase kinase (MEK) 3/6-p38 MAP kinase and MEK4/7-jun N-terminal kinase (JNK) 1/2 signaling modules. One result of this is the release of Bcl-2-associated death promoter (Bad) from 14-3-3 scaffolding proteins and its interaction with mitochondrial antiapoptotic B-cell leukemia/lymphoma 2 (Bcl-2) proteins. A number of other proapoptotic proteins, including BH3-only protein (Bid) and Bcl-2 interacting mediator of cell death (Bim), are also activated. Ultimately, cytochrome C is released from mitochondria through Bcl-2-associate X protein (Bax)/ Bcl-2 homologous antagonist/killer (Bak) channels and the mitochondrial permeability transition pore, resulting in activation of apoptosis. (B) GIP, and probably GLP-1, activation of PKB leads to phosphorylation of Ask1 at S83 and its inactivation. The downstream MAP kinase pathways are down-regulated and Bad is sequestered by scaffolding proteins, thus reducing or stopping apoptosis.

are measured are important determining factors in assessing the effects of incretin-mediated protection against cell death. Activation of CREB by both GLP-1R- and GIPR-activation has been implicated in their anti-apoptotic effects. GIP (Kim *et al.*, 2008b) and GLP-1 (Hui *et al.*, 2003) increased levels of the antiapoptotic proteins Bcl-2 and Bcl_{XL}. In the case of GIP, *bcl-2* gene expression was increased through a PKA-mediated pathway involving dephosphorylation of AMP activated protein kinase (AMPK), increased nuclear entry of cAMP-responsive CREB coactivator 2 (TORC2), and phosphorylation of CREB (Kim *et al.*, 2008b). Sustained effects of the incretins on apoptosis are indicated by their ability to modulate β-cell chromatin structure through posttranslational modification of core H3 histones (Fig. 2.6; Kim *et al.*, 2009b). Both acetylation and phosphorylation were increased via pathways involving elevated histone H3 acetyltransferase and reduced histone deacetylase activities (Kim *et al.*, 2009b). Upstream signaling was mediated via activation of PKA, as well as Erk1/2 (p44/42 MAPK) and p38 MAPK signaling modules, resulting in the activation of mitogen- and stress-activated kinase-1 (MSK-1). Inhibitor studies showed that such modification contributed to incretin-mediated CREB-related increases in *Bcl-2* transcription (Kim *et al.*, 2009b), but the effects of incretins on chromatin structure are likely to have wider implications.

As discussed in Section IV.E.1, GLP-1R activation increases β-cell IGF-1 receptor expression and IGF-2-mediated PKB phosphorylation and activation (Cornu *et al.*, 2009, 2010). Antiapoptotic effects of PKB are also activated by GIP (Kim *et al.*, 2005a; Trümper *et al.*, 2001, 2002; Widenmaier *et al.*, 2009a,b). In both human islets and INS-1 β-cells exposed to staurosporine, GIP activation of PKB altered the phosphorylation state of the apoptosis signal regulating kinase 1 (ASK1), resulting in suppression of p38 MAPK and jun N-terminal kinase (JNK; Widenmaier *et al.*, 2009a; Fig. 2.5). Ex-4 activation of CREB, and increased expression of inhibitor islet-brain 1(IB1), was also shown to induce suppression of the JNK pathway during β-cell apoptosis activated by interleukin-1β (Ferdaoussi *et al.*, 2008). In addition to promotion of β-cell proliferation, phosphorylation and nuclear exclusion of Foxo1 also results in the promotion of cell survival, since nuclear Foxo1 is required for expression of proapoptotic proteins, such as Bax (Kim *et al.*, 2005b). Incretin activation of the Mek1/2-Erk1/2 module probably plays a relatively minor role in short-term modulation of apoptosis in β-cells (Ehses *et al.*, 2003; Trümper *et al.*, 2002), although it is involved in the regulation of β-cell chromatin structure (Kim *et al.*, 2009b). However, cAMP-dependent reduction in p38 MAPK phosphorylation is important (Ehses *et al.*, 2003; Widenmaier *et al.*, 2009a).

The effects of incretins on β-cell proliferation and survival in rodents suggest that they may be beneficial in the prevention of islet loss and regeneration of islets in human diabetes, as well as prolonging islet transplant


GIP
GLP-1
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survival. However, there is uncertainty as to whether human islets will demonstrate equivalent responses. For example, GLP-1 increased expression of Pax4 in both human and rat islets, but only the latter demonstrated increased β-cell proliferation (Brun *et al.*, 2008) and FACS sorted β-cells demonstrated minimal capacity for proliferation (Parnaud *et al.*, 2008). Nevertheless, preserving or increasing β-cell mass in diabetic humans is an important target for diabetes therapeutics and there is a strong potential for manipulation of the incretin system with such an objective.

V. Effects of GLP-1 on Food Intake and Satiety

Food intake and energy expenditure are regulated by a complex interplay between peripheral hormones and autonomic and CNS neural circuits (Drucker, 2007; Huda *et al.*, 2006; Woods and D'Alessio, 2008). GLP-1 has been shown to influence food intake and satiety in a number of species, whereas there is no strong evidence supporting a role for GIP. Intracerebroventricular administration of GLP-1 was shown to strongly inhibit feeding behavior in fasted normal weight rats (Gunn *et al.*, 1996; Navarro *et al.*, 1996; Tang-Christensen *et al.*, 1996; Turton *et al.*, 1996) or obese Zucker rats (Gunn *et al.*, 1996), whereas blockade of GLP-1Rs with ex (9–39) increased food intake in satiated rats and augmented feeding responses to the orexigenic peptide neuropeptide Y (NPY; Turton *et al.*, 1996). GLP-1 administered i.c.v. additionally reduced water intake (Tang-Christensen *et al.*, 1996). Injection of GLP-1 agonists into the PVN also reduced food intake (McMahon and Wellman, 1997; Turton *et al.*, 1996). These studies suggested that central pathways known to be involved in the regulation of food intake are activated by GLP-1, and repeated i.c.v. administration of GLP-1 resulted in weight loss in rats (Meeran *et al.*,

Figure 2.6 Proposed pathways by which incretins regulate β-cell chromatin structure and gene transcription. GIP and GLP-1 binding to the GIP receptor (GIPR) or GLP-1 receptor (GLP-1R), respectively, activate PKA, MEK 1/2, and p38 MAPK signaling modules, resulting in increases in activity of mitogen- and stress-activated kinase-1 (MSK-1) and histone 3 acetyltransferase (H3AT) and decreased histone deacetylase (HDAC) activity. Pathways for activation of the MEK 1/2 and p38 MAPK signaling modules (broken arrows) are unclear. Active PKA catalytic subunits (PKA-C), released following cAMP binding to the PKA regulatory subunits (R), enter the nucleus and phosphorylate cyclic AMP response element binding protein (CREB) at Ser 133. Incretin-mediated activation of H3AT and inactivation of HDAC leads to the post-translational modification of histone H3 core proteins, allowing increased accessibility and recruitment of phospho-CREB and cAMP-responsive CREB coactivator 2 (TORC2) to target DNA. The topographical relationship between the histone proteins is controversial. The transcription coactivator paralogs, CREB binding protein (CBP)/p300, possess HAT activity and bind to phospho-CREB phosphorylated.

1999). However, the majority of data support a physiological role for GLP-1 as a short-term satiety signal in rodents, rather than long-term regulation of caloric intake and body weight (van Dijk and Thiele, 1999). In agreement with this proposal, GLP-1R knockout mice have normal body weights, but exhibit subtle changes in their pattern of food intake (Scrocchi *et al.*, 1996). Nevertheless, GLP-1R agonists have subsequently been shown to reduce food intake and decrease body weight in a number of other species, including pigs (Raun *et al.*, 2007), nonhuman primates (Scott and Moran, 2007), and humans (DeFronzo *et al.*, 2008). Intravenous or s.c. infusions of GLP-1 in humans produced dose-dependent reductions in appetite and caloric intake (Flint *et al.*, 1998; Gutzwiller *et al.*, 1999a; Meier *et al.*, 2002a; Verdich *et al.*, 2001), even with doses that only raised circulating levels to the physiological range (Flint *et al.*, 2001). Obese (Näslund *et al.*, 1999) and type 2 diabetic (Gutzwiller *et al.*, 1999b) subjects also exhibited decreased food intake in response to GLP-1, with increases in periods of postprandial satiety in obese humans (Näslund *et al.*, 1998a) and reductions in body weights (Näslund *et al.*, 2004). Long-acting GLP-1R agonists, including ex-4 (Exenatide) and Liraglutide, produce sustained weight loss in rodents (Larsen *et al.*, 2001) and humans (DeFronzo *et al.*, 2008; Vilsbøll, 2009).

It has been suggested that some effects observed with GLP-1 agonists in rodents are due to malaise-inducing effects, since conditioned taste aversion was observed following i.c.v. administration of GLP-1 (Thiele *et al.*, 1997). Patterns of c-Fos activation following lithium chloride administration were similar to those seen with GLP-1 treatment (Lachey *et al.*, 2005) and i.c.v. administration of a GLP-1 antagonist attenuated effects of i.p. LiCl (Seeley *et al.*, 2000). However, intraparaventricular injection of GLP-1 in doses that reduced food intake effectively did not demonstrate taste aversion effects (McMahon and Wellman, 1998). It now appears that GLP-1 interacts with two separate pathways, involving hypothalamic receptors for the satiety effects and extrahypothalamic receptors, such as those in the amygdala (Woods and D'Alessio, 2008), mediating the taste aversion/visceral illness effects (Kinzig *et al.*, 2002; van Dijk and Thiele, 1999).

There are ongoing studies focusing on the pathways by which GLP-1 impacts on food intake and satiety (Hellström and Näslund, 2001b; Sandoval, 2008), as discussed in Section II, but there is no general consensus. Interaction between leptin and GLP-1-mediated pathways has been demonstrated in rodents. Leptin receptors are present on GLP-1 NTS neurons in mice (Goldstone *et al.*, 1997) and the GLP-1R antagonist ex (9–36) inhibited leptin-induced reductions in food intake and body weight in mice (Goldstone *et al.*, 1997). Leptin treatment of mice reversed food restriction-induced reductions in NTS proglucagon expression (Huo *et al.*, 2009) and hypothalamic GLP-1 content (Goldstone *et al.*, 2000). STAT3 signaling in proglucagon-expressing NTS neurons was also activated by

leptin treatment (Huo *et al.*, 2009). There is also evidence that GLP-1 interacts with anorexigenic CRH-producing neurons (Larsen *et al.*, 1997; Sarkar *et al.*, 2003). Both gastric emptying-dependent and -independent events appear to contribute to GLP-1 effects on food intake in rodents (Chelikani *et al.*, 2005; Hayes *et al.*, 2009). An important role for a brainstem/vagal pathway is supported by the demonstration that bilateral truncal vagotomy or brainstem–hypothalamic transsectioning abolished the effects of both GLP-1 and $PYY_{3–36}$ on food intake (Abbott *et al.*, 2005). However, recent studies indicated that neither GLP-1 actions on hepatic portal vagal afferent nerves (Kim *et al.*, 2009a; Rüttimann *et al.*, 2009) nor signaling via the arcuate nucleus are involved in its ability to reduce food intake (Sandoval *et al.*, 2008). In humans, gastric emptying has been suggested to contribute significantly to GLP-1-induced satiety responses (Hellström and Näslund, 2001b), but definitive studies are needed.

VI. Gastrointestinal Effects of GIP and GLP-1

A. Gastric emptying

GLP-1, administered by i.v. infusion or s.c. injection, has been shown to inhibit gastric emptying of a liquid meal in normal human volunteers (Nauck *et al.*, 1997; Wettergren *et al.*, 1993), as well as in obese individuals (Näslund *et al.*, 1998b) and T2DM patients (Nauck *et al.*, 1996; Willms *et al.*, 1996), and reduced gastric emptying contributes significantly to the beneficial effects of incretin mimetics in T2DM (Drucker and Nauck, 2006; McIntosh, 2008; Williams, 2009). GIP has not been shown to exert major effects on gastric emptying in humans (Meier *et al.*, 2004). Inhibition of gastric emptying by GLP-1 has also been demonstrated in rodents (Willms *et al.*, 1996; Young *et al.*, 1996), pigs (Wettergren *et al.*, 1998), and dogs (Anvari *et al.*, 1998). During a meal, the proximal stomach undergoes accommodation, increasing in volume with little change in intragastric pressure, and GLP-1 appears to contribute to this response (Delgado-Aros *et al.*, 2002; Schirra *et al.*, 2002). In both humans (Schirra *et al.*, 2000) and dogs (Anvari *et al.*, 1998), GLP-1 reductions in gastric emptying were associated with inhibition of antral and duodenal contractions, and increased pyloric tone and phasic motility, responses expected to inhibit gastric emptying.

There is no compelling evidence for direct actions of GLP-1 on smooth muscle, since its administration affected neither isolated perfused antral preparations (Wettergren *et al.*, 1998) nor gastric muscle strips (Näslund *et al.*, 2001; Tolessa *et al.*, 1998). As discussed in Section II, peripheral information from the GI tract is sent to the brain via vagal afferents that project to the NTS, as well as ventral and dorsal segments of the medullary reticular nucleus (Hellström and Näslund, 2001a). In gastric fistula rats,

inhibitory effects of central or peripheral administration of GLP-1 on gastric emptying were reversed by vagal afferent denervation or peripheral administration of ex(9–39) (Imeryuz *et al.*, 1997). A capsaicin-sensitive component was involved, indicating GLP-1 activation of vagal afferent pathways (Imeryuz *et al.*, 1997). Studies with Albugon, a GLP-1 analog covalently bound to albumin, also supported a role for vagal afferent nerve activation in GLP-1 action (Baggio *et al.*, 2004b). There are also data supporting a role for GLP-1 modulation of efferent parasympathetic innervation to the stomach (Wettergren *et al.*, 1998; Williams, 2009). However, whether effects within the stomach are mediated via inhibition of a stimulatory pathway (Delgado-Aros *et al.*, 2002; Schirra *et al.*, 1998) or stimulation of an inhibitory pathway (Delgado-Aros *et al.*, 2002; Schirra *et al.*, 2000) is still controversial.

B. Gastric secretion

GIP was originally identified on the basis of its enterogastrone action. However, as discussed in detail elsewhere (McIntosh *et al.*, 2009; Pederson and McIntosh, 2004), despite strong evidence for a role in the regulation of rodent gastric secretion, the situation in other species, including humans, is unclear. There may also be species differences in the effects of GIP on gastrin secretion, with a stimulatory action in the rat, but inhibitory effects in the pig and dog (reviewed in McIntosh *et al.*, 2009). The parasympathetic innervation exerts strong inhibitory effects on GIP- and GLP-1-stimulated SS secretion in the rat stomach, and it has been suggested that sympathetic activition, during the enterogastric reflex, may normally facilitate gastric inhibitory effects of GIP and GLP-1 (McIntosh *et al.*, 2009). Such potential modulation is difficult to study, however, especially in humans. GLP-1 inhibits both gastric acid (Wettergren *et al.*, 1993) and lipase (Wøjdemann *et al.*, 1998) secretion in humans, and from studies on the isolated perfused pig pancreas it has been suggested that afferent vagal or central effects of GLP-1 are involved (Wettergren *et al.*, 1998), presumably via a similar route to effects on gastric emptying.

C. Exocrine pancreatic secretion

There is surprisingly little information available on the effects of G2IP and GLP-1 on the exocrine pancreas. Although GIP modulated secretagogue-induced rat pancreatic exocrine secretion, it is unclear as to whether responses were due to direct actions on the acinar cell or were secondary to insulin secretion (McIntosh *et al.*, 2009). GLP-1 decreased pancreatic enzyme secretion in both the isolated perfused pancreas (Wettergren *et al.*, 1998) and in humans, although in the latter case it was suggested to be secondary to decreased gastric emptying (Wettergren *et al.*, 1993). Since

GLP-1 actions on exocrine secretion in the rat exocrine pancreas have also been shown to involve activation of efferent vagal outflow (Wan *et al.*, 2007), a similar pathway may exist in humans.

There have been reports of an apparent association between increased incidence of acute pancreatitis and chronic therapeutic use of GLP-1R agonists in humans (Cure *et al.*, 2008). However, in the mouse, although ex-4 increased expression of a series of genes associated with acute pancreatitis, loss of GLP-1R signaling did not alter the severity of experimental pancreatitis, thus providing no support for deleterious functional effects of GLP-1R agonists (Koehler *et al.*, 2009).

D. Intestinal absorption, secretion, and motility

In vitro studies showed that GIP, but not GLP-1, increased intestinal glucose uptake in the rodent intestine through a mechanism involving increased trafficking of the Na^+-dependent glucose transporter 1 (SGLT1) into the brush-border membrane and GLUT2-dependent transport in the basolateral membrane (Cheeseman, 1997; Cheeseman and O'Neill, 1998; Cheeseman and Tsang, 1996), with a cAMP-mediated pathway involved (Singh *et al.*, 2008). GIP may also regulate mucosal alkalinization in the proximal duodenum (Konturek *et al.*, 1985) and intestinal water and electrolyte transport in more distal parts of the small intestine (Helman and Barbezat, 1977). Entry of nutrients into the small intestine initiates signaling events that result in the inhibition of gastric function. These responses are initiated either in the proximal bowel, through release of "enterogastrones" and activation of the enterogastric reflex or in the distal small intestine via the "ileal brake" (Holst, 2007). Fats and carbohydrates administered into the dog (Wen *et al.*, 1995) or human (Young *et al.*, 1996) ileum *in vivo* resulted in increases in GLP-1 secretion that correlated with inhibitory responses in duodenal motility (Wen *et al.*, 1995). Administration of the GLP-1R antagonist ex(9–39) to humans receiving an intraduodenal glucose load resulted in potentiated antro-duodenal motility, in agreement with GLP-1 acting as a component of the ileal brake (Schirra *et al.*, 2006). There have been few administration studies on the effects of GIP on GI motility, although inhibitory effects on antral and duodenal motor activity in dogs have been observed (Castresana *et al.*, 1978).

VII. Cardiovascular Effects of GIP and GLP-1

There is little known regarding the regulatory effects of gastrointestinal hormones on the vascular delivery of nutrients following absorption, although early evidence suggested that GIP plays a role in regulating

the hepatic and splanchnic vasculature. GIP infusion increased hepatic portal (Kogire *et al.*, 1992) and mesenteric arterial (Fara and Salazar, 1978; Kogire *et al.*, 1988) blood flow, whereas hepatic arterial flow was decreased (Kogire *et al.*, 1992). Increased pancreatic islet blood flow in response to GIP was also observed (Svensson *et al.*, 1997). These effects of GIP would be expected to assist in optimizing delivery of nutrients to the liver during a meal, as well as facilitating glucose-induced insulin secretion. Although GIP-R mRNA transcripts were detected in the heart (Usdin *et al.*, 1993), no cardiac effects of GIP have been reported. In contrast, GLP-1 receptors in mice have been localized to cardiomyocytes, coronary and vascular endothelial cells, and smooth muscle (Ban *et al.*, 2008), and there is now a large literature on cardiovascular effects of GLP-1R activation (reviewed in Abu-Hamdah *et al.*, 2009).

Improved cardiovascular performance has been observed in several clinical trials on incretin mimetics or DPP-IV inhibitors in obese and diabetic patients (Mafong and Henry, 2009). Although these beneficial effects have generally been attributed to weight loss, lowered glycemia, and reduced insulin resistance, additional effects are suggested by the marked cardiovascular actions of GLP-1R agonists observed in lower species. Surprisingly, in rodents, GLP-1R agonists administered peripherally (Barragán *et al.*, 1994; Yamamoto *et al.*, 2002) or centrally (Yamamoto *et al.*, 2002) were found to increase heart rate and mean arterial pressure. Ex-4 administration increased *c-fos* expression in a number of autonomic control sites in the rat brain (Yamamoto *et al.*, 2002) and evidence has been presented for both parasympathetic and sympathetic pathways mediating the effects of central GLP-1R activation. However, although bilateral vagotomy was reported to ablate responses to i.c.v. GLP-1 (Barragán *et al.*, 1999), the majority of data indicate a central role for the sympathetic system. GLP-1R activated neurons have been identified that project to sympathetic preganglionic neurons, ultimately innervating the adrenal medulla and heart (Yamamoto *et al.*, 2002) and sympathoadrenal-mediated cardiovascular effects of GLP-1R activation have been substantiated by pharmacological blockade (Gardiner *et al.*, 2006). However, effects mediated via activation of the sympathetic nervous system may be species-dependent (Abu-Hamdah *et al.*, 2009). For example, no increases in heart rate or arterial blood pressure were evident during administration of GLP-1 in normal conscious dogs (Nikolaidis *et al.*, 2004a). GLP-1 also exerts direct effects on cardiac glucose uptake, contractility, and heart rate (Zhao *et al.*, 2006) and the importance of these effects is demonstrated in GLP-1R$^{-/-}$ mice that exhibit reduced resting heart rate and elevated left ventricular end diastolic pressures (Gros *et al.*, 2003). Protective effects of GLP-1R activation have been observed in both cardiac tissue and the vasculature. In models of ischemia/reperfusion (Bose *et al.*, 2005a,b), as well as with *in vivo* (Noyan-Ashraf *et al.*, 2009) treatment, GLP-1R agonists decreased the size of infarctions and, in dogs with advanced dilated

cardiomyopathy, GLP-1 greatly improved left ventricular and systemic hemodynamics (Nikolaidis *et al.*, 2004a). From a wide range of studies on various animal models of cardiovascular dysfunction, a large number of beneficial effects of GLP-1R activation have now been reported, including increases in myocardial insulin sensitivity, glucose uptake, stroke volume, and cardiac output, and decreases in left ventricular and-diastolic volume, heart rate, and systemic vascular resistance (Nikolaidis *et al.*, 2004a; Poornima *et al.*, 2008; Zhao *et al.*, 2006). GLP-1 also exerted beneficial effects on myocardial infarction patients after angioplasty (Nikolaidis *et al.*, 2004b) or patients with congestive heart failure (Sokos *et al.*, 2006).

Mechanisms involved in GLP-1R-mediated cardiovascular effects are gradually being clarified. Increases in rat heart glucose uptake in response to GLP-1 were associated with elevations in GLUT1 and GLUT4 protein expression and increased p38 MAPK activity (Zhao *et al.*, 2006). The protective cardiac effects appear to be mediated, at least in part, via stimulation of cAMP production, as well as PKB activation (Bose *et al.*, 2005a,b). Pretreatment of mice with liraglutide prior to induction of myocardial infarction increased expression and activity of a number of cardioprotective genes (Noyan-Ashraf *et al.*, 2009). In contrast to the pressor effects of GLP-1R agonists observed *in vivo* in rodents, direct relaxatory actions on the vasculature have been observed *in vitro* that appear to be mediated mainly via the endothelium (Nyström, 2008). For example, GLP-1 induced both relaxation of rat pulmonary artery rings *in vitro* (Golpon *et al.*, 2001; Richter *et al.*, 1993) and flow-mediated vasodilation in humans (Nyström *et al.*, 2004). Signaling probably involves activation of eNOS and production of NO (Nyström, 2008), as well as activation of K_{ATP} channels (Green *et al.*, 2008). GIP has been shown to induce endothelin-1 secretion from endothelial cells from the canine hepatic artery and nitric oxide secretion from portal vein endothelial cells (Ding *et al.*, 2004a,b).

While examining the effects of GLP-1 on cardiovascular function, GLP-1(9–36), the metabolite of GLP-1(7–36) resulting from DPP-IV action, was found to mimic some of the effects of the intact peptide, including improved myocardial glucose uptake and vascular contractility in dogs with dilated cardiomyopathy (Nikolaidis *et al.*, 2005). Similar responses were observed in rodents, with evidence for a nonclassical GLP-1R-mediated action (Ban *et al.*, 2008). Subsequently, GLP-1(9–36), was shown to strongly inhibit hepatic glucose production in humans as well as exhibiting weak insulinotropic activity (Elahi *et al.*, 2008). The effects of GLP-1(9–36) may be effected through a non-GLP-1R-mediated pathway. An interesting hypothesis was recently proposed, suggesting that GLP-1 (9–36) is transported into target cells and further metabolized to form peptides that contain consensus mitochondrial targeting sequences, where they exert metabolic and antioxidant actions (Tomas and Habener, 2010). It will be interesting to follow progress of this field.

VIII. Effects of GIP and GLP-1 on Nutrient Storage and Flux

As a result of their islet actions, GIP and GLP-1 promote the anabolic actions of insulin. However, there is evidence that both hormones exert direct effects on the uptake, storage, and turnover of nutrients, although the literature is not without controversy.

A. Liver and skeletal muscle

Pancreatic hormone-mediated effects of the incretins on glucose homeostasis have been well established. However, based on a number of metabolic studies in humans, it has been suggested that GLP-1R activation suppresses hepatic glucose production and enhances tissue glucose utilization via non-insulin-dependent mechanisms (D'Alessio *et al.*, 1994, 2004; Egan *et al.*, 2002; Meneilly *et al.*, 2001; Prigeon *et al.*, 2003). This proposal has proven controversial for a number of reasons (Abu-Hamdah *et al.*, 2009; D'Alessio *et al.*, 2004; Holst, 2007; Toft-Nielson *et al.*, 1996). Much of the evidence is based on studies using glucose clamps with SS infused to reduce endogenous insulin and glucagon secretion, and the ability to finely control these parameters has been questioned (Holst, 2007). A direct effect of GLP-1R-mediated suppression of hepatic glucose output has also been considered unlikely, due to the extremely low levels, or complete lack of, detectable GLP-1R mRNA in extracts from mouse, rat, dog, or human liver (Blackmore *et al.*, 1991; Bullock *et al.*, 1996; Sandhu *et al.*, 1999; Wei and Mojsov, 1996; Wheeler *et al.*, 1993; Yamato *et al.*, 1997). *In vitro* studies have also provided conflicting results. Although there are reports of GLP-1-induced inhibition of glucagon-stimulated cAMP production and stimulation of glycogen synthase activity (Redondo *et al.*, 2003; Valverde *et al.*, 1994), others have reported a complete lack of effect of GLP-1 on hepatic cyclic AMP production (Ghiglione *et al.*, 1985) or glycogenolysis in the perfused liver (Murayama *et al.*, 1990). GLP-1 stimulated glucose transport (O'Harte *et al.*, 1997; Villanueva-Penacarrillo *et al.*, 1994) and glycogenesis (Acitores *et al.*, 2004; Morales *et al.*, 1997; O'Harte *et al.*, 1997) in skeletal muscle has also been observed. However, the physiological significance of such effects (Fürnsinn *et al.*, 1995; Hansen *et al.*, 1998; Holst, 2007) as well as the identity of the receptor involved (Yang *et al.*, 1998) have been questioned. Similarly, although GIP-R mRNA transcripts were not found in muscle or liver (Usdin *et al.*, 1993), GIP was also reported to increase muscle glucose uptake and glycogen production *in vitro* (O'Harte *et al.*, 1998) and to suppress glucagon-dependent hepatic glycogenolysis *in vivo* (Hartmann *et al.*, 1986).

The *in vitro* studies have not provided a consensus viewpoint on direct actions of the incretins on liver and muscle. However, *in vivo* glucose and hormone clamp studies on dogs and rodents have demonstrated noninsulin-dependent effects of GLP-1 on glucose uptake, although they introduce the possibility of neurally mediated pathways. In dog clamp studies, intraportal administration of GLP-1 increased whole body glucose disposal, similar to humans (Dardevet *et al.*, 2004, 2005), but responses were small and only observed in the presence of high intraportal glucose levels (Johnson *et al.*, 2008). Evidence both for (Ionut *et al.*, 2008) and against (Dardevet *et al.*, 2005) the involvement of GLP-1Rs in the dog portal vein for noninsulin-dependent lowering of glycemia has been presented. Recently, insulin clamps and exercise were used to compare insulin-independent GLP-1 effects in GLP-1-$R^{-/-}$ mice and controls (Ayala *et al.*, 2009). GLP-1R activation was concluded to regulate both hepatic glucose production and muscle glucose uptake under conditions of increased glucose flux (Ayala *et al.*, 2009) via activation of receptors in the arcuate nucleus (Ayala *et al.*, 2009; Sandoval *et al.*, 2008). Outflow from hypothalamic neurons to skeletal muscle (Knauf *et al.*, 2005, 2008) was speculated to involve endothelium-mediated effects on glycogen storage (Knauf *et al.*, 2005). In conclusion, although there is a significant literature on the noninsulin-dependent effects of GLP-1 on glucose metabolism, it is currently unclear as to whether such effects are mediated indirectly, through autonomic neural activation, or via direct actions on peripheral tissues and, if the latter, whether they are mediated via conventional GLP-1 receptors. Similar uncertainty pertains to the actions of GIP.

B. Adipose tissue

Although there is strong evidence supporting a role for GIP in the regulation of adipocyte function, the situation with GLP-1 is unclear. Fat ingestion promotes robust GIP and GLP-1 responses in humans and rodents (Holst, 2007; McIntosh *et al.*, 2009). In a number of studies, GIP has been shown to increase triglyceride clearance *in vivo* (reviewed in McIntosh *et al.*, 2009; Morgan, 1996) whereas, in rodents, GLP-1 decreased intestinal lymph flow and reduced absorption of triglycerides (Qin *et al.*, 2005). Infusion of GLP -1 also reduced postprandial triglyceride and FFA levels in humans, but this effect was attributed to inhibition of gastric emptying and insulin-induced suppression of lipolysis (Meier *et al.*, 2006).

Expression of the GIP receptor in adipocytes from humans and rodents has been established (McIntosh *et al.*, 1999; Weaver *et al.*, 2008; Yip *et al.*, 1998). GLP-1-binding sites have been identified in rat adipocyte membranes (Merida *et al.*, 1993) and 3T3-L1 cells (Montrose-Rafizadeh *et al.*, 1997), but the binding protein responsible exhibited differences in

characteristics from the GLP-1R (Montrose-Rafizadeh *et al.*, 1997; Yang *et al.*, 1999). GLP-1R expression in adipose tissue was also undetectable with RT-PCR or RNAse protection assay (Bullock *et al.*, 1996). GIP stimulates AC and lipolysis in differentiated 3T3-L1 cells and rat adipocytes (Getty-Kaushik *et al.*, 2006; McIntosh *et al.*, 1999; Weaver *et al.*, 2008; Yip *et al.*, 1998), whereas GLP-1 has been found to exert variable effects on cAMP levels (Miki *et al.*, 1996; Montrose-Rafizadeh *et al.*, 1997; Ruiz-Grande *et al.*, 1992; Wang *et al.*, 1997) and had no effect on lipolysis in human subcutaneous adipose tissue, as measured with an *in vivo* microdialysis technique (Bertin *et al.*, 2001).

Studies on the GIP-R knockout mouse led to the suggestion that GIP normally enhances adiposity (Miyawaki *et al.*, 2002). GIP receptor expression increases during differentiation of preadipocytes to adipocytes (Song *et al.*, 2007; Weaver *et al.*, 2008) and GIP promotes the differentiation process through activation of PKB (Song *et al.*, 2007). GIP (Song *et al.*, 2007), GLP-1 (Sancho *et al.*, 2005; Wang *et al.*, 1997), and ex-4 (Sancho *et al.*, 2005) have all been reported to stimulate insulin-mediated uptake of 2-[^{3}H] deoxyglucose in adipocytes, through modulation of GLUT-4 (Gao *et al.*, 2007; Song *et al.*, 2007), although responses to GLP-1R activation have not been universally positive (Idris *et al.*, 2002). GIP also stimulates lipogenesis by increasing adipocyte fatty acid uptake (Hauner *et al.*, 1988). In humans, hydrolysis of circulating triglycerides and liberation of FFAs for uptake and storage in the adipocyte are primarily mediated by lipoprotein lipase (LPL). GIP, but not GLP-1, enhanced LPL activity in cultured 3T3-L1 cells and subcutaneous human adipocytes (Kim *et al.*, 2007a,b), through a pathway involving reduced activity of AMP Kinase, release of resistin, and activation of SAPK/JNK and p38 MAP Kinase signaling modules (Fig. 2.7; Kim *et al.*, 2007b).

There are a number of areas of incretin-mediated effects on adipocyte function that need clarification, including the identity of the receptor responding to GLP-1 receptor agonists. There appear to be insulin-independent and -dependent adipocyte actions of GIP (McIntosh *et al.*, 1999, 2009) that result in lipolytic and lipogenic responses, respectively. It has been suggested that GIP may assist in maintaining appropriate levels of circulating FFAs during fasting, thus priming β-cells for subsequent glucose stimulation, while switching to a lipogenic action as insulin levels rise (McIntosh *et al.*, 2009). Alternatively, increased FFAs produced by GIP-induced lipolysis may be reesterified, thus contributing to lipogenesis (Getty-Kaushik *et al.*, 2006). The question as to whether or not GIP is involved in the development of human obesity has been previously discussed (McIntosh *et al.*, 2009). The role of GLP-1 is enigmatic, but the possible existence of a neural pathway mediating GLP-1-induced lipolysis is intriguing (Nogueiras *et al.*, 2009).

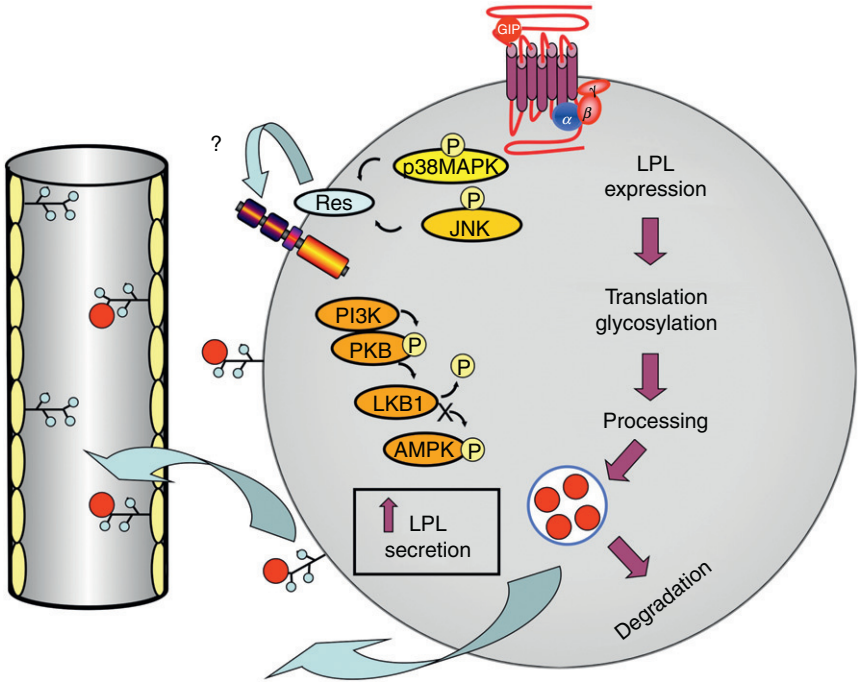

Figure 2.7 Proposed pathway by which GIP increases resistin secretion and lipoprotein lipase (LPL) activity in adipocytes. GIP receptor interaction results in activation of p38 MAPK and stress-activated protein kinase (SAPK)/JNK, leading to the secretion of resistin. Secreted resistin acts in either an autocrine or paracrine manner, on an as yet unidentified receptor, to increase PKB phosphorylation and decrease LKB1 and AMP-activated protein kinase (AMPK) phosphorylation, resulting in increased LPL activity. There is the potential for involvement of other growth factors/hormones in the regulatory pathway.

IX. Effects of GIP and GLP-1 on Bone

Both GIP and GLP-1 impact on bone metabolism, although the pathways by which they act differ significantly. Normal bone and osteoclast-like (SaSo2 and MG63) cell lines express GIPRs (Bollag *et al.*, 2000, 2001; Zhong *et al.*, 2007), and GIPR activation exerts anabolic and proliferative effects on bone. Among the *in vitro* responses to GIP were increased alkaline phosphatase activity and collagen type 1 mRNA levels, responses associated with new bone formation (Bollag *et al.*, 2000), and inhibition of osteoclast resorptive activity (Zhong *et al.*, 2007). The physiological relevance of these effects was reflected in the increased bone density induced by GIP treatment of ovariectomized rats (Bollag *et al.*, 2001).

Additionally, GIP-R$^{-/-}$ mice exhibited reduced bone size and mass, as well as altered bone microarchitecture, biomechanical properties, and turnover (Tsukiyama *et al.*, 2004; Xie *et al.*, 2005). In contrast, transgenic mice overexpressing GIP showed increases in markers of bone formation, decreases in bone resorption, and increased bone mass (Xie *et al.*, 2007), as well as a reduction in age-induced bone loss (Ding *et al.*, 2008). GLP-1R$^{-/-}$ mice exhibited bone fragility and increased numbers of osteoclasts and bone resorption (Yamada *et al.*, 2008). No direct effects of GLP-1 on bone have been identified, but administration of ex-4 to wild-type mice increased thyroid calcitonin expression and reduced markers of bone resorption (Yamada *et al.*, 2008). Therefore, it appears that GLP-1 acts indirectly to promote growth by increasing circulating calcitonin levels. Currently, there are no reliable data available on the effects of either GIP or GLP-1 on bone metabolism in humans.

X. The Future

As discussed in this review, the incretin hormones exhibit a remarkable spectrum of actions, many of which are related to the regulation of nutrient and mineral assimilation and metabolism. Incretin-related drugs currently in use as T2DM therapeutics are believed to act mainly through insulinotropic and glucagonostatic actions on the pancreatic islets, as well as suppression of food intake and inhibition of gastric emptying. However, in view of the pleiotropic actions of the incretins outlined, it seems likely that their β-cell proliferative and antiapoptotic effects can also be used to advantage, and recent reports also indicate that neuroprotective (Biswas *et al.*, 2008) and immunomodulatory (Hadjiyanni *et al.*, 2010; Kim *et al.*, 2010) actions could also prove clinically beneficial. Additional actions of GLP-1 have also been described for the lungs (Benito *et al.*, 1998) and kidneys (Moreno *et al.*, 2002). However, despite considerable research efforts toward understanding their mode of action, there are still many fundamental questions related to both the basic physiology and clinical applications of incretins that need answering, a few of which are listed here:

1. What are the relative functional contributions of GLP-1 delivered to target tissues via the vascular route compared to that acting on autonomic nerves?
2. How do GLP-1 and GIP act on noninsulin-dependent glucose metabolism?
3. Do the incretins exert tissue-selective effects via different receptor isoforms or through cell-specific signaling pathways? If so, is cell-specific targeting possible?

4. Are the signaling pathways identified as mediating β-cell proliferative and survival effects in rodents replicated in human islets? If so, can they be activated?
5. What is the contribution of incretins to improvements in diabetes observed following bariatric surgery?

Hopefully, answers to most of these questions will be forthcoming in the near future.

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CHAPTER THREE

Dietary Effects on Incretin Hormone Secretion

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Contents

Abstract

The delivery of nutrients from the stomach into the duodenum and their subsequent interaction with the small intestine to stimulate incretin hormone release are central determinants of the glycemic response. The incretin effect has hitherto been attributed to the secretion of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) from enteroendocrine cells in the intestinal epithelium. A number of recent studies have yielded fundamental insights into the

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influence of individual nutrients on incretin release and the mechanisms involved in the detection of carbohydrates, fats, and proteins by enteroendocrine cells, including the K_{ATP} channel, sodium–glucose cotransporter 1 (SGLT1), sweet taste receptors, G-protein-coupled receptors (GPRs), and oligopeptide transporter 1 (PepT1). Dietary modification, including modifying macronutrient composition or the consumption of "preloads" in advance of a meal, represents a novel approach to manipulate the incretin response and thereby regulate glucose homeostasis in patients with type 2 diabetes. This review focuses on the effects of individual nutrients on incretin hormone secretion, our current understanding of the signaling mechanisms that trigger secretion by enteroendocrine cells, and the therapeutic implications of these observations.

I. Introduction

The "incretin effect," whereby oral glucose elicits a much greater insulin response than an isoglycemic intravenous glucose infusion, has been recognized for over 40 years (Perley and Kipnis, 1967). To date, the incretin effect has been attributed to two hormones—glucose-dependent insulinotropic polypeptide (GIP, also known as gastric inhibitory polypeptide), first identified in the 1970s in extracts of the upper small intestine (Brown *et al.*, 1975), and glucagon-like peptide-1 (GLP-1), uncovered during the next decade (Drucker *et al.*, 1987). The combined action of these peptides accounts for approximately 50–70% of the total insulin release in healthy humans after ingestion of oral glucose. Furthermore, these two peptides induce glucose-stimulated insulin secretion in a synergetic manner, and appear to contribute approximately equally to the incretin effect in health (Baggio and Drucker, 2007). The delivery of nutrients from the stomach into the duodenum and their subsequent interaction with the small intestine to stimulate incretin hormone release are central determinants of the glycemic response. An improved understanding of the incretin response to food ingestion is, therefore, of fundamental importance for management of type 2 diabetes. In this review, we summarize current knowledge related to the effects of individual nutrients on incretin hormone release, and the mechanisms by which nutrients interact with the small intestine to induce GLP-1 and GIP secretion.

II. Physiology of the Incretin Hormones

GLP-1 is released from intestinal L-cells, the majority of which are located in the distal small intestine and colon. In contrast, GIP is secreted by intestinal K-cells, distributed mainly in the upper small intestine (duodenum

and proximal jejunum). However, in pigs, rats, and humans, there also exists a large population of "K/L-cells," expressing both GIP and GLP-1, located mainly in the upper small intestine (Fujita *et al.*, 2008; Mortensen *et al.*, 2003; Theodorakis *et al.*, 2006). In response to a variety of stimuli, biologically active GLP-1 is secreted as a 7–37 or 7–36 amide peptide, and GIP as a 42 amino acid sequence. Both incretins are then rapidly inactivated at the site of an alanine residue by an aminopeptidase, dipeptidyl peptidase IV (DPPIV) (Drucker and Nauck, 2006). More than 50% of GLP-1 is inactivated before it reaches the systemic circulation, and the half-life of circulating GLP-1 is less than 2 min (Baggio and Drucker, 2007). Similarly, the half-life of biologically active GIP is short, at approximately 7 and 5 min in healthy and type 2 diabetic individuals, respectively (Deacon *et al.*, 2000); the decreased half-life in the latter group has been attributed to increased DPPIV activity (Ryskjaer *et al.*, 2006). Despite their short plasma half-life, the incretin hormones have been found, by the application of GLP-1 and GIP receptor agonists and antagonists and DPPIV inhibitors, as well as receptor knockout animal models, to mediate a wide range of biological effects via binding to their specific receptors.

A. Biological actions of GLP-1

GLP-1 contributes to the regulation of glucose homeostasis in a number of ways, including pancreatic and extrapancreatic actions on insulin secretion, improvement of insulin sensitivity and peripheral glucose disposal, inhibition of gastric emptying, and suppression of glucagon secretion and food intake.

In the pancreas, GLP-1 triggers insulin secretion from β-cells in a glucose-concentration dependent manner (Yu and Jin, 2010). Activation of the GLP-1 receptor (GLP-1R) by exendin-4 enhances β-cell proliferation and neogenesis, and inhibits β-cell apoptosis in a rodent model of diabetes, leading to expansion of β-cell mass and improvement of glucose tolerance (Maida *et al.*, 2009). Furthermore, GLP-1−/− mice exhibit defective regeneration of the β-cell, and are more susceptible to streptozotocin-introduced β-cell apoptosis (Maida *et al.*, 2009). In addition, GLP-1 is able to enhance glucose sensitivity by upregulating the expression of glucose transporters and glucokinases (Holz Iv *et al.*, 1993), thereby improving the capacity of β-cell to respond to glucose.

Rodent studies indicate that GLP-1 mediated stimulation of insulin secretion can occur indirectly via a neural pathway. In the portal vein of rats, GLP-1 activates hepatic vagal afferent fibers via the nodose ganglion, which in turn increases the activity of pancreatic vagal efferents, leading to glucose-stimulated insulin secretion and glucose disposal (Balkan and Li, 2000). GLP-1 also inhibits hepatic glucose production (Prigeon *et al.*, 2003)

and stimulates glucose uptake in fat and muscle (Villanueva-Penacarrillo *et al.*, 2001).

Among the multiple actions of GLP-1 on glucose homeostasis, inhibition of gastric emptying (the "ileal brake"), as demonstrated in studies involving "physiological" infusion of exogenous GLP-1 (Nauck *et al.*, 1997), or the specific GLP-1 antagonist, exendin(9–39) (Deane *et al.*, 2010), is of particular importance, given that it may outweigh the insulinotropic property of GLP-1. This is highlighted by studies demonstrating that exogenous GLP-1 infusion, in the presence of lower blood glucose induced by delayed gastric emptying, leads to less insulin secretion after a meal compared to control (Meier *et al.*, 2005). Slowing of gastric emptying also plays a dominant role in the action of the GLP-1 analog, exenatide, at least acutely, to reduce postprandial glycemia in type 2 patients (Cervera *et al.*, 2008).

GLP-1 has the capacity to suppress glucagon secretion, when infused in physiological concentrations in patients with type 2 diabetes (Meier *et al.*, 2003a). The glucagonostatic action of GLP-1 is also glucose-dependent, but is not dependent on the stimulation of insulin (Baggio and Drucker, 2007). GLP-1 also appears to regulate feeding behavior—both central and peripheral administration of GLP-1R agonists reduce short-term food and water intake, leading to decreased body weight (Szayna *et al.*, 2000; Turton *et al.*, 1996). That the effects persist after subdiaphragmatic bilateral vagotomy or surgical transaction of the brainstem-hypothalamic pathway indicates the importance of the central actions of GLP-1 (Abbott *et al.*, 2005).

B. Biological actions of GIP

The actions of GIP on the pancreatic β-cell are similar to those of GLP-1 in health, but diminished in diabetes. In contrast, GIP promotes energy storage via direct action on adipose tissue and is likely to exacerbate insulin resistance.

GIP is able to act synergistically with glucose to stimulate insulin secretion (Baggio and Drucker, 2007) and enhance the survival of pancreatic β-cell lines (Kim *et al.*, 2005). In contrast to the suppression of glucagon seen with GLP-1, GIP stimulates glucagon secretion in healthy humans, at least under euglycemic conditions (Meier *et al.*, 2003b). Although GIP release is intact, or even increased, in response to nutrient ingestion in type 2 diabetic patients, its insulinotropic effect has been reported to be markedly diminished in this group (Ma *et al.*, 2009c). Some studies have proposed that this impairment of GIP action is attributable to desensitization and/or downregulation of GIPR expression during long-standing hyperglycemia (Hojberg *et al.*, 2009; Meier *et al.*, 2004a; Nauck *et al.*, 2004a), since a period of strict glycemic control has the capacity to restore the

insulinotropic effects of GIP (Hojberg *et al.*, 2009). In contrast to GLP-1, GIP has no effect on gastric emptying (Meier *et al.*, 2004b).

In addition to its insulinotropic properties, GIP exhibits unique physiological actions in extrapancreatic tissues. In adipose tissue, GIP acts in concert with insulin to increase lipoprotein lipase activity and lipogenesis (Irwin and Flatt, 2009). Rodent studies also suggest that GIP has roles in neuroprotection (Nyberg *et al.*, 2005) and bone formation (Ding *et al.*, 2008; Xie *et al.*, 2007). GIPR mRNA is also detectable in the heart, testis, lung, and several other tissues, where its physiologic actions are largely unknown.

III. Dietary Influence on Incretin Hormone Secretion

Plasma concentrations of both GLP-1 and GIP are low (around 10 pmol/L) in the fasting state (Herrmann *et al.*, 1995), but increase approximately two- to threefold after nutrient ingestion (Elliott *et al.*, 1993; Orskov *et al.*, 1994; Vilsboll *et al.*, 2001), with peak values being highly dependent on the rate (Chaikomin *et al.*, 2005) and the load (Pilichiewicz *et al.*, 2007) of nutrient entry to the small intestine (Fig. 3.1), the flow patterns within it (Chaikomin *et al.*, 2007), and the length of small intestine exposed (Little *et al.*, 2006). Following meal ingestion, GLP-1 and GIP display distinct secretory profiles—the former characteristically exhibits a biphasic profile with an "early" phase occurring within 15 min and lasting for 15–30 min, and a "late" phase persisting for 1–2 h or longer (Herrmann *et al.*, 1995), while secretion of the latter usually occurs with a short time delay, and characteristically remains elevated for several hours (Elliott *et al.*, 1993). These differences are likely to relate, at least in part, to the locations of K-cells and L-cells in the small intestine, as discussed. Although several mechanisms (e.g., nutrient-mediated, neural, and hormonal) have been suggested in the regulation of incretin release (Baggio and Drucker, 2007), the direct interaction of luminal nutrients with K-cells and L-cells appears likely to be fundamental, as these enteroendocrine cells are "open-type" and, therefore, configured to sense the presence of nutrients at the mucosal surface (Dumoulin *et al.*, 1998).

Meal properties, including macronutrient composition, are of central importance to the incretin response. For example, GLP-1 release can be triggered by each of the macronutrients—carbohydrates, fats, and proteins (Baggio and Drucker, 2007)—with fat and carbohydrate generally being the most potent stimuli (Elliott *et al.*, 1993; Herrmann *et al.*, 1995). Similarly, GIP secretion is stimulated strongly by fat and carbohydrate (Elliott *et al.*, 1993; Meier and Nauck, 2004), while protein appears to be less potent

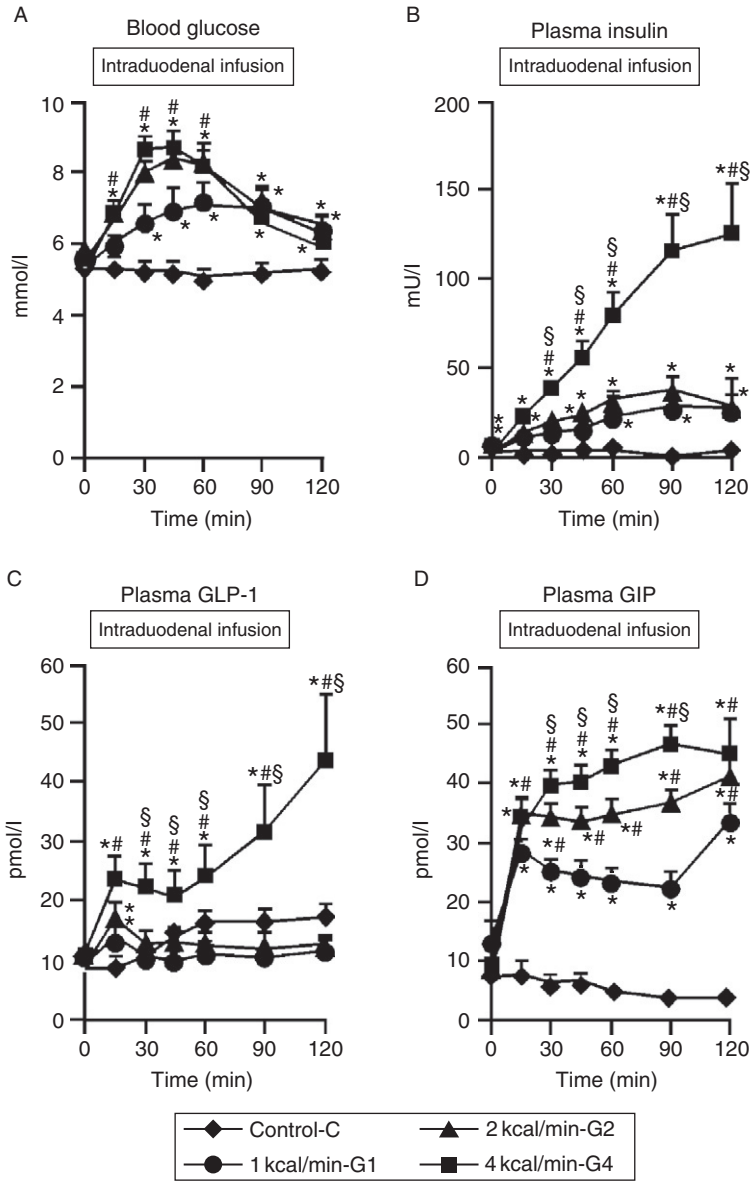

Figure 3.1 Blood glucose (A), plasma insulin (B), GLP-1 (C) and GIP (D) in response to intraduodenal glucose (25%, 1390 mOsmol/L) infused over 120 min at rates of 1 ("G1"), 2 ("G2"), or 4 ("G4") kcal/min, or saline (4.2%, 1390 mOsmol/L) control ("C"), in 10 healthy males. (A) ⋆ versus control: $P < 0.05$, # versus G1: $P < 0.05$, § versus G2: $P < 0.05$. (B) ⋆ versus control: $P < 0.05$, # versus G1: $P < 0.05$, § versus G2: $P < 0.05$. (C) ⋆ versus control: $P < 0.05$, # versus G1: $P < 0.05$, § versus G2: $P < 0.05$. (D) ⋆ versus control: $P < 0.01$, # versus G1: $P < 0.01$. Data are means ± SEM. Adapted from Pilichiewicz *et al.*, 2007.

(Elliott *et al.*, 1993; Herrmann *et al.*, 1995), although certain amino acids induce GIP release (Thomas *et al.*, 1978). The combination of fat and carbohydrate appears to have an additive effect on incretin secretion, at least in rodent models (Lu *et al.*, 2007; Shimotoyodome *et al.*, 2009).

A. Effects of carbohydrates on incretin hormone release

Carbohydrates are potent stimuli for both GLP-1 and GIP release, consistent with their role as incretins, and their effect on incretin secretion is dose-dependent. In health, the interaction of nutrients with the small intestine generates feedback to modulate the rate of gastric emptying at about 2–3 kcal/min (Brener *et al.*, 1983; Macdonald, 1996). Although one study suggested that the stimulation of GLP-1 requires a rate of small intestinal glucose delivery in excess of 1.4 kcal/min (Schirra *et al.*, 1996), we have shown that there is an early, transient rise in GLP-1 in response to intraduodenal glucose at 1 kcal/min (Kuo *et al.*, 2008)(Fig. 3.2), consistent with the observation that there is a sufficient density of L-cells in the human

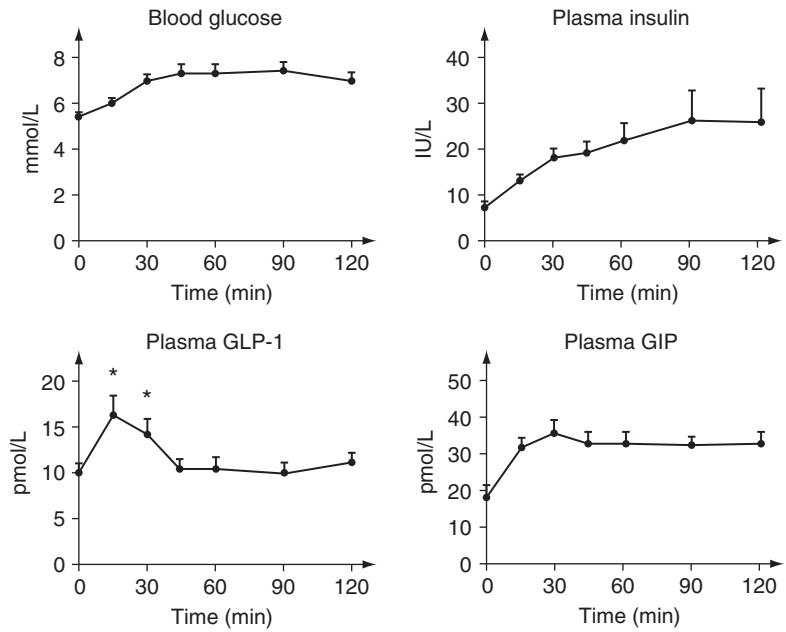

Figure 3.2 Blood glucose and plasma insulin, GLP-1 and GIP levels, during intraduodenal infusion of glucose at 1 kcal/min for 120 min. Blood glucose and plasma insulin, GLP-1, and GIP—all increased significantly from baseline ($P < 0.001$). For GLP-1, only the values at 15 and 30 min differed significantly from baseline ($^{\star}P < 0.001$). Data are means ± SEM. From Kuo *et al.*, 2008.

duodenum to account for the early phase of GLP-1 secretion (Theodorakis *et al.*, 2006). The transient rise in GLP-1 in response to low glucose loads may also be explained by initial rapid transit of glucose to interact with L-cells in the jejunum, with subsequent inhibition of transit by the release of GLP-1; the latter has been shown to slow small intestinal transit in rats (Tolessa *et al.*, 1998). In health, the magnitude of GLP-1 and GIP responses to intraduodenally infused glucose is dose-dependent—GIP increases in approximately linear fashion with increasing glucose loads, whereas the GLP-1 response is nonlinear, being modest at 1–2 kcal/min, and substantially greater at 4 kcal/min (Pilichiewicz *et al.*, 2007). This GLP-1 response to intraduodenal glucose parallels the subsequent reduction in food intake, leading to an improved glycemic response to the following meal (Pilichiewicz *et al.*, 2007). Furthermore, the GLP-1 response is greater with an initially rapid and subsequently slower rate of glucose delivery into the small intestine, when compared to delivery of the same total glucose load at a constant infusion rate (O'Donovan *et al.*, 2004b)(Fig. 3.3).

Both the length and the region of small intestine exposed to carbohydrate are crucial determinants of GLP-1 release; in humans, GLP-1 was stimulated when glucose was allowed to access to the entire small intestine, but not when restricted to the proximal 60 cm (Little *et al.*, 2006)(Fig. 3.4). Furthermore, secretion of GLP-1 in response to sucrose is increased when malabsorption is induced by the α-glucosidase inhibitor, acarbose (Qualmann *et al.*, 1995), which presumably allows stimulation of a greater length, or more distal region of the gut by ingested sugar (Gentilcore *et al.*, 2005).

The incretin response profiles vary with different carbohydrates. Observations *in vitro* and in perfused animal ileum demonstrate that monosaccharides, including glucose, galactose, and 3-O-methylglucose (3OMG), stimulate GLP-1 release, while 2-deoxyglucose does not (Reimann *et al.*, 2008; Ritzel *et al.*, 1997; Sugiyama *et al.*, 1994). A similar pattern of GIP responses to these sugars was observed in ob/ob mice (Flatt *et al.*, 1989). These specificities could relate to glucose transporters (to be discussed later). Fructose stimulates GLP-1 release from both perfused rat ileum (Ritzel *et al.*, 1997) and the small intestine in healthy humans (Rayner *et al.*, 2000), but does not induce GIP release (Ganda *et al.*, 1979; Tazawa *et al.*, 2005), indicating that signaling pathways other than glucose transporters are likely to be involved in GLP-1 secretion. Whether 3OMG, a nonmetabolized substrate for glucose transporters, stimulates release of the incretin hormones in humans is not known.

The glycemic index (GI) of carbohydrates, a measure of glycemic response to ingestion of a carbohydrate, when compared to a standard load of oral glucose or white bread, also influences incretin hormone secretion. Diets with a low-GI, especially those rich in fiber content (Wolever, 1990), have beneficial effects on long-term glycemic control in

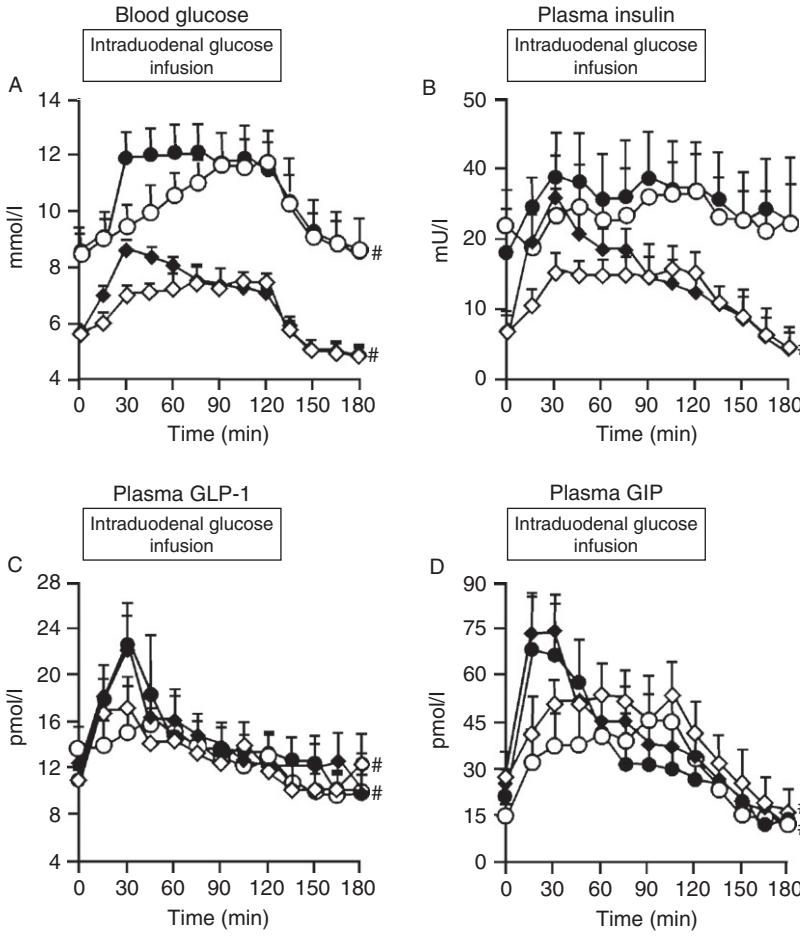

Figure 3.3 Effect of initially more rapid intraduodenal glucose infusion (3 kcal/min between $t = 0$ and 15 min and 0.71 kcal/min between $t = 15$ and 120 min) (closed symbols) compared to constant infusion (1 kcal/min between $t = 0$ and 120 min) (open symbols) in healthy subjects (squares) and patients with type 2 diabetes (circles) on blood glucose, plasma insulin, plasma GLP-1, and plasma GIP. Each pair of curves differs between 0 and 30 min for variable versus constant intraduodenal infusion ($P < 0.05$). Adapted from O'Donovan *et al.*, 2004b.

type 2 diabetes (Esposito *et al.*, 2010). It is possible that an increase in GLP-1 contributes to these metabolic improvements, as low-GI carbohydrates exhibit a lower absorption rate and, therefore, have the potential to give rise to interaction of nutrients with more distal regions of the small intestine. For example, incorporation of indigestible carbohydrates (barley fiber and resistant starch) into an evening meal (white wheat bread) was found to

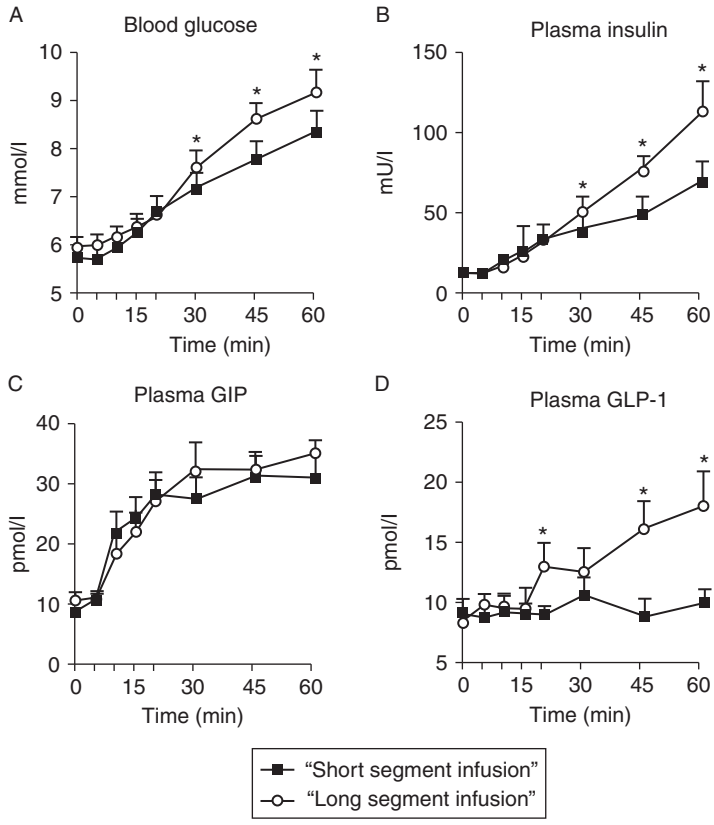

Figure 3.4 Blood glucose (A), plasma insulin (B), GIP (C), and GLP-1 (D) concentrations during a 60 min infusion of 1 M glucose to either (1) a 60 cm segment of the proximal small intestine (short-segment infusion) or (2) >60 cm of the small intestine (long-segment infusion). A: there was a treatment-by-time interaction for blood glucose ($P < 0.05$). Although blood glucose increased progressively on both days between $t = 15$ and 60 min, the magnitude of the rise was greater during the long-segment infusion between $t = 30$ and 60 min than during the short-segment infusion ($P < 0.05$). B: there was a treatment-by-time interaction for plasma insulin concentrations ($P < 0.001$). Although plasma insulin concentrations progressively increased from baseline during both treatments between $t = 20$ and 60 min, the magnitude of the rise in plasma insulin was greater during the long-segment infusion between $t = 30$ and 60 min than during the short-segment infusion ($P < 0.05$). C: there was no effect of treatment on plasma GIP concentrations. D: there was a treatment-by-time interaction for plasma GLP-1 concentrations ($P < 0.001$). At $t = 20$ and between $t = 45$ and 60 min, plasma GLP-1 was greater during the long- than during the short-segment infusion ($P < 0.01$). Data are means ± SEM; $n = 8$. *Short-segment infusion versus long-segment infusion, $P < 0.05$. From Little *et al.*, 2006.

induce greater and more prolonged GLP-1 release and improved glucose tolerance after the following breakfast, when compared to an evening meal with white wheat bread only (Nilsson *et al.*, 2008).

B. Effect of fat on incretin release

Fats are strong stimuli for both GLP-1 and GIP secretion, although their release is often delayed after fatty meals when compared to carbohydrates (Elliott *et al.*, 1993). Direct fat infusion into the small intestine, bypassing the influence of the stomach in the regulation of nutrient delivery, has established that lipid (e.g., triacylglycerol) is able to induce a prompt GLP-1 response, in both healthy and obese subjects (Feinle-Bisset *et al.*, 2002). The GIP and GLP-1 responses to intraduodenal lipid are load-dependent in rats (Yoder *et al.*, 2009). Whether this is also the case in humans remains to be determined, although this has been shown to be the case in relation to the effects of lipid on peptide YY (PYY) and cholecystokinin (CCK) (Pilichiewicz *et al.*, 2006). In healthy young subjects, incorporation of fat into either a carbohydrate-containing drink (Houghton *et al.*, 1990) or a solid meal (Cunningham and Read, 1989), or direct intraduodenal infusion of lipid into the small intestine (Welch *et al.*, 1987), slows gastric emptying and attenuates the blood glucose and insulin responses, in proportion to the stimulation of incretin hormones, especially GLP-1 (Gentilcore *et al.*, 2006). Slowing of gastric emptying and stimulation of GLP-1 and GIP are dependent on the digestion of fat to fatty acids. Accordingly, administration of the lipase inhibitor, orlistat, accelerates gastric emptying of high-fat carbohydrate-containing meals, attenuates the release of GIP and GLP-1, and exacerbates the glycemic response in both health and type 2 diabetics (O'Donovan *et al.*, 2004a; Pilichiewicz *et al.*, 2003). Among different fats, olive oil apparently induces a greater GLP-1 response than butter in both healthy subjects (Thomsen *et al.*, 1999) and patients with type 2 diabetes (Thomsen *et al.*, 2003), suggesting that postprandial GLP-1 release might correlate inversely with the saturation of fatty acids. The chain length of fatty acids has also been proposed as a determinant of the GLP-1 response (Feltrin *et al.*, 2004); when fatty acids were introduced intraduodenally in humans, lauric acid (C12) stimulated GLP-1 release, whereas decanoic acid (C10) did not (Feltrin *et al.*, 2004). The droplet size of fat emulsions is another determinant of gut hormone release, at least for CCK and PYY, whose secretion correlates inversely with the droplet size of infused fat emulsions (Seimon *et al.*, 2009). Even though incretin measurements were not reported in the latter study, it seems likely that GLP-1 would be affected similarly to PYY, given that the latter is also released from L-cells.

C. Effect of protein on incretin release

The effects of protein on incretin release are less well studied than those of carbohydrates or fats. Protein appears to require digestion into peptides or amino acids to stimulate gut feedback responses (Meyer *et al.*, 1976). Some studies have indicated that intraduodenal administration of specific amino acids stimulates GIP release (Thomas *et al.*, 1978), although intact proteins appear to be less effective (Elliott *et al.*, 1993). In healthy humans, the effects of protein (milk and egg) on GLP-1 release were comparable with fat (oleic acid), while GIP levels in the early postprandial period were greater (Carr *et al.*, 2008); while a meal rich in protein (milk and egg) was reported to trigger a greater GLP-1 response than a carbohydrate-rich meal (corn flakes and white bread) (Raben *et al.*, 2003). In contrast, protein (gelatin) supplementation of an oral glucose load in healthy subjects had no effect on the GLP-1 response, and was associated with a reduction in GIP secretion than with glucose alone, but the addition of gelatin was associated with delayed gastric emptying (Karamanlis *et al.*, 2007). Among different protein sources, whey protein has been shown to stimulate GLP-1 and satiety more than casein (Hall *et al.*, 2003). These different effects are likely to be related to their differing amino acid profiles (Hall *et al.*, 2003) and rates of absorption (Tessari *et al.*, 2007). We found in type 2 diabetic patients that, when given acutely, whey protein can stimulate GLP-1 and improve postprandial glycemia (Ma *et al.*, 2009d)(Fig. 3.5), although the latter was also likely regulated by other mechanisms, including slowing of gastric emptying and direct stimulation of insulin release by absorbed amino acids.

IV. Mechanisms by Which Nutrients Stimulate Incretin Release

Due to the limited availability of validated experimental models, the mechanisms underlying nutrient detection in the small intestine and consequent stimulation of incretin release are poorly understood. As discussed, the enteroendocrine cells that release GLP-1 and GIP are "open-type," and are, accordingly, able to sample the luminal nutrients directly. Because of the insulinotropic actions of GLP-1 and GIP and the associated importance of the incretins in glucose homeostasis, much recent research has focused on the mechanisms of carbohydrate sensing by L- and K-cells. However, it is clear that other macronutrients can also stimulate hormone release from these cells, probably via different mechanisms. Carbohydrate, fat, and protein are usually consumed together. Therefore, the relative contributions of the mechanisms described below will vary with meal composition.

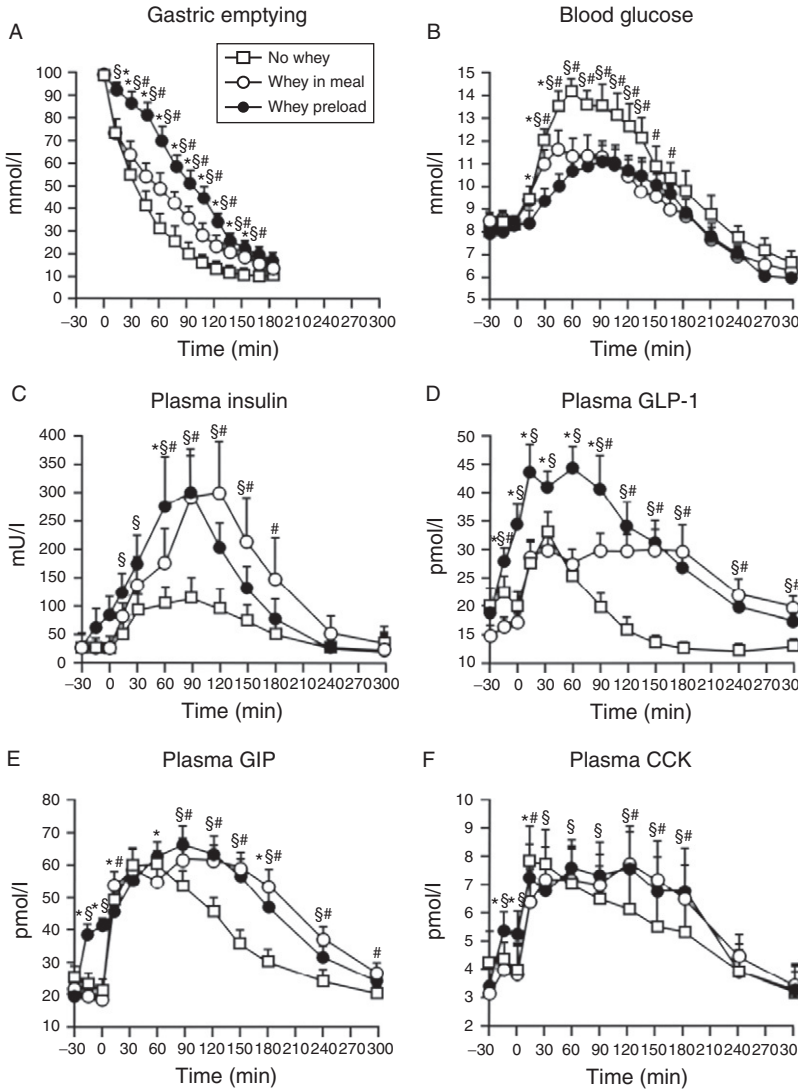

Figure 3.5 Gastric emptying (A), concentration of blood glucose (B), plasma insulin (C), plasma GLP-1(D), plasma GIP (E), and plasma CCK (F) in response to a mashed potato meal in eight type 2 diabetic patients. On each study, subjects ingested 350 mL beef-flavored soup 30 min before a radiolabeled mashed potato meal; 55 g whey protein was added either to the soup (whey preload) or to the potato (whey in meal) or no whey was given (no whey). Data are means ± SEM. $*P < 0.05$, whey preload versus whey in meal; # $P < 0.05$, whey in meal versus no whey; § $P < 0.05$, whey preload versus no whey. From Ma *et al.*, 2009d.

A. Mechanisms underlying glucose sensing

A variety of signaling mechanisms have been proposed to explain how enteroendocrine cells might sense glucose, including ATP-sensitive potassium channel closure, sodium–glucose cotransporter activity, and activation of sweet taste receptors (Tolhurst *et al.*, 2009). It has also been suggested that a proximal–distal neural or hormonal loop might explain how GLP-1 release can occur rapidly after nutrient digestion despite the predominantly distal location of L-cells (Holst, 2007). GIP appears to fulfill this latter function in rodents, but does not stimulate GLP-1 secretion in humans (Hansen and Holst, 2002; Nauck *et al.*, 1993). However, as discussed, it is possible that the density of L-cells in the human duodenum or proximal jejunum may be sufficiently great to account for the early phase of the GLP-1 response (Theodorakis *et al.*, 2006).

1. K_{ATP} channel

As in the pancreatic beta-cell, the closure of ATP-sensitive K^+ channels (K_{ATP} channels) in enteroendocrine cells occurs in response to glucose exposure, and gives rise to changes in the proportions of ATP and Mg^{2+}-ADP, leading to reduction of background K^+ flux and triggering membrane depolarization. Both the K_{ATP} channel subunits Kir6.2 and SUR1 are expressed at high levels in purified mouse K- and L-cells (Parker *et al.*, 2009; Reimann *et al.*, 2008). Expression of Kir6.2 and SUR1 is also detectable in human K- and L-cells by immunostaining (Nielsen *et al.*, 2007). Nevertheless, one study in healthy subjects indicated that sulfonylureas, which induce closure of K_{ATP} channels, do not stimulate GLP-1 or GIP responses to oral glucose (El-Ouaghlidi *et al.*, 2007), suggesting that additional mechanisms are responsible for glucose-mediated incretin release. Furthermore, Kir6.2(−/−) mice exhibit increased, rather than decreased, plasma GIP levels after administration of oral glucose (Miki *et al.*, 2005). Therefore, a role for K_{ATP} channels in incretin secretion remains uncertain.

2. Sodium–glucose cotransporter

The sodium–glucose cotransporter 1 (SGLT1), encoded by the SLC5A1 gene, is the principle transporter for glucose absorption at the luminal surface (Ritzel *et al.*, 1997), transporting glucose and Na^+ in a 1:2 fashion (Wright and Turk, 2004) and, thereby, generating sufficient inward current to excite K- and L-cells (Gribble *et al.*, 2003). Studies in both cell lines and rodent models confirmed the expression of SGLT1 in K- and L-cells and established that monosaccharides that are substrates for SGLT1, regardless of whether they can be metabolized (e.g., glucose) or not (e.g., 3-OMG), stimulate incretin secretion in the presence of sodium. Phoridzin, which blocks SGLT1, inhibits the release of GLP-1 in response to monosaccharides in animal models (Moriya *et al.*, 2009; Sugiyama *et al.*, 1994). Similarly, in

ob/ob mice, GIP is secreted in response to glucose, galactose, and 3OMG (Flatt *et al.*, 1989). SGLT1 may well be involved in GIP secretion in humans, since glucose and galactose release GIP, while fructose and mannose, which are not SGLT1 substrates, do not (Ganda *et al.*, 1979; Tazawa *et al.*, 2005). Nevertheless, SGLT1 is probably not the only signaling pathway for GLP-1 release, for example, fructose stimulates GLP-1 release in both perfused rat ileum (Ritzel *et al.*, 1997) and in healthy humans (Rayner *et al.*, 2000).

3. Sweet taste receptor

In the tongue, the molecular mechanism of sweet taste detection involves activation of a specific sweet taste G-protein coupled receptor (GPCR), a heterodimer of the T1R2 and T1R3 subtypes, located on the apical membranes of taste cells. Activation of the T1R2/T1R3 complex initiates a signal transduction cascade involving the G-protein alpha-gustducin and the transient receptor potential ion channel TRPM5, resulting in cellular depolarization and release of intracellular calcium stores (Nelson *et al.*, 2001). Expression of mRNA for the sweet taste molecules T1R2, T1R3, alpha-gustducin, and TRPM5 has been demonstrated in the rodent intestine and in gut enteroendocrine cell lines (Jang *et al.*, 2007), and the same elements were recently observed in the proximal human small intestine (Young *et al.*, 2009). Stimulation of sweet taste receptors has been linked to incretin hormone release. For example, gustducin-null mice exhibit a defective GLP-1 response and impaired glucose tolerance after oral glucose administration (Kokrashvili *et al.*, 2009). Stimuli of the sweet taste receptor, including the artificial sweetener sucralose, were reported to release GLP-1 from L-cells *in vitro* (Jang *et al.*, 2007), although sucralose apparently fails to release GLP-1 or GIP in either rodents *in vivo* (Fujita *et al.*, 2009) or humans (Ma *et al.*, 2009a). Nonetheless, other ligands of the sweet taste receptor could potentially stimulate GLP-1 secretion, for example, diet soda sweetened with both sucralose and acesulfame K was reported to stimulate GLP-1 release synergically with glucose in healthy humans (Brown *et al.*, 2009). Rodent primary K-cells in culture exhibit low levels of gene expression for sweet taste receptors and no responsiveness to sucralose (Parker *et al.*, 2009), indicating that stimulation of sweet taste receptors appears to be of lesser importance for GIP secretion.

B. Mechanisms underlying fat sensing

Fat is a potent stimulus for both GLP-1 and GIP release. Digestion of fats into fatty acids appears to be essential for stimulating incretin secretion, as demonstrated by the attenuation of GIP and GLP-1 responses to triglyceride in the presence of the lipase inhibitor, orlistat (Ellrichmann *et al.*, 2008; O'Donovan *et al.*, 2004a; Pilichiewicz *et al.*, 2003).

G-protein-coupled receptors (GPRs), including GPR119 (Overton *et al.*, 2006), GPR40 (Edfalk *et al.*, 2008), and GPR120 (Tanaka *et al.*, 2008),

have been identified as important mediators of fatty acid sensing by enteroendocrine cells. The expression of these lipid-sensing receptors has been well established in murine L- and K-cells (Chu *et al.*, 2009; Lauffer *et al.*, 2009; Parker *et al.*, 2009). In humans, one study reported a high density of GPR119 in the human duodenum, ileum, and jejunum when evaluated by quantitative PCR (Chu *et al.*, 2008), while another found relatively high GPR40 mRNA expression in the ileum (Itoh *et al.*, 2003), and colocalization of GPR120 with GLP-1 bearing cells has been demonstrated in the ileum, colon, and rectum (Hirasawa *et al.*, 2005). The relevance of these receptors to GLP-1 and GIP secretion has been examined intensively in gene-modified rodent and enteroendocrine cell models. GPR119 is Gs-coupled, and administration of a GPR119 agonist in the rodent model induced prompt elevation of plasma GLP-1 and GIP, and substantial improvements in postprandial glycemia (Chu *et al.*, 2008). *In vitro* studies indicate that elevation of intracellular cyclic AMP (cAMP) is fundamental to GPR119-mediated incretin release (Lauffer *et al.*, 2009; Parker *et al.*, 2009). GPR40 and GPR120 are G_q coupled and are proposed as pathways for enhanced GLP-1 and GIP responses to long chain unsaturated fatty acids (Edfalk *et al.*, 2008; Tanaka *et al.*, 2008), possibly via downstream activation of PKC and inositol (1,4,5)-trisphosphate-dependent Ca^{2+} release from intracellular stores (Parker *et al.*). Consistent with this role, GPR40 knockout mice exhibit diminished GIP and GLP-1 responses to a high-fat diet (Edfalk *et al.*, 2008), and administration of the GPR120 ligand, α-linolenic acid, potently elevates plasma GLP-1 levels in rodent models (Tanaka *et al.*, 2008). However, as mentioned previously, species differences should be taken into consideration and more human evidence is required to determine whether ligands of these receptors will be useful therapeutically.

Recently, another G-protein-coupled receptor, TGR5, was identified in the small intestine and is expressed in the enteroendocrine L-cells in rodents (Reimann *et al.*, 2008). Its stimulation by bile acids potentiates GLP-1 release from STC-1 cells *in vitro* (Katsuma *et al.*, 2005), and in isolated perfused rat colon (Plaisancie *et al.*, 1995). *In vivo* studies, using either a TGR5 agonist (6alpha-ethyl-23(S)-methyl-cholic acid) or gene knockout/overexpressing rodent models, indicate that TGR5 signaling is able to induce substantial GLP-1 release, leading to enhanced glucose tolerance in mice (Thomas *et al.*, 2009), and suggesting a therapeutic role for the targeting of TGR5 in diabetes management.

C. Mechanisms underlying protein sensing

The mechanisms mediating protein-induced incretin secretion remain largely unknown. The oligopeptide transporter 1 (PepT1), a proton-coupled di- and tri-peptide transporter, is a candidate for this role, since it is distributed widely on the intestinal surface and is central to absorption of

oligopeptides arising from protein digestion (Adibi, 2003). Studies using the enteroendocrine cell lines GLUTag, NCI-H716, and STC-1 have shown that protein hydrolysates (peptones) can release incretins dose-dependently (Cordier-Bussat *et al.*, 1998; Reimer, 2006), and this effect is further enhanced in PepT1-transfected STC-1 cells (Matsumura *et al.*, 2005), but inhibited partially by nifedipine (Matsumura *et al.*, 2005), suggesting a role of voltage-gated Ca^{2+}-channels subsequent to oligopeptide transport by PepT1. In addition, activation of the glycine receptor (a ligand-gated Cl^- channel) by glycine and alanine triggers substantial GLP-1 secretion in GLUTag cells (Gameiro *et al.*, 2005), while glutamine and asparagine trigger GLP-1 release from these cells via Na^+-coupled electrogenic uptake, leading to depolarization (Reimann *et al.*, 2004). These observations suggest that changes in cellular electrical status might be fundamental to protein-induced incretin responses. However, the secretory characteristics of these cell models can differ from the primary cells, not to mention the *in vivo* responses. For example, in healthy humans, ileal perfusion with peptone induced only a weak plasma GLP-1 response (Layer *et al.*, 1995). Therefore, the mechanisms underlying protein sensing in the gut remain to be clarified.

V. Incretin Responses in Obesity and Diabetes

Some studies have reported lower postprandial GLP-1 concentrations in patients with type 2 diabetes compared to healthy controls, and that GIP secretion was relatively intact (Toft-Nielsen *et al.*, 2001; Vilsboll *et al.*, 2003), suggesting that impaired secretion of GLP-1 contributes to postprandial hyperglycemia in type 2 diabetes. Defective GLP-1 secretion is not apparent in relatives of type 2 diabetic patients (Nauck *et al.*, 2004b; Nyholm *et al.*, 1999) and therefore appears to be secondary to the development of diabetes, perhaps due to hyperglycemia. However, normalization of glycemia over 4 weeks (i.e., HbA_{1c} was reduced from $8.0 \pm 0.4\%$ to $6.6 \pm 0.3\%$), while improving the insulin response to GLP-1, does not correct the deficiency of GLP-1 release (Hojberg *et al.*, 2008). Furthermore, obesity is associated with reduced postprandial GLP-1 (Lugari *et al.*, 2004; Naslund *et al.*, 1998), and it has been reported that body mass index and the degree of impaired glucose tolerance act independently, and additively, to reduce GLP-1 secretion (Muscelli *et al.*, 2008). It seems extraordinary that none of these studies has controlled for potential differences in gastric emptying associated with diabetes or obesity. As both type 1 and type 2 diabetic patients are reported, a high prevalence of delayed gastric emptying (Horowitz *et al.*, 2002) and obese subjects may have a greater initial rate of gastric emptying (Verdich *et al.*, 2000). By using an intraduodenal glucose

infusion, thus bypassing any effect of gastric emptying, we observed that glucose ingestion triggers a similar maximal, but deficient early phase, GLP-1 response in relatively well-controlled type 2 diabetic patients when compared to healthy subjects (Ma *et al.*, 2009b). Further investigation is needed in these with poor glycemic control, in order to identify whether GLP-1 is deficient, and if so, why.

Genetic variations are well recognized as important in the pathogenesis of obesity and diabetes. It is interesting to consider whether similar mechanisms underlie impaired incretin secretion and action in these pathophysiological states, although to date, genetic association studies with GLP-1 and GIP levels are relatively few. The diabetes-related transcription factor 7-like 2 (TCF7L2) gene was the first candidate proposed to affect GLP-1 secretion due to its influence on intestinal proglucagon gene expression (Grant *et al.*, 2006), although the principle effect of TCF7L2 is on the pancreatic islets themselves (Schafer *et al.*, 2007). Recently, polymorphisms in KCNQ1, another gene associated with diabetes, were reported to correlate with the plasma GLP-1 and GIP response to oral glucose (Mussig *et al.*, 2009). Furthermore, incretin release seems to differ between individuals from different racial backgrounds, for example, African-American children and adolescents exhibit lower fasting and postprandial concentrations of GLP-1 than Caucasians (Higgins *et al.*, 2008; Velasquez-Mieyer *et al.*, 2008).

VI. Therapeutic Implications

A growing number of studies have yielded insights into potential strategies based on the incretin axis for the prevention and management of obesity and diabetes. These approaches have focused on GLP-1, rather than GIP, since, as discussed, the insulinotropic effect of the latter is impaired in type 2 diabetes (Nauck *et al.*, 1993). The concept of stimulating endogenous GLP-1 release, either by modifying macronutrient composition or by changing dietary habits, is an attractive alternative to the use of exogenous GLP-1 analogs as a way of minimizing postprandial glucose excursions.

A. Modifying macronutrient composition

Modifying the macronutrient composition of a meal represents one therapeutic strategy in diabetes prevention and management that potentially impacts on the incretin axis. For example, substitution of low- for high-GI carbohydrates may result in slowing of small intestinal carbohydrate absorption (Bjorck and Elmstahl, 2003), allowing exposure of the more

distal gut to carbohydrate and subsequent stimulation of greater, and more prolonged, GLP-1 release (Little *et al.*, 2006). Similar effects are evident with the use of the α-glucosidase inhibitor, acarbose (Qualmann *et al.*, 1995).

Incorporation of fat, particularly monounsaturated fatty acid (Thomsen *et al.*, 1999; Thomsen *et al.*, 2003), into either a carbohydrate-containing drink or a solid meal, slows the rate of gastric emptying and increases incretin hormone levels, especially GLP-1, reducing the postprandial glycemic excursion (Gentilcore *et al.*, 2006). Nevertheless, diets rich in fat will inevitably increase the overall caloric load and raise a possibility of weight gain in the long term.

Supplementation of a carbohydrate-rich meal (mashed potato) with protein lowers postprandial glucose excursion by slowing the rate of gastric emptying and stimulating incretin hormone secretion (Ma *et al.*, 2009d). A rodent study also suggested that digested whey protein fragments can inhibit the activity of small intestinal DPPIV (Gunnarsson *et al.*, 2006). A 5-week dietary intervention in type 2 diabetic patients, in which the percentage of dietary protein was increased from 15 to 30%, reported a reduction in postprandial glycemia and a modest decrease in glycated hemoglobin (Gannon *et al.*, 2003).

B. The "preload" concept

A novel strategy to minimize postprandial glycemia could be to administer a small load of a macronutrient before a meal, so that the presence of nutrients in the small intestine induces the release of GLP-1 and GIP, and other gut peptides such as CCK and PYY, to slow gastric emptying, and potentially stimulate insulin secretion in advance of the main nutrient load. For example, giving fat at an interval (e.g., 30 min) before the meal appears more efficacious than incorporation of fat into the meal (Gentilcore *et al.*, 2006). Furthermore, the "preload" may also have the advantage of suppressing appetite and reducing energy intake at the subsequent meal. We recently reported that acute administration of a whey protein preload markedly reduced the postprandial glycemic excursion (both peak and AUC glucose) in type 2 patients by these mechanisms (Ma *et al.*, 2009d). However, a potential disadvantage of this strategy is the provision of additional energy intake, which might not be compensated for by a reduction in energy intake at the subsequent meal. A preload that stimulates incretin release and induces slowing of gastric emptying, but contributes minimal additional energy intake, would represent a major advance. Nonnutrient ligands of the various receptors involved in incretin detection that were discussed earlier would be obvious targets of such an approach. Nevertheless, there is as yet little published evidence that this goal can be achieved.

Given the short half-life of incretin hormones in the circulation (Baggio and Drucker, 2007) and moderate effect of monotherapy with GLP-1 analogs (e.g., exenatide) on glucose reduction (Moretto *et al.*, 2008), the combination of DPPIV inhibitors with the dietary strategies discussed above may be a useful maneuver to optimize this approach to diabetes management.

C. Targeting GIP

GIP is associated with fat deposition in adipocytes by acting in concert with insulin to increase lipoprotein lipase activity and lipogenesis (Irwin and Flatt, 2009), and might be an important contributor in the development of obesity. GIPR gene-deficient mice are resistant to diet-induced obesity, without reduction of lean mass or food intake when compared with wild-type controls (Yamada *et al.*, 2007), indicating an anabolic effect of GIPR signaling in lipid metabolism, and implying that GIP antagonists could be used for the prevention and treatment of obesity. Indeed, in mice, the GIP antagonist (Pro3)GIP effectively decreased body weight, reducing adipose tissue mass and the deposition of triglyceride in liver and muscle, and improving insulin sensitivity and glucose tolerance in mice (McClean *et al.*, 2007). As discussed, in humans with type 2 diabetes, the impaired incretin effect is partly attributable to a diminished insulinotropic action of GIP, in addition to impaired GLP-1 secretion, although a period of strict glycemic control can restore the insulinotropic effect of GIP (Hojberg *et al.*, 2008; Hojberg *et al.*, 2009). It is, therefore, interesting to ask whether the use of GIP agonists in type 2 patients would have an overall beneficial effect by increasing the incretin effect or whether it would drive more fat deposition into adipocytes with a consequent deleterious effect on obesity. Further exploration is required to address this therapeutic issue.

VII. Conclusions

The macronutrient composition of a meal, together with the physical properties of food, influences incretin hormone release, and is accordingly a fundamental determinant of the postprandial glycemic response. Recent insights into the mechanisms by which nutrients modulate incretin hormone secretion have led to the recognition of a number of potential therapeutic strategies that require further evaluation for their use in the management, and potentially prevention, of type 2 diabetes. Incretin-based strategies involving dietary manipulation are promising additions to pharmacological treatments in diabetes management.

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CHAPTER FOUR

K-cells and Glucose-Dependent Insulinotropic Polypeptide in Health and Disease

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Abstract

In the 1970s, glucose-dependent insulinotropic polypeptide (GIP, formerly gastric inhibitory polypeptide), a 42-amino acid peptide hormone, was discovered through a search for enterogastrones and subsequently identified as an incretin, or an insulinotropic hormone secreted in response to intraluminal nutrients. Independent of the discovery of GIP, the K-cell was identified in small intestine by characteristic ultrastructural features. Subsequently, it was realized that K-cells are the predominant source of circulating GIP. The density of K-cells may increase under conditions including high-fat diet and obesity, and generally correlates with plasma GIP levels. In addition to GIP, K-cells secrete xenin, a peptide with as of yet poorly understood physiological functions, and GIP is often colocalized with the other incretin hormone glucagon-like peptide-1 (GLP-1). Differential posttranslational processing of proGIP produces 30 and 42 amino acid versions of GIP. Its secretion is elicited by intraluminal nutrients, especially carbohydrate and fat, through the action of SGLT1, GPR40, GPR120, and GPR119. There is also evidence of regulation of GIP secretion via neural pathways and somatostatin. Intracellular signaling mechanisms of GIP secretion are still elusive but include activation of adenylyl cyclase, protein kinase A (PKA), and protein kinase C (PKC). GIP has extrapancreatic actions on adipogenesis, neural progenitor cell proliferation, and bone metabolism. However, the clinical or physiological relevance of these extrapancreatic actions remain to be defined in humans. The application of GIP as a glucose-lowering drug is limited due to reduced efficacy in humans with type 2 diabetes and its potential obesogenic effects demonstrated by rodent studies. There is some evidence to suggest that a reduction in GIP production or action may be a strategy to reduce obesity. The meal-dependent nature of GIP release makes K-cells a potential target for genetically engineered production of satiety factors or glucose-lowering agents, for example, insulin. Transgenic mice engineered to produce insulin from intestinal K-cells are resistant to diabetes induced by a beta-cell toxin. Collectively, K-cells and GIP play important roles in health and disease, and both may be targets for novel therapies.

I. History of K-cells and GIP

The intestine is a critical organ for maintaining life in animals, from sponges to humans. From an evolutionary perspective, it is intuitive that gut mucosa relay signals for the regulation of nutrient metabolism. As a result of

the discovery of secretin in 1902, the conception of gastrointestinal endocrinology, Bayliss and Starling speculated that signals arising from the gut after ingestion of nutrients might elicit pancreatic endocrine responses and affect the metabolism of carbohydrates (Bayliss and Starling, 1902). Thereafter, scientists tried to find specific hormones regulating gastrointestinal physiology and nutrient metabolism. The term "enterogastrone" was proposed by Kosaka and Lim in 1930 to describe a putative hormone that was secreted in response to fat or its digestive products in the intestinal lumen and inhibited gastric acid secretion (Kosaka and Lim, 1930). By using the canine Heidenhain pouch, Brown *et al.* purified an active substance with enterogastrone properties from hog upper small intestinal mucosa and named it gastric inhibitory polypeptide (GIP) (Brown, 1971; Brown and Dryburgh, 1971). In parallel with the enterogastrone hunt, there was an enormous effort to find the so-called incretins. La Barre introduced the term "incretin" to describe the humoral activity of the gut that might enhance the endocrine secretion of the pancreas (La Barre and Still, 1930). However, there was no success in finding the conceptual incretin until the development of a reliable radioimmunoassay (RIA) for insulin in the 1960s by Yalow and Berson (1960). The insulin RIA provided solid evidence for the presence of gut signals augmenting insulin secretion with the observation that oral or intrajejunal glucose induced a significantly greater insulin response than intravenous glucose, even with higher blood glucose levels during the intravenous glucose load (Elrick *et al.*, 1964; McIntyre *et al.*, 1964). Perley and Kipnis compared the plasma insulin responses to oral and intravenous glucose and estimated the alimentary component to be close to 50% (Perley and Kipnis, 1967). In 1969, Unger and Eisentraut (1969) named the connection between the gut and the pancreatic islets the "enteroinsular axis". Creutzfeldt defined the criteria for fulfillment of the hormonal or incretin part of the enteroinsular axis as: (1) it must be released by nutrients, particularly carbohydrates, and (2) at physiological levels, it must stimulate insulin secretion in the presence of elevated blood glucose levels (Creutzfeldt, 1979).

In 1973, Dupre *et al.* determined that GIP may be a long sought gut-derived hormone that augments meal-dependent insulin secretion. They demonstrated that a purified preparation of porcine GIP infused intravenously in humans with glucose stimulated the secretion of significantly greater amounts of insulin than when the same dose of glucose was administered without GIP (Dupre *et al.*, 1973). The insulin response was sustained during the GIP infusion but was not observed during the euglycemic state (Dupre *et al.*, 1973). The glucose-dependent nature for the insulinotropic activity of GIP was also replicated *in vivo* in dogs (Pederson *et al.*, 1975) and humans (Elahi *et al.*, 1979) and in the perfused rat pancreas (Pederson and Brown, 1976). GIP secretion is elicited by oral ingestion of nutrients including glucose and fat (Andersen *et al.*, 1978;

Konturek *et al.*, 1986; Kuzio *et al.*, 1974; Morgan, 1979; Pederson *et al.*, 1975; Varner *et al.*, 1980). However, GIP released in response to the oral ingestion of fat does not increase plasma insulin levels unless intravenous glucose is also administered (Brown, 1974; Cleator and Gourlay, 1975; Ross and Dupre, 1978). The glucose-dependent nature of GIP-stimulated insulin secretion appears to provide an important safety measure against inappropriate stimulation of insulin release during a high-fat, low-carbohydrate meal. In the light of this important function of GIP as an incretin hormone, perhaps its main physiological function, the new name, glucose-dependent insulinotropic polypeptide, was adopted (Brown and Pederson, 1976), thereby retaining the acronym GIP. GIP was the first incretin to obviously fulfill the criteria suggested by Creutzfeldt (1979).

In 1975, Buffa *et al.* found that the cells in small intestinal mucosa reacting with GIP antisera were the K-cells previously classified by their ultrastructural features without knowing the content of their secretory granules (Buffa *et al.*, 1975). Subsequent ultrastructural studies of human immunoreactive GIP cells indicated a characteristic appearance of the K-cell: intracellular secretory granules having a small electron-dense core surrounded by a concentric electron-lucent halo (Buchan *et al.*, 1978). In the dog, however, immunoreactive GIP cells identified in the duodenum contain uniformly electron-dense secretory granules consistent with the cell type recognized as the I-cell instead of the K-cell according to the endocrine cell classification (Usellini *et al.*, 1984). Thus, some species specificity exists with regard to the ultrastructure of the immunoreactive GIP cells. Although it was proposed that the term GIP-cell be used in place of K-cell (Buffa *et al.*, 1975), the two names are currently used interchangeably.

Interestingly, studies employing GIP antisera to immunoneutralize endogenous GIP (Ebert and Creutzfeldt, 1982; Ebert *et al.*, 1979) and intestinal extracts after removal of GIP by immunoadsorption (Ebert *et al.*, 1983) indicated that intestinal hormones other than GIP contribute substantially to the incretin effect. Subsequently, in 1983, the presence of glucagon-like peptide-1 (GLP-1) was discovered following the cloning and sequencing of mammalian proglucagon genes (Bell *et al.*, 1983; Lopez *et al.*, 1983). In 1987, the meal-induced nature of GLP-1 secretion was found in humans (Kreymann *et al.*, 1987) and its insulinotropic action was discovered in rats (Mojsov *et al.*, 1987), pigs (Holst *et al.*, 1987), and humans (Kreymann *et al.*, 1987), which eventually proved that GLP-1 is a second incretin hormone. To date, only GIP and GLP-1 fulfill the definition of the incretin hormone in humans (Baggio and Drucker, 2007; Kieffer and Habener, 1999; Kim and Egan, 2008). Here, we focus on the K-cell and GIP biology in health and disease.

II. The GIP Gene and Regulation of its Expression

A. The GIP gene and its evolutionary perspective

The human proGIP is a 153-amino acid precursor that is encoded by a 459-bp open reading frame and whose gene is localized to chromosome 17q (Inagaki *et al.*, 1989; Takeda *et al.*, 1987). It is encoded by six exons, and the majority of GIP-encoding sequences are in exon 3 (Inagaki *et al.*, 1989). The rat proGIP cDNA has a 432-bp open reading frame encoding a 144-amino acid polypeptide (Higashimoto *et al.*, 1992). There is a >90% amino acid homology of GIP among human, porcine, bovine, mouse, and rat (McIntosh *et al.*, 2009).

GIP immunoreactivity has been found by RIA in extracts of the gut of hagfish (*Myxine glutinosa*) (Falkmer *et al.*, 1980), which suggests the possibility of a functional enteroinsular axis at this evolutionary level. Phylogenetic analysis of the GIP amino acid substitutions in birds, reptiles, and mammals showed that GIP has evolved in an episodic fashion, and has accumulated a large number of amino acid substitutions on the mammalian lineage after the divergence of the platypus but before the radiation of placental mammals, a divergence which occurred about 100 million years ago (Irwin, 2009). A rapid rate of sequence evolution suggests that GIP adapted to a new role in early mammalian evolution. Within placental mammals, the sequences of GIP, a 42-amino acid peptide, are largely conserved, with most of the variation confined to the C-terminal extension between residues 30 and 42 (Irwin, 2009). This is in contrast to GLP-1 and glucagon, which are extremely well-conserved sequences between vertebrate species (Irwin, 2009).

Since GIP secretion is triggered by meal intake and GIP promotes fat storage in the adipose tissue (Kieffer, 2003; Wideman and Kieffer, 2004), the GIP gene could be considered as a "thrifty gene" defined by Neel (1962). GIP secretion during times of feast may increase to favor enhanced storage of excess nutrients as fat to facilitate survival during times of famine. Although it is not known whether mammalian GIP is functionally different from that of fish, reptiles, and birds, a rapid rate of sequence evolution in mammals after the divergence of the platypus suggests some functional benefit against selection pressure (Irwin, 2009). Interestingly, a $GIP_{1–30}$ variant exhibits equivalent potency to $GIP_{1–42}$ on beta-cell function and survival, but has a decreased capacity to increase lipoprotein lipase activity in adipocytes (Widenmaier *et al.*, 2010), which suggests that the C-terminus of GIP contributes the lipogenic action or "thrifty" function.

B. Transcriptional control of GIP gene

Since intraluminal nutrients regulate GIP secretion from K-cells, they could also play an important role in GIP gene transcription. Indeed, glucose and lipid administrations into the rat gastrointestinal tract increased GIP mRNA levels, whereas prolonged fasting decreased GIP mRNA levels (Higashimoto *et al.*, 1995; Tseng *et al.*, 1994). The precise mechanisms by which nutrients regulate GIP expression levels remain to be elucidated. Studies of the rodent GIP promoter revealed that the first 193 bp upstream of the transcription initiation site is sufficient to induce specific expression of GIP transcription through the binding of transcription factors including GATA4, Isl1, and Pdx1 (Boylan *et al.*, 1997; Jepeal *et al.*, 2003, 2005, 2008). Pdx1 is capable of binding to the second *cis*-regulatory element located between base pairs −156 and −151 of the murine GIP promoter, and overexpression of Pdx1 leads to a specific increase in the activity of GIP/Luc reporter constructs (Jepeal *et al.*, 2005). The first intron of human GIP gene contains essential *cis*-acting elements for its cell-specific expression (Hoo *et al.*, 2010). The activity of the human GIP promoter is regulated by Pdx1 along with Pax6 (Fujita *et al.*, 2008). Moreover, the human GIP promoter contains a TATA motif, consensus Sp1, Ap-1, and Ap-2 sites, as well as two CRE elements (Inagaki *et al.*, 1989; Someya *et al.*, 1993), thus suggesting regulation by protein kinase A (PKA) and protein kinase C (PKC) (McIntosh *et al.*, 2009).

C. Posttranslational processing of proGIP

As is the case in other peptide hormones, posttranslational processing is required to produce GIP. The predicted amino acid sequence for both the rat and human GIP cDNAs indicates that GIP is derived from a larger prohormone precursor that encodes a signal peptide, an N-terminal peptide, GIP, and a C-terminal peptide (Baggio and Drucker, 2007). Studies using specific prohormone convertase (PC) knockout mice or PC overexpressing cell lines revealed that the mature 42-amino acid bioactive form of GIP ($GIP_{1–42}$), which is encoded by exons 3 and 4 (Inagaki *et al.*, 1989), is released from its 153-amino acid proGIP precursor via PC1/3-dependent posttranslational cleavage at flanking single arginine residues (Ugleholdt *et al.*, 2006). Moreover, PC1/3 colocalizes with GIP in K-cells (Ugleholdt *et al.*, 2006). The peptides encoded within the GIP N- or C-terminal sequences have no known biological function (Baggio and Drucker, 2007). In pancreatic alpha-cells, proGIP is processed to yield $GIP_{1–31}$ by PC2 and subsequently likely converted to $GIP_{1–30NH2}$ by peptidyl-glycine alpha-amidating monooxygenase (Fujita *et al.*, 2010b). In addition, 5–15% of K-cells in the small intestine express PC2 and produce $GIP_{1–30NH2}$ (Fujita *et al.*, 2010a). It is known that $GIP_{1–30NH2}$ has equipotent insulinotropic

action with $GIP_{1–42}$ (Fujita *et al.*, 2010b), whereas a $GIP_{1–30}$ variant has a decreased capacity to increase lipoprotein lipase activity in adipocytes (Widenmaier *et al.*, 2010). Further studies are warranted to explore the potential divergent actions of GIP isoforms.

III. Anatomical Localization and Development of K-cells

A. Anatomical localization of GIP-producing cells

The surface of the intestine consists of villus and crypt structures. The villus is a finger-like projection protruding into the lumen and the crypt is an invaginated structure at the base of the villi. Gut stem cells reside in crypts and are differentiated into absorptive enterocytes, mucin-producing goblet cells, and enteroendocrine cells, as they migrate upward, and Paneth cells, as they migrate downward to the crypt base. With the exception of the Paneth cells, the epithelial cells lining the villi turn over every 3–5 days by exfoliating from the tips of the villi (Barker *et al.*, 2008).

In accordance with the roles of GIP as an incretin, immunoreactive GIP cells have been located in the upper small intestine of humans, pigs, dogs (Buffa *et al.*, 1975), ruminants (Bunnett and Harrison, 1986), and rats (Buchan *et al.*, 1982). In the gastrointestinal tract of dog and man, immunoreactive GIP is present in cells predominantly in the duodenum and, to a lesser extent, in the jejunum (Polak and Bloom, 1982). The K-cell density in human duodenal mucosa has been estimated at 13 per 1000 cells (Theodorakis *et al.*, 2006). Other studies have found a few immunoreactive GIP cells as far as the terminal ileum in rats (Buchan *et al.*, 1982) and humans (Ferri *et al.*, 1983), and GIP transcripts in the ileal mucosa of rats (Tseng *et al.*, 1993). Release of immunoreactive GIP by selective small bowel perfusion of human subjects using an occluding balloon perfusion technique has confirmed the histological localization of the GIP cells. The integrated immunoreactive GIP levels (ng min^{-1} ml^{-1}) were: duodenum, 111 ± 21; proximal jejunum, 69 ± 5; midjejunum, 47 ± 7; and ileum, 25 ± 6 (Thomas *et al.*, 1977).

It was traditionally thought that the incretin hormones, GIP and GLP-1, are produced in separate endocrine cells located predominantly in the proximal and distal gut, respectively. However, GIP and GLP-1 are sometimes colocalized, apparently with a frequency as high as 55–75% in the mid-small intestine (Mortensen *et al.*, 2003). Coexpression of GIP and GLP-1 is regulated by unique transcriptional control. For example, cells expressing GLP-1 contain the transcription factor Pax6, while coexpression of GIP requires the additional transcription factor Pdx1 (Fujita *et al.*, 2008). Cells coexpressing GIP and GLP-1 are typically referred to as K/L-cells or

L/K-cells for convenience (Fujita *et al.*, 2008; Theodorakis *et al.*, 2006). In support of coproduction of GIP and GLP-1, transgenic mice expressing a rat GIP promoter/attenuated diphtheria toxin A chain transgene (GIP/DT) tended to have decreased expression of proglucagon gene in the small bowel and decreased plasma GLP-1 levels in response to an oral glucose load (Althage *et al.*, 2008).

In addition to GLP-1, a subset of K-cells express xenin, a 25-amino acid peptide hormone (Anlauf *et al.*, 2000). Xenin is a cleavage product derived from proxenin, which was revealed to be identical with the ubiquitously expressed COPA (coatomer protein complex, subunit alpha) (Hamscher *et al.*, 1996). Although supraphysiological concentrations of xenin increased glucose-stimulated insulin release in perfused rat pancreas (Silvestre *et al.*, 2003), xenin alone has no impact on glucose homeostasis in wild-type and GIP/DT mice (Wice *et al.*, 2010). In contrast to GIP and GLP-1, xenin is also released in response to sham feeding or during the cephalic phase of feeding (Feurle *et al.*, 2003), which does not meet the classic definition of an incretin by Creutzfeldt (1979). In a recent study, however, xenin was reported to potentiate GIP action in GIP/DT mice by increasing acetylcholine receptor signaling in pancreatic beta-cells (Wice *et al.*, 2010). Based on these observations, xenin might be useful to enhance the insulinotropic action of GIP in diabetes, where GIP resistance is commonly found (Lynn *et al.*, 2001; Nauck *et al.*, 1993a). It also remains possible that K-cells produce additional products with glucose-lowering activity, since GIP/DT mice revealed a near absence of insulin response 15 min after an oral glucose load (Althage *et al.*, 2008), while GIP receptor (GIPR) knockout ($GIPR^{-/-}$) mice have a reduced, but still present insulin response to oral glucose (Hansotia *et al.*, 2007; Miyawaki *et al.*, 1999; Preitner *et al.*, 2004).

In the Burmese python (*Python molurus*), the pancreas—rather than the intestinal K-cells—is the primary source of circulating GIP (Secor *et al.*, 2001). In mammals including mice and humans, GIP protein is also localized to islet alpha-cells, although unlike in the snake, mammalian pancreatic GIP likely contributes little to the total GIP found in the circulation (Fujita *et al.*, 2010b). GIP produced by pancreatic alpha-cells appears to be in the form of $GIP_{1–30NH2}$ instead of $GIP_{1–42}$ and is speculated to have a paracrine effect modulating islet function and development (Fujita *et al.*, 2010b).

Interestingly, expression of the GIP gene has been detected in other tissues, including the salivary gland, eye, and brain. RNA analysis, radioimmunoassay, and *in situ* hybridization localized GIP expression in ductal cells of rat salivary gland (Tseng *et al.*, 1993). Furthermore, an increase in expression of the GIP gene was demonstrated in the submandibular gland following oral glucose ingestion (Tseng *et al.*, 1995). GIP secretion into saliva was also demonstrated in humans, with its concentration in saliva reduced after feeding (Messenger *et al.*, 2003). In rats, GIP is reported to be

present in lens epithelial cells in the eyes (Nakajima *et al.*, 2002) and in a subpopulation of adult hippocampal cells, where it may have a role in proliferation of neural progenitor cell turnover (Nyberg *et al.*, 2005). Otherwise, RNA blot analysis has not revealed the presence of preproGIP mRNA in any other tissues (Inagaki *et al.*, 1989; Sharma *et al.*, 1992; Takeda *et al.*, 1987; Tseng *et al.*, 1993).

B. Development of K-cells in the gut

Immunoreactive GIP cells in the intestine are observed in the 14th week of gestation in humans, initially appearing in the duodenal region and distally with increased gestational age (Bryant *et al.*, 1982). An ontogenic study in rats reported that GIP mRNA is detected as early as day 20 of embryonic development and very low levels remain until postnatal day 3, at which time there is a rapid increase in GIP mRNA levels up to postnatal day 5, followed by a gradual increase toward adult levels (Higashimoto and Liddle, 1994). These observations suggest that GIP may play an important role in early postnatal development probably associated with suckling, but the precise role of developmental expression of GIP remains to be determined.

The development of the enteroendocrine system has not been extensively studied, but there is a growing body of knowledge through studies adopting genetic manipulation in mice (Lee and Kaestner, 2004; Rindi *et al.*, 2004; Schonhoff *et al.*, 2004). Conditional ablation of secretin cells in mice showed nearly complete ablation of enteroendocrine cells expressing cholecystokinin (CCK) and protein YY (PYY)/proglucagon (L-cells) as well as secretin cells, suggesting a close developmental relationship between these three cell types (Rindi *et al.*, 1999). In addition, the number of enteroendocrine cells producing GIP, substance-P, somatostatin, and serotonin were reduced in these mice. These results suggest the existence of multipotent endocrine progenitor cells. In contrast, Hes-1 knockout mice revealed excessive differentiation of multiple endocrine cell types in the developing stomach and gut, including cells expressing GIP, proglucagon, CCK, gastrin, and somatostatin (Jensen *et al.*, 2000). Therefore, Hes-1 plays a key role in enteroendocrine cell differentiation as a general negative regulator.

Specific transcription factor inactivation studies in mice have identified Math1, Ngn3, Pax4, Pax6, and Pdx1 as crucial factors for the differentiation of intestinal endocrine cells, including K-cells. Math1 is a key factor in the differentiation of intestinal secretory lineages as $Math1^{-/-}$ mice are lacking three of the four gastrointestinal epithelial cell types—goblet, Paneth, and enteroendocrine cells (Yang *et al.*, 2001). The dependence on Math1 expression distinguishes enteroendocrine differentiation from the pancreas, since islet differentiation is normal in $Math1^{-/-}$ mice. $Ngn3^{-/-}$ mice fail to develop any endocrine cells in the small intestine as well as pancreatic

endocrine cells (Gradwohl *et al.*, 2000; Jenny *et al.*, 2002; Lee *et al.*, 2002). However, in the glandular stomach serotonin cells, enterochromaffin-like cells and ghrelin-expressing cells are unaffected in the absence of Ngn3 in mice, suggesting that the differentiation of these lineages is not regulated by Ngn3 (Lee *et al.*, 2002). Interestingly, intestinal specific knockout of Ngn3 in mice to eliminate all enteroendocrine cells reduced postnatal survival, impaired lipid absorption, reduced weight gain, and improved glucose homeostasis. (Mellitzer *et al.*, 2010). Deletion of the Pax4 gene virtually eliminates duodenal and jejunal hormone-secreting cells, as well as serotonin and somatostatin cells of the distal stomach, while deletion of the Pax6 gene eliminates duodenal GIP cells as well as gastrin and somatostatin cells of the distal stomach (Larsson *et al.*, 1998). In $Pdx1^{-/-}$ mice, there is a 98% reduction in the number of GIP-expressing cells and no change in the number of serotonin-positive cells (Jepeal *et al.*, 2005). Severe reductions in the numbers of other endocrine cells in $Pdx1^{-/-}$ newborn mice have been noted, including gastrin-producing cells (Larsson *et al.*, 1996; Offield *et al.*, 1996).

IV. Secretion, Degradation, and Elimination of GIP

A. Regulation of GIP secretion

The K-cell is an open type endocrine cell with an obvious connection to the gut lumen (Buchan, 1999). Therefore, it appears by design to have GIP secretion mainly controlled by luminal contents of the gut (Baggio and Drucker, 2007; Kim and Egan, 2008; Krarup, 1988; McIntosh *et al.*, 2009). The K-cell is highly polarized with the hormone containing secretory granules concentrated at the basal pole of the cell, ready to be released from secretory granules through the basolateral membrane (Buchan *et al.*, 1978; Sykes *et al.*, 1980). In addition, K-cells are found in close association with the capillary network running through the lamina propria. The receptors for neural modulators are believed to be located on the basolateral membrane, and nerve fibers are found in close spatial proximity to the cells (Buchan, 1999). Based on these morphological features, GIP secretion from K-cells is regulated by intraluminal contents, neural stimuli, and hormones. A schematic representation on the morphology and GIP secretion machinery of the K-cell is shown in Fig. 4.1.

1. Nutritional stimuli

As introduced above, GIP secretion is chiefly regulated by intraluminal nutrients. Intravenous glucose administration does not increase plasma GIP levels, but plasma GIP levels markedly increase in response to orally

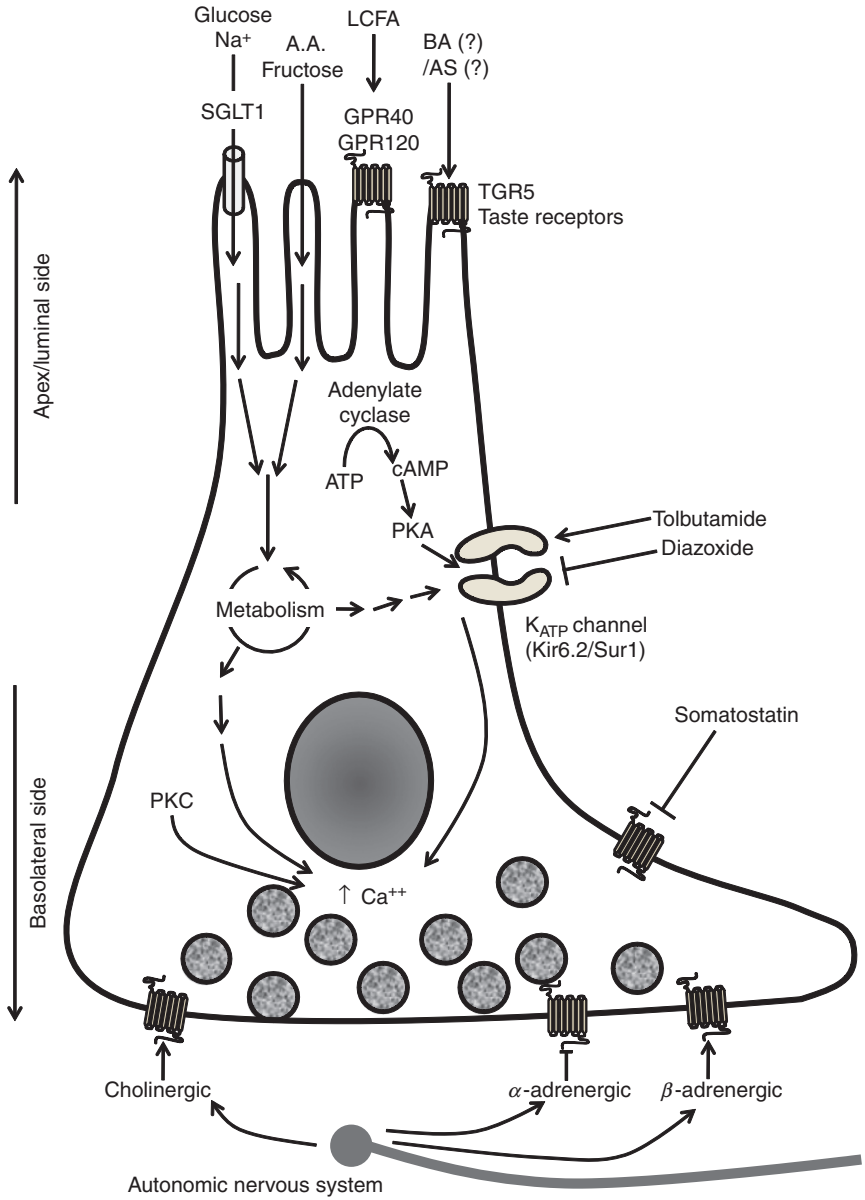

Figure 4.1 A schematic representation of the GIP secretion machinery of the K-cell. See text for details. SGLT1, sodium-dependent glucose transporter 1; A.A., amino acids; LCFA, long-chain fatty acids; AS, artificial sweeteners; PKA, protein kinase A; PKC, protein kinase C; BA, bile acids. TGR5 is a G-protein coupled receptor and also known as GPR131.

administered glucose (Andersen *et al.*, 1978). Likewise intraduodenal fat or protein administration increases GIP secretion, whereas intravenous infusion of these nutrients does not (Konturek *et al.*, 1986; Varner *et al.*, 1980). The circulating levels of GIP increase several fold shortly after ingestion of a meal containing fat (Brown, 1974; Cleator and Gourlay, 1975; Ross and Dupre, 1978) or glucose (Kuzio *et al.*, 1974; Morgan, 1979; Pederson *et al.*, 1975), usually within 10–20 min. It appears glucose may act directly at the level of the GIP-secreting K-cells to stimulate GIP release (Kieffer *et al.*, 1994, 1995a). In primary intestinal crypt cultures, GIP release was stimulated by glucose, glutamine, and linoleic acid (Parker *et al.*, 2009). Oral or intraduodenal infusion of glucose and/or fat increases GIP secretion in a dose-dependent fashion (Schirra *et al.*, 1996). More specifically, it is the rate of nutrient absorption rather than the mere presence of nutrients in the intestine that stimulates GIP release, since GIP secretion is reduced in individuals with intestinal malabsorption syndrome or after the administration of pharmacologic agents that reduce nutrient absorption (Besterman *et al.*, 1979; Fushiki *et al.*, 1992).

Studies on isolated perfused rodent intestine revealed that carbohydrate detection by K-cells involves the sodium-dependent sugar uptake pathway through the intestinal Na^+-coupled glucose transporters, particularly sodium-dependent glucose transporter 1 (SGLT1) (Flatt *et al.*, 1989; Sykes *et al.*, 1980). The SGLT inhibitor phloridzin impairs glucose-stimulated GIP secretion in rodent intestine (Sykes *et al.*, 1980) and GIP secretion is increased by alpha-methylglucopyranoside (αMG), a substrate of SGLT1 (Parker *et al.*, 2009). Furthermore, fluorescent cells purified by flow cytometry from the intestine of transgenic mice expressing the yellow fluorescent protein Venus under the control of the GIP promoter (GIP/Venus mice) have high levels of mRNA for SGLT1 compared to adjacent fluorescent negative cells (Parker *et al.*, 2009). However, the fact that the potency of αMG in eliciting GIP secretion is one third of glucose indicates that further metabolism of glucose in the K-cell is essential in GIP secretion (Parker *et al.*, 2009).

As enteroendocrine cells are likely the first line of integration of information from the gut lumen, it has been postulated that they might be equipped with chemoreceptors like taste receptors in the tongue. Indeed, α-subunit of gustducin (α-gustducin), a key G-protein involved in taste sensing, is expressed throughout the surface epithelium of the gut (Hofer *et al.*, 1996). Interestingly, α-gustducin knockout mice are characterized by deficiencies in secretion of GIP as well as GLP-1, accompanied by decreased insulin responses and impaired glucose tolerance (Margolskee *et al.*, 2007). In addition, sucralose, an artificial sweetener, was reported to induce GIP release from murine gut endocrine GLUTag cells (Margolskee *et al.*, 2007). Furthermore, some K-cells contain sweet receptors, and their activation by sugars and sweeteners was reported to induce GIP secretion (Egan and

Margolskee, 2008; Jang *et al.*, 2007). However, Fujita *et al.* found that oral gavage of artificial sweeteners did not increase GIP or GLP-1 levels, or reduce the blood glucose excursion to a subsequent intraperitoneal glucose tolerance test (Fujita *et al.*, 2009). They also found that the immunoreactivity of α-gustducin is colocalized with only ~15% of total GIP-expressing cells in rat duodenum. Consistent with the findings by Fujita *et al.*, it has been reported that the gene expression levels of sweet taste receptor subunits (TAS1R1, TAS1R2, and TAS1R3) and GNAT3 (encoding α-gustducin) are very low in K-cells and not different compared with non-K-cells in the upper small intestine of GIP/Venus mice (Parker *et al.*, 2009). Furthermore, in primary intestinal crypt cultures, GIP release was unaffected by the artificial sweetener sucralose, whereas glucose-triggered GIP secretion was SGLT1-dependent and modulated by K_{ATP} channel activity (Parker *et al.*, 2009). In a human study with type 2 diabetic patients, there was no effect of acute oral stevioside, an artificial sweetener marketed as Stevia®, on release of GIP and GLP-1 (Gregersen *et al.*, 2004). Therefore, the results are conflicting and further studies are required to evaluate whether the low expression levels of taste receptor components play a role in GIP (and GLP-1) secretion *in vivo*.

Recently, it was suggested that $G_{\alpha q}$-coupled lipid receptors, GPR40 and GPR120, as well as $G_{\alpha s}$-coupled lipid receptor, GPR119, play an important role in regulating incretin hormone release (Chu *et al.*, 2008; Hirasawa *et al.*, 2005; Overton *et al.*, 2006). In GIP/Venus mice, K-cells express higher levels of mRNAs for GPR40, GPR120, and GPR119 (Parker *et al.*, 2009). These data raise the possibility that these and/or related receptors might provide novel targets for promoting the endogenous release of incretin hormones.

2. Neural regulation of GIP secretion

It was reported that GIP responses to oral glucose are exaggerated in patients following a vagotomy and pyloroplasty (Thomford *et al.*, 1974), suggesting the possibility that GIP secretion might be regulated by the autonomic nervous system. Administration of atropine was reported to reduce the immediate postabsorptive GIP secretion in humans (Ahren and Holst, 2001). In healthy humans intravenously infused with various adrenergic modulators (isoproterenol, a beta-agonist; propranolol, a beta-blocker; epinephrine, a nonselective alpha- and beta-agonist; phentolamine, an alpha-blocker), the sympathetic nervous system was found to exert a differential effect on GIP secretion, increasing plasma GIP concentrations after beta-adrenergic stimulation of K-cells (Flaten *et al.*, 1982) and decreasing GIP levels in response to alpha-adrenergic activation (Salera *et al.*, 1982a). However, caution should be exercised in interpreting the results of all these studies, as the methods used may indirectly impact GIP secretion through effects on gastric emptying (McIntosh *et al.*, 2009).

3. Hormonal regulation

There are some lines of evidence suggesting that GIP secretion may be under hormonal input. It appears that somatostatin reduces GIP and GLP-1 secretion in a paracrine manner. Somatostatin-containing D-cells are located throughout the small intestine (Damholt *et al.*, 1999) and are found in close proximity to both K- and L-cells, and somatostatin inhibits GIP secretion *in vitro* (Kieffer *et al.*, 1995a) and *in vivo* (Ho *et al.*, 1987; Martin and Faulkner, 1996; Salera *et al.*, 1982c). Insulin infusion reduced GIP secretion stimulated by intraduodenal glucose in humans (Sirinek *et al.*, 1978) or oral gavage in rats (Bryer-Ash *et al.*, 1994), and glucagon infusion reduced GIP responses to carbohydrate (Ranganath *et al.*, 1999). Interestingly, intravenous infusion of C-peptide also reduced GIP release in response to fat (Dryburgh *et al.*, 1980), which suggests a metabolic function of C-peptide. Further evidence for possible feedback inhibition can be found from studies on dipeptidyl peptidase 4 (DPP4) inhibitor administration to dogs (Deacon *et al.*, 2002), normal human subjects (El-Ouaghlidi *et al.*, 2007), and type 2 diabetes patients (Herman *et al.*, 2006), where reductions in total GIP and GLP-1 levels occurred while active GIP and GLP-1 increased. However, related to the effects of DPP4 inhibitors, it is not clear whether incretin hormones directly inhibit their respective enteroendocrine cells, or whether other mediators such as insulin or neural pathways may be involved (McIntosh *et al.*, 2009).

4. Intracellular mechanisms of GIP secretion from K-cells

The intracellular mechanisms controlling GIP secretion from K-cells are poorly understood, but may have some similarities with that of pancreatic beta-cells (Baggio and Drucker, 2007; Kim and Egan, 2008; McIntosh *et al.*, 2009). Like beta-cells, K-cells express the glucose sensor glucokinase (Cheung *et al.*, 2000; Parker *et al.*, 2009) and purified K-cells also express high levels of mRNA for subunits of K_{ATP} channels (Kir6.2 and Sur1 genes) (Parker *et al.*, 2009). In cultured canine endocrine cells, GIP secretion was stimulated by glucose, potassium depolarization, a calcium ionophore, forskolin (an adenylyl cyclase activator to increase cAMP levels), and gastrin-releasing peptide (GRP) (Kieffer *et al.*, 1994). In a recent study using primary intestinal crypt cultures, GIP release was potentiated by forskolin plus 3-isobutyl-1-methylxanthine (IBMX), which inhibits phosphodiesterase to increase cAMP levels, and the presence of functional K_{ATP} channels in K-cells was demonstrated by the findings that GIP release was triggered by tolbutamide, a potassium channel blocker, under basal conditions and attenuated by diazoxide, a potassium channel activator, in the presence of forskolin/IBMX (Parker *et al.*, 2009). In that study, glucose (10 mmol/l) triggered GIP secretion 1.3-fold under basal conditions, but threefold in the presence of forskolin/IBMX, indicating a synergistic interaction between

these two stimulatory signals. Since tolbutamide responsiveness was lost but diazoxide still inhibited GIP secretion in the presence of forskolin/IBMX, it appears that elevated cAMP brings about the closure of K_{ATP} channels in K-cells (Parker *et al.*, 2009). In addition, a PKC activator, phorbol 12-myristate 13-acetate (PMA), increased GIP secretion from cultured intestinal crypt cells (Parker *et al.*, 2009). Recently, TGR5, a G-protein coupled receptor (GPCR), was reported to be present in L-cells and play an important role in regulating intestinal GLP-1 secretion in response to bile acid (Reimann *et al.*, 2008; Thomas *et al.*, 2009). Likewise, TGR5 expression can be detected in flow cytometry sorted K-cells with expression levels similar to the ones reported for the L-cells (Dr. F. Reimann, personal communication). Data from recent studies suggest that harnessing the pathways coupled to adenylyl cyclase, PKC, and TGR5 might provide novel strategies to modulate GIP secretion *in vivo*.

B. Degradation and elimination of GIP

Once released, GIP is rapidly subjected to degradation by DPP4, which is bound to endothelial cells of blood vessels of gut and liver and lymphocytes (where it is designated as CD-26) or present as a soluble form in the circulation (Baggio and Drucker, 2007; Kim and Egan, 2008; McIntosh *et al.*, 2009). The half-life of intact biologically active GIP is less than 2 min in rodents (Kieffer *et al.*, 1995b), and approximately 5 and 7 min in type 2 diabetes patients or healthy subjects, respectively (Deacon *et al.*, 2000). The first two amino acids (Tyr and Ala) at the amino terminus of GIP are cleaved to produce N-terminally truncated GIP, which functions as a weak antagonist of the GIPR *in vitro* and in rodents at a pharmacologic concentration but does not antagonize the insulinotropic effects of GIP *in vivo* at a physiological concentration (Deacon *et al.*, 2000; Kieffer *et al.*, 1995b). Studies with rodents and both healthy and diabetic humans indicate that DPP4 is the primary enzyme responsible for inactivating GIP *in vivo* (Deacon *et al.*, 2000; Kieffer *et al.*, 1995b). Interestingly, DPP4 activity is 20-fold lower in intestinal lymphatic fluid than that of portal venous blood (D'Alessio *et al.*, 2007). In this regard, along with DPP4 inhibitor treatment or DPP4-deficient rat models (Deacon *et al.*, 2001; Kieffer *et al.*, 1995b), the measurement of intestinal lymphatic GIP levels might provide a valuable tool to examine the *in vivo* secretion kinetics and/or local action of GIP (Lu *et al.*, 2008).

In terms of plasma GIP elimination, the measurement of arteriovenous differences in GIP levels across various organ beds in pigs has identified the kidney as the major site of GIP elimination, and the liver and extremities also contribute to GIP extraction (Deacon *et al.*, 2001). It was shown that $GIP_{1–42}$ levels are not different between patients with chronic renal insufficiency and normal subjects (Meier *et al.*, 2004b). However, the concentration of the degradation product, $GIP_{3–42}$, is higher in patients with renal

insufficiency, which indicates that the kidney plays an important role in final elimination of GIP degradation products (Deacon, 2004). Since the elimination rates of $GIP_{1–42}$ and $GIP_{3–42}$ are similar in patients with type 2 diabetes and healthy subjects (Vilsboll *et al.*, 2006), it is unlikely that more rapid degradation/elimination of GIP is a factor contributing to the pathogenesis of type 2 diabetes.

V. Biological Actions of GIP

A. GIP receptor (GIPR)

The human GIPR gene comprises 14 exons that span approximately 13.8 kb (Yamada *et al.*, 1995) and is localized to chromosome 19q13.3 (Gremlich *et al.*, 1995), whereas the rat GIPR gene spans approximately 10.2 kb and contains 13 exons (Boylan *et al.*, 1999). Similar to the GLP-1R, the GIPR belongs to the 7-transmembrane-spanning, heterotrimeric GPCR superfamily (Usdin *et al.*, 1993). The GIPR gene is expressed in the pancreas, stomach, small intestine, adipose tissue, adrenal cortex, pituitary, heart, testis, endothelial cells, bone, trachea, spleen, thymus, lung, kidney, thyroid, and several regions in the central nervous system (CNS) (Baggio and Drucker, 2007; Kim and Egan, 2008; McIntosh *et al.*, 2009). In humans, the GIPR appears to be most highly expressed in pancreatic beta-cells, consistent with the primary role of GIP as an incretin hormone (Saxena *et al.*, 2010).

B. GIP actions on the pancreatic islets

The principle physiological role of GIP is to increase insulin secretion from the pancreatic beta-cells in response to intestinally absorbed nutrients. GIP stimulation of insulin secretion is mediated by activation of both cAMP/PKA and cAMP/Epac2, in addition to phospholipase A2 and specific protein kinase signaling pathways (Ehses *et al.*, 2001, 2002; Kashima *et al.*, 2001). GIP also upregulates insulin gene transcription and biosynthesis, as well as the expression of components of beta-cell glucose sensors (Wang *et al.*, 1996). The physiological importance of GIP as an incretin hormone has been demonstrated in rodent studies eliminating GIPR signaling by peptide antagonists, antisera, and targeted genetic inactivation, where impaired oral glucose tolerance and defective glucose-stimulated insulin secretion were observed (Baggio *et al.*, 2000; Gelling *et al.*, 1997; Lewis *et al.*, 2000; Miyawaki *et al.*, 1999; Tseng *et al.*, 1996). In a recent human genome-wide association study (Saxena *et al.*, 2010), an intronic single nucleotide polymorphism in the GIPR (rs10423928), in strong linkage disequilibrium ($r^2 = 0.93$) with a missense mutation in GIPR (E354Q), was found to be associated with elevated plasma glucose levels

2 h after oral glucose challenge, decreased insulin secretion, and a diminished incretin effect. All of these findings are consistent with the established role of GIP as an incretin hormone.

GIP stimulates cell proliferation and improves survival of pancreatic beta-cells in various conditions such as treatment with wortmannin, streptozotocin, glucolipotoxicity, or serum or glucose deprivation. The molecular signaling pathways that mediate the proliferative and antiapoptotic actions of GIP include activation of cAMP/PKA, PKA/CREB, MAPK, PI-3K-dependent activation of Akt/PKB, nuclear exclusion of FoxO1, reductions in caspase 3 activity, downregulation of Bax gene transcription, upregulation of the antiapoptotic Bcl-2 gene, and reducing endoplasmic reticulum (ER) stress (extensively reviewed in Baggio and Drucker, 2007; Kim and Egan, 2008; McIntosh *et al.*, 2009). Pdx1 and cyclin D1 are known to play a key role in GIP-induced beta-cell proliferation (Friedrichsen *et al.*, 2006). Although GIP clearly can influence proliferation and apoptosis of beta-cells, no markedly abnormal islet morphology was found in GIPR$^{-/-}$ mice, double incretin receptor knockout mice as well as GLP-1R$^{-/-}$ mice, which indicates that GIP (and GLP-1) is not essential in pancreatic beta-cell development (Pamir *et al.*, 2003; Preitner *et al.*, 2004).

The actions of GIP on pancreatic beta-cells are generally analogous to those of GLP-1. However, at least two notable differences have been reported. First, GLP-1 but not GIP can induce insulin secretion in Kir6.2$^{-/-}$ mice (Miki *et al.*, 2005). Second, a recent study revealed that GLP-1R signaling by exendin-4 exerts more robust control of beta-cell survival relative to GIPR activation by D-Ala^2-GIP (Maida *et al.*, 2009). Furthermore, GIP has an opposite action from GLP-1 on pancreatic alpha-cell function. GIP has been shown to increase glucagon secretion from the isolated perfused rat pancreas at glucose concentrations less than 5.5 mM, while it increases insulin secretion at glucose levels greater than 5.5 mM (Pederson and Brown, 1978). In addition, at supraphysiological concentrations, GIP increases glucagon secretion in human subjects with type 2 diabetes, which offsets its insulinotropic effect (Chia *et al.*, 2009). This is in contrast to GLP-1, which decreases glucagon secretion in perfused pancreas and patients with type 1 or type 2 diabetes at or above normal fasting glucose levels (Creutzfeldt *et al.*, 1996; Holst, 2007; Nauck *et al.*, 1993b). Therefore, these divergent actions of GIP and GLP-1 on pancreatic islets may have implications in developing strategies to treat diabetes by fine-tuning islet function.

C. Extrapancreatic actions of GIP

GIP was originally named gastric inhibitory polypeptide for its inhibition of gastric acid secretion (Brown, 1971; Brown and Dryburgh, 1971). However, its inhibitory action on gastric acid secretion is only seen at pharmacological concentrations in dogs and humans (Nauck *et al.*, 1992; Wolfe

et al., 1983). At supraphysiological concentrations, GIP significantly decreases the interval of migrating myoelectric complexes (commonly dubbed MMC) in dogs (Thor *et al.*, 1987), but it does not slow the gastric emptying rate in healthy human subjects (Meier *et al.*, 2004a),

Functional GIPRs are expressed on isolated rat adipocytes and 3T3-L1 cells (Yip *et al.*, 1998), indicative of an additional physiological role of GIP beyond a simple incretin action. Effects of GIP on lipid metabolism include increasing plasma triglyceride clearance following meals (Wasada *et al.*, 1981), increasing lipoprotein lipase activity (Eckel *et al.*, 1979), and promoting fat storage by adipocytes (Miyawaki *et al.*, 2002). Interestingly, interrupting GIP signaling appears to be beneficial in resisting high-fat diet-induced obesity and insulin resistance in GIPR$^{-/-}$ mice (Miyawaki *et al.*, 2002), GIP/DT mice lacking GIP-producing cells (Althage *et al.*, 2008), rodents treated with Pro^3-GIP, a purported peptide-based GIPR antagonist (Gault *et al.*, 2005; Irwin *et al.*, 2007), and vaccination against GIP (Fulurija *et al.*, 2008). Although fat ingestion is a potent stimulator of GIP secretion in humans and GIP plasma levels are increased in some obese individuals (Creutzfeldt *et al.*, 1978; Salera *et al.*, 1982b), it is unknown whether increased GIP signaling causally contributes to human obesity.

Given the broad tissue distribution of GIPR, it is not surprising that GIP actions have been reported on several tissues other than stomach, islets, and adipocytes. In the CNS, GIP may play a role in neural progenitor cell proliferation and behavior modification (Nyberg *et al.*, 2005). Functional GIPR is present on bone cells, and GIP increases bone mineral density in ovariectomized rats (Bollag *et al.*, 2001). However, acute administration of GIP does not alter markers of bone turnover in humans (Henriksen *et al.*, 2003), although it is unknown whether more long-term GIP treatment affects bone turnover. Abnormal expression of the GIPR in adrenocortical adenomas is known to be associated with the development of food-dependent Cushing's syndrome (Lacroix *et al.*, 1992). The GIPR is present in the vascular endothelium and GIP increases intracellular Ca^{2+} levels in endothelial cell cultures (Zhong *et al.*, 2000). Although GIPR mRNA is also detected in the heart, testis, lung, and several other tissues in rodents, the physiological actions of GIP in these tissues are still elusive. Furthermore, it is unclear whether the extrapancreatic effects of GIP referred to above are relevant in humans.

VI. GIP and K-cells in Health and Disease

A. K-cells and GIP in obesity/diabetes

Consistent with the known adipogenic effect of GIP (Kieffer, 2003; Wideman and Kieffer, 2004), there are some changes in K-cell density and/or GIP content in the small intestine of obese animals. Long-term

administration of a high-fat diet increases intestinal GIP mRNA and peptide levels in rats (Tseng *et al.*, 1994), as well as the circulating amount of plasma GIP in mice (Miyawaki *et al.*, 2002). The K-cell number and/or GIP content in the small intestine are increased in obese *ob/ob* mice compared with lean control mice (Flatt *et al.*, 1983; Polak *et al.*, 1975). Furthermore, chronic high-fat diet feeding increased the density of K-cells in the upper jejunum in *ob/ob* mice by 54% compared with control diet (Bailey *et al.*, 1986). The increased K-cell density and circulating GIP levels found in obese animals and high-fat fed animals might be the result of chronically increased stimuli from the gut lumen. Assuming that GIP is shown to contribute to obesity in humans, measures to prevent K-cell hyperplasia or reduce GIP production may be a new therapeutic option to treat obesity.

In patients with type 2 diabetes, there is typically a defective incretin response—insulin secretion by oral glucose is no longer substantially greater than the response to intravenous glucose (Nauck *et al.*, 1986). However, defective GIP secretion does not appear to play a causal role in this defect, since most human studies have shown that the secretion of GIP is normal or even higher in patients with type 2 diabetes compared with healthy control subjects (Crockett *et al.*, 1976; Ebert and Creutzfeldt, 1980; Jones *et al.*, 1989; Nauck *et al.*, 1986; Ross *et al.*, 1977; Vilsboll *et al.*, 2001). However, in some studies, the insulinotropic effect of GIP in patients with type 2 diabetes is only 10–20% of the normal response, while that of GLP-1 still remains intact (Krarup *et al.*, 1987; Meier *et al.*, 2001; Nauck *et al.*, 1986, 1993a). Interestingly, the GIP effects in first-degree relatives of the patients with type 2 diabetes were reported to be diminished compared with control subjects (Meier *et al.*, 2001), suggesting that genetic defects in the GIP signaling pathway might confer a risk for type 2 diabetes. Indeed, a recent genome-wide association study in humans revealed that a single nucleotide polymorphism in GIPR is associated with increased plasma glucose levels 2 h after oral glucose challenge, decreased insulin secretion, and a diminished incretin effect (Saxena *et al.*, 2010). In addition, a variant in TCF7L2, an established type 2 diabetes gene with allele frequency ranging from 0.03 to 0.3 depending on populations (Ng *et al.*, 2008; Staiger *et al.*, 2009), is reported to contribute to decreased insulinotropic effects of GIP and GLP-1 (Lyssenko *et al.*, 2007; Pilgaard *et al.*, 2009; Schafer *et al.*, 2007; Shu *et al.*, 2009; Villareal *et al.*, 2010). In a recent study, decreased GIPR and GLP-1R expression was observed in islets from patients with type 2 diabetes and human islets treated with siRNA to TCF7L2 (Shu *et al.*, 2009). The compromised insulinotropic effect of GIP in some subjects with type 2 diabetes might also in part be due to receptor desensitization, as has been described in Vancouver diabetic fatty (VDF) Zucker rats in association with decreased mRNA expression of GIPR in pancreatic islets (Lynn *et al.*, 2001, 2003). The decreased GIPR expression in pancreatic islets might be the result of hyperglycemia, since it can be induced by hyperglycemia (Xu *et al.*, 2007) and reversed by alleviating

hyperglycemia (Piteau *et al.*, 2007). Taken together, the GIP resistance found in some patients with type 2 diabetes can be explained by both genetic and environmental (e.g., hyperglycemia) factors.

B. GIP and K-cells after bariatric surgery

Certain types of bariatric surgeries performed in obese patients with type 2 diabetes dramatically reverse hyperglycemia without the need of antidiabetic medications. A meta-analysis showed that diabetes was completely resolved in a considerable number of patients following bariatric surgery, where gastric banding, gastric bypass, and biliopancreatic diversion induced diabetes remission in 47.9%, 83.7%, and 98.9%, respectively (Buchwald *et al.*, 2004). The diabetes remission after gastric bypass is often observed within days after operation, even before significant weight loss occurs, whereas it takes several months with restrictive surgery (e.g., gastric banding) (Dixon *et al.*, 2008; Pories *et al.*, 1995; Schauer *et al.*, 2003). Therefore, the mechanisms of diabetes remission after gastric bypass and biliopancreatic diversion are considered to be independent of weight loss or calorie restriction (Thaler and Cummings, 2009; Vetter *et al.*, 2009), although long-term improvement of glycemic control is dependent on the amount of weight loss (Dixon *et al.*, 2008). Currently, the antidiabetes mechanisms of gastric bypass are believed to be complex interactions among calorie restriction and altered signals from stomach, foregut, and hindgut (Thaler and Cummings, 2009; Vetter *et al.*, 2009).

It is expected that exclusion of the upper small intestine from contact with nutrients results in decreased GIP secretion. Indeed, fasting and postprandial GIP levels decrease after gastric bypass or biliopancreatic diversion, which exclude the duodenum and upper jejunum from intraluminal nutrients, while GLP-1 levels increase apparently due to expedited delivery of nutrients to the distal intestine (Clements *et al.*, 2004; Korner *et al.*, 2007; Mingrone *et al.*, 2009; Rubino *et al.*, 2004; Salinari *et al.*, 2009). Although there is a view that the decreased GIP levels might reduce obesity and insulin resistance (Irwin and Flatt, 2009), it is unclear whether decreased GIP secretion is a major mechanism of diabetes remission in this setting, as there are a few studies reporting remarkable improvement of hyperglycemia with no decrease in plasma GIP levels after gastric bypass surgery (Laferrere *et al.*, 2007; Whitson *et al.*, 2007).

C. GIP in reactive hypoglycemia after gastrectomy or gastric bypass

Reactive hypoglycemia or late dumping syndrome is frequently encountered after gastrectomy. Exaggerated insulin secretion associated with increased incretin levels in response to accelerated gastric emptying is

regarded as a possible mechanism of reactive hypoglycemia (Gebhard *et al.*, 2001; Toft-Nielsen *et al.*, 1998), although this is not consistent with the glucose-dependent insulinotropic property of incretins. However, there is evidence that GLP-1, not GIP, might induce hypoglycemia in some patients. When GLP-1 or GIP was concomitantly infused with glucose in the patients with postgastrectomy reactive hypoglycemia, only GLP-1 was shown to reproduce the hypoglycemia (Toft-Nielsen *et al.*, 1998). In addition, the neuroglycopenic symptoms due to severe hypoglycemia (altered mental status or level of consciousness, with or without seizure, requiring assistance of others) after gastric bypass surgery were also more related to the increased GLP-1 levels than the GIP levels (Goldfine *et al.*, 2007). However, it is unknown whether increased plasma GIP or GLP-1 levels might differently contribute to the development of hypoglycemia related to nesidioblastosis (Service *et al.*, 2005) or islet hyperplasia (Patti *et al.*, 2005) after gastric bypass surgery. Interestingly, a recent case report demonstrated that postgastric bypass hypoglycemia may result from altered nutrient delivery rather than hyperfunction of beta-cells by comparing the blood glucose, insulin, GIP, and GLP-1 levels between oral feeding (bypassed route) and gastrostomy tube feeding (original route) (McLaughlin *et al.*, 2010). In that case, oral feeding evoked hypoglycemia associated with exaggerated insulin, GIP, and GLP-1 responses, while gastrostomy tube feeding abated both hypoglycemia and the exaggerated hormone responses. Ultimately, elucidation of the mechanisms by which bariatric surgery resolves diabetes could lead to novel and effective nonsurgical approaches to combat the disease.

D. Effect of total parenteral nutrition on GIP and K-cells

Since total parenteral nutrition (TPN) induces intestinal mucosal atrophy (Buchman *et al.*, 1995), a decrease in enteroendocrine cell populations might be expected to occur during prolonged TPN. While the number of CCK cells in the duodenum and jejunum was significantly reduced after 7-day TPN in rats, surprisingly there was no change in the number of cells producing GIP, somatostatin, and enteroglucagon (Buchan *et al.*, 1985). Likewise, another study reported no alteration in the number or distribution of K-cells following 6-day TPN in rats and normal basal and glucose-stimulated GIP responses (Pederson *et al.*, 1985). However, the authors noted an exaggerated insulin response to an oral glucose challenge, which they attributed to increased beta-cell sensitivity to GIP, perhaps as a result of the chronically low (i.e., basal) GIP levels during the TPN (Pederson *et al.*, 1985). To the contrary, the GIP/DT mice with no GIP-producing cells exhibit a blunted insulin response to exogenously administered GIP (Wice *et al.*, 2010). Therefore, the increased GIP sensitivity during TPN might be explained by other factors than decreased GIP secretion.

E. Effect of aging on K-cells and GIP

Aging is one of the major risk factors for impaired glucose metabolism. By age 75, up to 20% of the U.S. population has diabetes (Harris *et al.*, 1998). In normal elderly subjects, glucose-stimulated insulin secretion from pancreatic beta-cells is impaired, and this defect is more noticeable in elderly patients with diabetes (Meneilly and Elliott, 1999; Meneilly *et al.*, 1998). The responses of GIP and GLP-1 to oral glucose are normal or increased in healthy elderly subjects when compared with healthy young controls (Elahi *et al.*, 1984; Korosi *et al.*, 2001; MacIntosh *et al.*, 1999; McConnell *et al.*, 1983; Meneilly *et al.*, 2000; Ranganath *et al.*, 1998), while the sensitivity of the beta-cell to both GIP and GLP-1 is decreased in normal elderly subjects and is more profoundly decreased in elderly patients with diabetes (Elahi *et al.*, 1984; Meneilly *et al.*, 1998; Ranganath *et al.*, 1998). In addition, there is no difference in the number of K-cells between young adults and elderly subjects (Sandstrom and El-Salhy, 1999). Taken together, the age-associated impairment in glucose-stimulated insulin secretion can be largely explained by decreased beta-cell sensitivity to GIP and GLP-1.

F. K-cells and GIP in autoimmune diseases and inflammatory bowel diseases

Autoimmune polyendocrine syndrome type 1 (APS-I) is characterized by multiple autoimmune endocrine diseases, including type 1 diabetes, hypoparathyroidism, and primary adrenal insufficiency as well as immunodeficiency, particularly susceptible to a certain fungal infection (candidiasis) (Eisenbarth and Gottlieb, 2004). Type 1 diabetes associated with APS-I is reported to be manifested by gastroenteric dysfunction consisting of malabsorption, constipation, or diarrhea, which has been considered as nonendocrine manifestation of APS-I presumably by an autoimmune destruction of responsible gastrointestinal cells (Gianani and Eisenbarth, 2003). Autoantibodies directed to tryptophan hydroxylase (Hedstrand *et al.*, 2000) and histidine decarboxylase (Skoldberg *et al.*, 2003) were reported to be associated with gastrointestinal dysfunction in APS-I. However, there is no evidence of autoimmune destruction of K-cells or L-cells in APS-I.

In celiac disease, which is characterized by gluten-induced, reversible architectural abnormalities of the intestinal mucosa, the K-cell number was initially reported to be increased (Sjolund *et al.*, 1979), but one study employing methods taking into account alterations of crypt morphology concluded that the K-cell number was reduced (Johnston *et al.*, 1988). The latter finding is consistent with the reduced GIP levels in celiac disease (Cooper *et al.*, 1981; Creutzfeldt *et al.*, 1976). Although plasma GIP levels during oral glucose tolerance tests were lower in the patients with celiac disease than in control subjects, insulin secretion was not different between

groups (Lauritsen *et al.*, 1982). It was reported that 4 out of 173 patients (2.3%) with celiac disease were positive for anti-GIP antibodies (Mirakian *et al.*, 1980). However, anti-GIP antibodies were also detected in patients with Crohn's disease, ulcerative colitis, irritable bowel syndrome, and even in subjects without any evident bowel diseases (Jones *et al.*, 1983). Interestingly, the plasma GIP levels at both fasting and 2 h after a test meal were significantly lower in the subjects with anti-GIP antibodies (Jones *et al.*, 1983), although there was no difference in plasma insulin levels between the two groups. In addition, circulating GIP levels were increased in the patients with Crohn's disease and ulcerative colitis, but there was no difference in insulin and glucagon levels compared to the control subjects (Besterman *et al.*, 1983). Collectively, the clinical relevance is uncertain with relation to the presence of anti-GIP antibodies and altered plasma GIP levels in autoimmune or inflammatory bowel diseases.

G. GIP-producing tumor

Although the L-cell tumors of the appendix and large intestine have been occasionally reported (Williams, 2007), to our knowledge, there is currently no report of a GIP-producing K-cell tumor in the intestine. One case with a pancreatic tumor producing both GIP and vasoactive intestinal polypeptide (VIP) has been reported (Kunert *et al.*, 1976). In this case, the clinical manifestations were typical of VIPoma (i.e., diarrhea and flushes), although the plasma GIP level was reportedly very high.

VII. Clinical Application of GIP and K-cells

A. GIP, friend or foe of diabetes/obesity?

Although the action of GIP on the pancreatic beta-cell is analogous to that of GLP-1, current therapeutic strategies have been focused on the use of GLP-1R agonists for the treatment of type 2 diabetes. This is based on the apparent lack of GIP effect in some patients with type 2 diabetes and presumed obesogenic effect of GIP observed in animal models (Irwin and Flatt, 2009; Kieffer, 2003; Wideman and Kieffer, 2009). GIP secretion is normal or higher in patients with type 2 diabetes compared with healthy control subjects (Crockett *et al.*, 1976; Ebert and Creutzfeldt, 1980; Jones *et al.*, 1989; Ross *et al.*, 1977; Vilsboll *et al.*, 2001), while its insulinotropic effect is markedly decreased (Krarup *et al.*, 1987; Meier *et al.*, 2001; Nauck *et al.*, 1986, 1993a). Therefore, alleviating GIP resistance of pancreatic beta-cells may be a therapeutic option to treat type 2 diabetes. In this regard, it is noteworthy that GIP resistance associated with type 2 diabetes might be

reversible by decreasing hyperglycemia. In the VDF Zucker rat, a model of type 2 diabetes, reducing hyperglycemia with phloridzin or a DPP-4 inhibitor, restored GIP sensitivity of pancreatic beta-cells (Piteau *et al.*, 2007). In patients with type 2 diabetes, 1-month glyburide (a sulfonylurea) treatment reduced blood glucose levels and increased GIP sensitivity (Meneilly *et al.*, 1993). In addition, it was recently reported that peroxisome proliferator-activated receptor γ regulates GIPR expression in the beta-cell (Gupta *et al.*, 2010), suggesting that thiazolidinediones can be used to alleviate GIP resistance. Further elucidation of the mechanism of GIP resistance may help to treat type 2 diabetes.

Unlike GLP-1, GIP was reported to increase glucagon secretion from the isolated perfused rat pancreas (Pederson and Brown, 1978). In a recent study (Chia *et al.*, 2009), supraphysiological GIP levels (fivefold of that normally observed during postmeal period) showed a short-lived insulinotropic effect in human type 2 diabetic patients. However, with a concomitant increase in plasma glucagon, the glucose-lowering effect of GIP was not observed in these patients. The glucagonotropic effect of GIP in patients with type 2 diabetes may present a caveat of GIP as a therapeutic agent.

It has been suggested that attenuating GIP signaling could be a strategy for treating or preventing high-fat diet-induced obesity and insulin resistance (Irwin and Flatt, 2009; Kieffer, 2003). Obese diabetic animals treated with the putative GIP antagonist Pro^3-GIP (Gault *et al.*, 2005; Irwin *et al.*, 2007) or vaccination against GIP (Fulurija *et al.*, 2008); or manipulated by genetic knockout of GIPR (Miyawaki *et al.*, 2002) or targeted K-cell destruction (Althage *et al.*, 2008) are reported to be protected against obesity and associated metabolic disturbances. GIP signaling blockade induces preferential oxidation of fat and clears triglyceride deposits from liver and muscle, which eventually contributes to suppression of hepatic glucose output and improvement in insulin sensitivity (Hansotia *et al.*, 2007; Miyawaki *et al.*, 2002). Despite this evidence in rodents, it is unclear whether modulation of GIP signaling may be equally beneficial in humans. Although GIP antagonism appears to be an attractive approach to treat obesity and insulin resistance, it should be kept in mind that GIP antagonism will reduce glucose-stimulated insulin secretion (Ebert and Creutzfeldt, 1982; Lewis *et al.*, 2000; Tseng *et al.*, 1996). In this regard, it is noteworthy that D-Ala^2-$GIP_{1–30}$ exhibits equivalent potency to $GIP_{1–42}$ on beta-cell function and survival, but might have decreased adipogenic capacity (Widenmaier *et al.*, 2010). Therefore, it might be feasible to modify the GIP structure to preserve the glucose-dependent insulinotropic effect while reducing the adipogenic and glucagonotropic effects.

B. K-cells as a useful target for gene therapy for metabolic diseases

Since K-cells secrete large quantities of GIP in response to glucose and the secretion kinetics of GIP closely parallels that of insulin (Cataland *et al.*, 1974; Fujita *et al.*, 2004; Kieffer *et al.*, 1994, 1995a), it has been proposed that K-cells may be suitable targets to engineer as a non beta-cell source of meal-regulated insulin production. Transgenic mice expressing the human insulin gene linked to rat GIP promoter (GIP/Ins) produced human insulin specifically in intestinal K-cells and were resistant to the development of diabetes induced by the beta-cell toxin streptozotocin (Cheung *et al.*, 2000). Aside from germ-line manipulation, *in vivo* transfection of GIP/Ins plasmid into rat small intestine K-cells by using liposomes was reported (Palizban *et al.*, 2007). Therefore, utilizing a patient's own K-cells to reestablish endogenous meal-regulated insulin secretion represents a potential strategy to treat or ultimately cure diabetes. If safe, effective gene transfer technologies can be developed to target gut K-cells, similar engineering approaches could be used to boost the meal-regulated production of other glucose-lowering products (e.g., GLP-1) or satiety factors (e.g., PYY) as therapies for diabetes and obesity.

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CHAPTER FIVE

The Emerging Role of Promiscuous 7TM Receptors as Chemosensors for Food Intake

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Contents

Abstract

In recent years, several highly promiscuous seven transmembrane (7TM) receptors have been cloned and characterized of which many are activated broadly by amino acids, proteolytic degradation products, carbohydrates, or free fatty

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acids (FFAs) and are expressed in taste tissue, the gastrointestinal (GI) tract, endocrine glands, adipose tissue, and/or kidney. This has led to the hypothesis that these receptors may act as sensors of food intake modulating, for example, release of incretin hormones from the gut, insulin/glucagon from the pancreas, and leptin from adipose tissue. In the present review, we describe the molecular mechanisms of nutrient-sensing of the calcium-sensing receptor (CaR), the G protein-coupled receptor family C, group 6, subtype A (GPRC6A), and the taste1 receptor T1R1/T1R3-sensing L-α-amino acids; the carbohydrate-sensing T1R2/T1R3 receptor; the proteolytic degradation product sensor GPR93 (also termed GPR92); and the FFA sensing receptors FFA1, FFA2, FFA3, GPR84, and GPR120. Due to their omnipresent nature, the natural ligands have had limited usability in pharmacological/physiological studies which has hampered the elucidation of the physiological function and therapeutic prospect of their receptors. However, an increasing number of subtype-selective ligands and/or receptor knockout mice are being developed which at least for some of the receptors have validated them as promising drug targets in, for example, type II diabetes.

Abbreviations

7TM	seven transmembrane
CaR	calcium-sensing receptor
CCK	cholecystokinin
CRD	cysteine-rich domain
FFA	free fatty acid
$GABA_B$	γ-aminobutyric acid type B
GI	gastrointestinal
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide-1
GPRC6A	G protein-coupled receptor, family C, group 6, subtype A
IMP	5′-inosine monophosphate
L-Asp	L-aspartate
L-Glu	L-glutamate
L-Lys	L-lysine
L-Phe	L-phenylalanine
L-Trp	L-tryptophan
mGlu	metabotropic glutamate
T1R	taste1 receptor
VFT	Venus flytrap

I. Introduction

In recent years, a number of seven transmembrane (7TM) receptors have been cloned and characterized that are highly promiscuous and thus respond to a range of natural agonists. Furthermore, several promiscuous taste receptors have been identified in tissues other than taste buds, such as the gut, suggesting that they have other functions than sensing tastants. Interestingly, these promiscuous receptors respond to organic nutrients or their immediate breakdown products, that is, fatty acids, sugars, amino acids, and proteolytic products, and thus possibly serve as chemosensors for food intake (Conigrave and Brown, 2006; Egan and Margolskee, 2008; Engelstoft *et al.*, 2008). For decades, it has been known that the organic nutrients cause release of hormones from the gut, pancreas, and other organs, but the molecular nature of the chemosensors has been enigmatic. The identification of promiscuous 7TM receptors responding to the organic nutrients located in the relevant tissues has potentially identified the missing links. In that regard, it makes perfect sense that these nutrient-sensing receptors have evolved to be promiscuous in order to respond to the wide range of foods digested by humans, and it opens for the perspective that mixtures of ligands work in a concerted fashion to activate one receptor, as has been shown for the calcium-sensing receptor (CaR) (Conigrave *et al.*, 2000, 2004).

Given the potential role of these receptors with respect to the metabolic syndrome, diabetes, and obesity, they have lately been the subject of intense research. We here review the current status of promiscuous 7TM receptors as potential chemosensors with particular focus on their molecular pharmacology, mechanisms(s) of receptor–ligand interaction, and physiological function.

II. Family C Receptors as Promiscuous Sensors for L-α-Amino acids, Peptides, Divalent cations, and Carbohydrates

The family C of 7TM receptors is intriguing both from a structural and a functional point of view. They are believed to evolve from the linking of two separate entities (Conklin and Bourne, 1994); a class of bacterial periplasmic-binding proteins (Felder *et al.*, 1999; O'Hara *et al.*, 1993), involved in nutrient uptake (Quiocho and Ledvina, 1996), and the archetypical 7TM structure found in all G protein-coupled receptors (Rosenbaum *et al.*, 2009). In fact, most members of family C 7TM receptors have a preserved ability to respond to nutrient-like compounds being it

amino acids, ions, or sugars, but they do so by different modes of interaction: The mGlu and γ-aminobutyric acid type B ($GABA_B$) receptors respond exclusively to one agonist (either L-glutamate (Frauli *et al.*, 2006) or GABA (Wellendorph and Bräuner-Osborne, 2009)), probably reflecting the important roles of these receptors in neurotransmission, whereas the subgroup of receptors formed by the CaR, T1Rs, and GPRC6A, phylogenetically distinct within family C receptors (Bjarnadóttir *et al.*, 2005; Kuang *et al.*, 2006; Wellendorph and Bräuner-Osborne, 2009) are promiscuous by nature and respond to subsets of L-α-amino acids and divalent cations (CaR, GPRC6A, and the heterodimeric receptor T1R1/T1R3) or sugars and D-amino acids (the heterodimeric receptor T1R2/T1R3). Due to the ingenious differential L-amino acid preference for each of these receptors, they potentially cover stimuli from all of the 20 proteinogenic L-amino acids (Conigrave and Hampson, 2006; Wellendorph and Bräuner-Osborne, 2009) (Fig. 5.1) which together with their expression in relevant tissues (Fig. 5.2) allows for a nutrient-sensing capacity of emerging physiological significance (for reviews see (Conigrave and Hampson, 2006, 2010; Rozengurt and Sternini, 2007; Sternini *et al.*, 2008)). Thus, this subgroup of

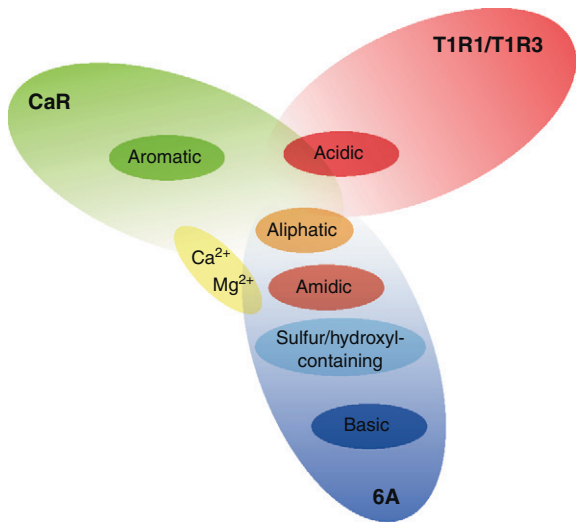


Figure 5.1 Illustration of L-α-amino acid/divalent cation responsiveness and promiscuity of CaR, GPRC6A (6A), and the T1R1/T1R3 heterodimer. Amino acids are grouped according to side chain charge and polarity. Data represent amino acid sensitivities reported for the human receptors (Conigrave *et al.*, 2004; Li *et al.*, 2002; Wellendorph *et al.*, 2005). Preferential responsiveness of the receptors is represented as proximity to the receptor names: CaR responds most strongly to aromatic amino acids, T1R1/T1R3 most strongly to acidic amino acids, and GPRC6A most strongly to basic amino acids.

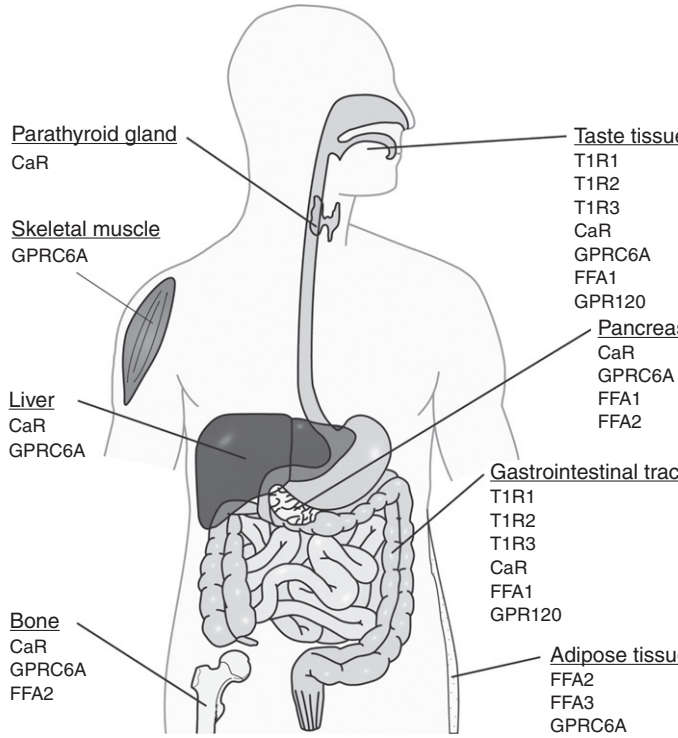


Figure 5.2 Illustration of human tissues displaying predominant expression of the promiscuous organic nutrient-sensing 7TM receptors (see text for details).

receptors are able to sense an impressive range of nutrients already from food enters the mouth, via receptors in the taste buds, to the additional proteolytic breakdown and chemosensing in the stomach and gut (Table 5.1), where the concentration of free L-amino acids may notably rise as high as to approach millimolar concentrations (Adibi and Mercer, 1973).

A. Structural and molecular features of family C nutrient-sensing receptors

The ability of nutrient-sensing of family C receptors resides in their large extracellular Venus FlyTrap (VFT) domain which has been shown by X-ray crystallography to contain the orthosteric binding site for the endogenous agonist as well as a dimerization interface (Kunishima *et al.*, 2000; Muto *et al.*, 2007; Tsuchiya *et al.*, 2002). The VFT domain is connected to the 7TM, G protein-activating domain via a cysteine-rich domain (CRD) (for a structural overview, refer to Fig. 5.3). Following on from

Table 5.1 Substances derived from the primary sources of nutrients (protein, lipid, and carbohydrate) are ligands for 7TM receptors, largely in a promiscuous fashion and comprehensively covering the spectrum of nutrients

Nutrient substance	L-α-amino acids peptides	Free fatty acids (FFAs)	Monosaccharides
Nutrient sensing receptor	T1R1/T1R3 GPRC6A CaR GPR93	GPR120 GPR34 FFA1 FFA2 FFA3	T1R2/T1R3

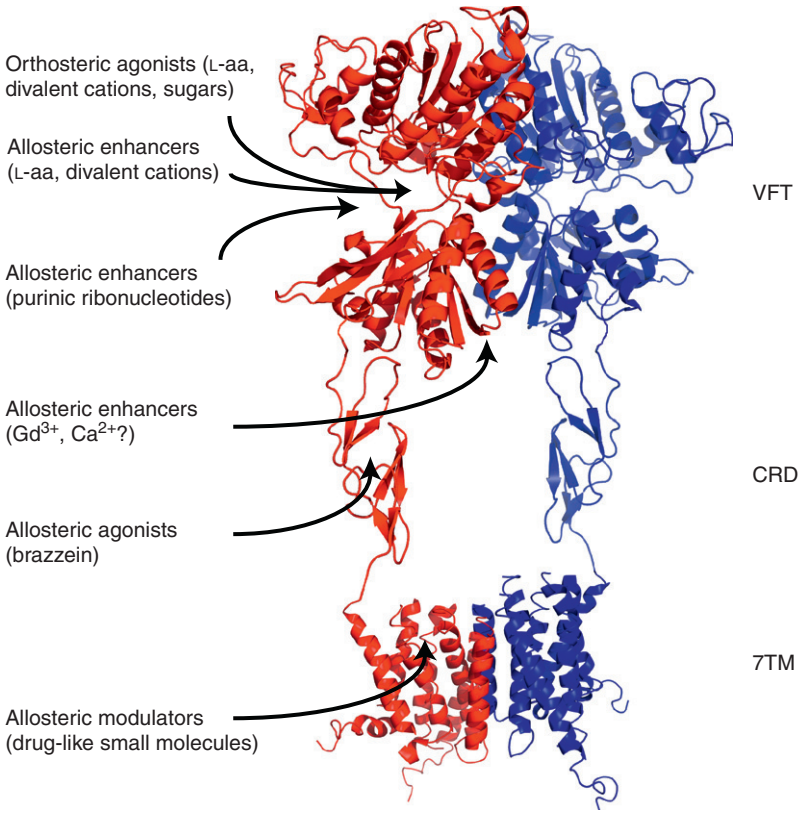


Figure 5.3 Model of a dimeric family C 7TM receptor in its open–closed/active conformation. The localizations of the Venus flytrap (VFT) domain, cysteine-rich domain (CRD) and 7 transmembrane (7TM) domain, and ortho- and allosteric ligand-binding sites are indicated (see text for details). The model was constructed with the program MacPyMol using coordinates from PDB files 1EWK ($mGlu_1$ open–closed/Active VFT), 2E4U ($mGlu_3$ CRR) and 2R4S (β_2-adrenergic receptor 7TM).

the crystallographic data, the binding of agonist in the VFT domain leads to domain closure of the VFT and an activating twist in the 7TM domain, thereby promoting G protein interaction and downstream signaling (Wellendorph and Bräuner-Osborne, 2009).

Appreciation of L-amino acid binding in the VFT orthosteric-binding pocket of family C receptors has come from crystal structures of the L-Glu-bound $mGlu_1$ and $mGlu_3$ VFT domains (Kunishima *et al.*, 2000; Muto *et al.*, 2007). This has convincingly led to the identification of five residues in the mGlu receptors that are particularly important for binding of the L-α-amino acid moiety of L-Glu, and two basic residues that are vital for binding of the distal carboxylic acid of L-Glu. Whereas the five residues responsible for L-α-amino acid recognition are highly conserved, the two basic residues, for binding the distal end of L-Glu are not conserved to CaR, GPRC6A, and T1R1 (Acher and Bertrand, 2005; Conigrave and Hampson, 2006; Wellendorph and Bräuner-Osborne, 2009). This observation is consistent with the ability of the latter three receptors to accommodate many different amino acid side chain functionalities in their binding pockets. However, some level of selectivity in amino acid-binding profiles of the individual receptors is introduced by differences in the distal end of the binding pocket (Wellendorph and Bräuner-Osborne, 2009).

In addition to the endogenous binding site in the VFT domain, several compounds binding at allosteric sites in both the VFT domain, the CRD and the 7TM domain have been identified for receptors of family C, allowing for fine-tuning of the agonist response. Every one of these three domains contains ligand-binding sites, allowing for receptor activation by a vast repertoire of chemically distinct molecules (refer to Fig. 5.3 for a generalized illustration of ligand-binding sites). The unique topology of family C receptors indeed represents great opportunities for drug discovery especially via allosteric modulation of the 7TM domain (Bräuner-Osborne *et al.*, 2007; Conn *et al.*, 2009). For many of these receptors, allosteric modulators acting in this region are now known (details given below).

B. L-Amino acid, Ca^{2+}, and peptide sensing by CaR

The primary physiological agonist for CaR is extracellular Ca^{2+} which is sensed by tissues relevant for maintaining calcium homeostasis such as the kidney and the parathyroid gland (Brown, 2007; Tfelt-Hansen and Brown, 2005). However, in addition to Ca^{2+}, and other divalent cations (Brown *et al.*, 1993), L-α-amino acids and di- and tripeptides work as allosteric enhancers at CaR in the presence of a certain level of extracellular Ca^{2+}. Experiments have detailed that *in vivo* L-amino acid sensing *per se* is enabled at physiological concentrations of Ca^{2+} (around 1 mM) (Conigrave and Hampson, 2006; Conigrave *et al.*, 2004). In terms of nutrient sensing, the L-amino acid sensing is investigated the most and is known to occur in organs

expressing CaR but not involved in regulating calcium homeostasis, such as the stomach, the lower gastrointestinal (GI) tract, liver, and pancreas (Fig. 5.2) (Geibel and Hebert, 2009). Several reports have demonstrated a role for CaR in protein/amino acid sensing, including CaR-mediated L-amino acid-stimulated gastric acid release from stomach parietal cells (Busque *et al.*, 2005), cholecystokinin (CCK) release from duodenal enteroendocrine cells (Hira *et al.*, 2008), and inhibition of parathyroid hormone release from human parathyroid cells (Conigrave *et al.*, 2004). Most recently, CaR expression has been reported in taste tissue, suggesting a role for the receptor in calcium and amino acid taste (Bystrova *et al.*, 2010; San Gabriel *et al.*, 2009). Interestingly, evidence is accumulating for a role for di- and tripeptides in the potentiation of calcium-induced responses both in relation to taste (so-called "kokumi" taste) (Ohsu *et al.*, 2010), and thus possibly also via chemosensing of proteolytic breakdown products in the gut.

The most potent amino acids at CaR *in vitro* are the aromatic amino acids such as L-phenylalanine (L-Phe) and L-tryptophan (L-Trp). Some aliphatic and polar amino acids are moderately effective, however dependent on the calcium concentration, whereas acidic, basic, and branched-chain amino acids are weak or inactive (Fig. 5.1; Conigrave *et al.*, 2000, 2004).

As mentioned, family C receptors, including CaR, contain a highly conserved 5-residue motif-binding site in their VFT domain which is predicted to bind the α-amino acid moiety of L-α-amino acids. However, whereas molecular pharmacology techniques have confirmed that the amino acid-binding site resides in the VFT (Mun *et al.*, 2004; Zhang *et al.*, 2002a), it has been inherently difficult to pinpoint residues specifically involved in amino acid-binding and activation, as the amino acids require the presence of Ca^{2+} to work and many of the examined mutations simultaneously reduce Ca^{2+} sensitivity due to partly overlapping binding sites (Silve *et al.*, 2005; Wellendorph and Bräuner-Osborne, 2009) (Fig. 5.3). Mutation studies have predicted several Ca^{2+}-binding sites in the cleft of the VFT (Bräuner-Osborne *et al.*, 1999; Huang *et al.*, 2007), but Ca^{2+} could also potentially bind between the two bilobed VFT domains stabilizing the closed conformation, as has been shown for Gd^{3+} in $mGlu_1$ (Fig. 5.3; Tsuchiya *et al.*, 2002). Mun *et al.* identified two mutations, T145A and S170T that specifically impair amino acid sensing while leaving Ca^{2+} sensing intact (Mun *et al.*, 2005). Others have also identified the three serines S169–171 as being important for amino acid binding (Lee *et al.*, 2007; Zhang *et al.*, 2002b). With regards to the distal end of the binding pocket, it remains to be investigated which residues participate in binding of, for instance, the aromatic moieties of L-Phe/L-Trp. In fact, the binding is flexible and large enough to accommodate even the small peptide glutathione (Wang *et al.*, 2006b) and γ-glutamyl peptides (Ohsu *et al.*, 2010). Altogether, both aromatic L-amino acids and peptides, that is,

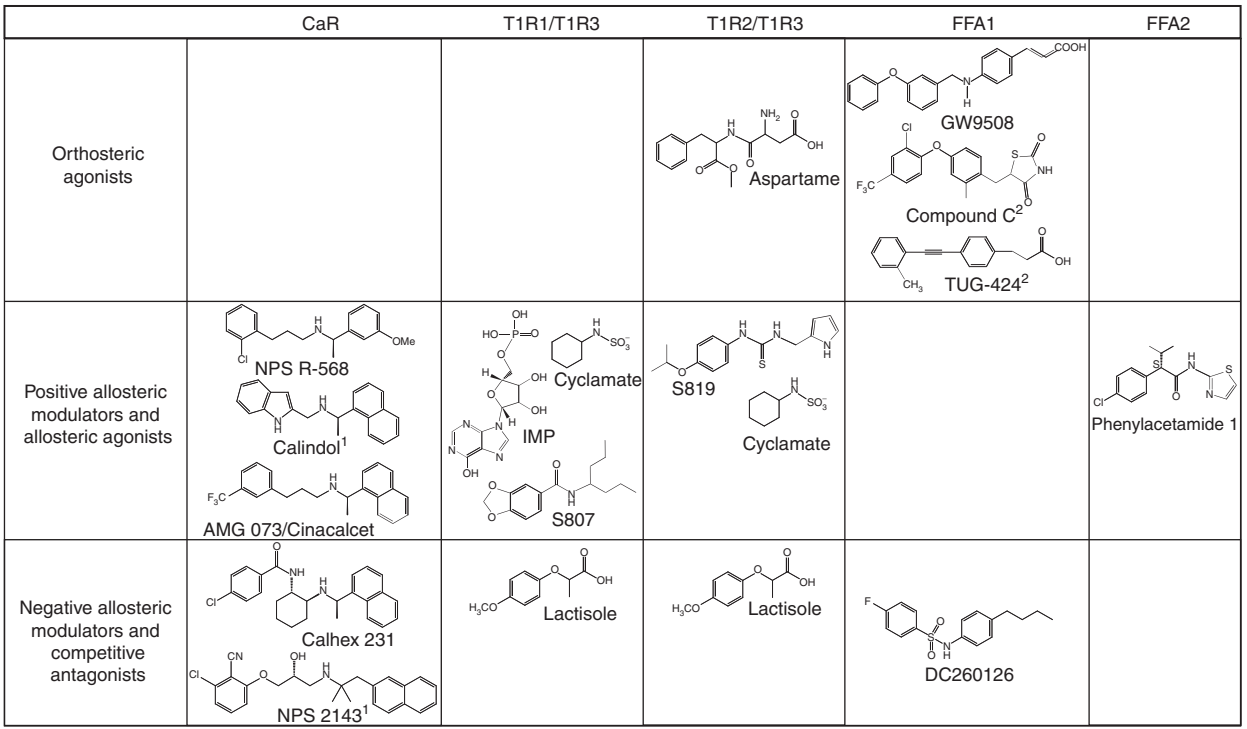
γ-glutamylcysteinylglycine (γ-Glu-Cys-Gly; GSH) are dependent on the presence of orthosteric agonist (Ca^{2+}) and referred to as positive allosteric modulators and, in contrast to the large class of calcimetics and calcilytics described below, these bind in the VFT domain (Wang *et al.*, 2006b).

Notable success has been obtained in relation to development of small molecule CaR allosteric modulators acting at the 7TM domain (Figs. 5.3 and 5.4), Both positive modulators, termed calcimimetics (e.g., NPS R-568, calindol, and cinacalcet), and negative modulators, termed calcilytics (e.g., NPS 2143 and Calhex 231), have been described (Kessler *et al.*, 2006; Nemeth *et al.*, 1998, 2001; Petrel *et al.*, 2003). The success is underlined by the registration of cinacalcet for the treatment of secondary hyperparathyroidism and also as the first GPCR positive allosteric modulator to enter the market (Brown, 2010). Binding sites identified thus far for this type of CaR modulating compounds are overlapping, residing within a crevice formed by transmembrane helices TM3, TM5, TM6, and TM7 (Miedlich *et al.*, 2004; Petrel *et al.*, 2003, 2004). Potentially, compounds of this sort may present novel drugs for affecting nutrient sensing by CaR and subsequently regulating physiological processes such as motility, digestion, absorption, and exocrine secretion of various hormones.

C. L-Amino acid sensing by GPRC6A

The most closely related human homologue of CaR is GPRC6A which as CaR also responds to both L-amino acids and divalent cations. GPRC6A is stereoselectively activated by 6–8 of the 20 proteinogenic L-α-amino acids, with some preference for basic amino acids, but also by small and neutral amino acids (Christiansen *et al.*, 2007; Kuang *et al.*, 2005; Wellendorph *et al.*, 2005, 2007) (Fig. 5.1). GPRC6A is the most recent family C receptor member, and also the least investigated in terms of physiological relevance.

Given the strikingly broad sensitivity to L-amino acids and the expression of GPRC6A in organs and tissues such as taste tissue, pancreatic islets, liver, skeletal muscle, bone, and fat (Fig. 5.2) (Bystrova *et al.*, 2010; Kuang *et al.*, 2005; Pi *et al.*, 2005; Regard *et al.*, 2007; Wellendorph and Bräuner-Osborne, 2004; Wellendorph *et al.*, 2007, 2009), GPRC6A represents a plausible taste/nutrient-sensing receptor (Conigrave and Hampson, 2006). In further support of this, the closely related goldfish odorant receptor 5.24 functions as a nutrient sensor for L-amino acids in sensory tissue of the goldfish (Speca *et al.*, 1999). Pertaining to a possible role for GPRC6A in metabolism, mice lacking GPRC6A, have been reported in one instance to have complex metabolic abnormalities including hepatic steatosis, hyperglycemia, glucose intolerance, and insulin resistance, reduced testosterone levels and obvious feminization of male mice, and an osteopenic phenotype (Pi *et al.*, 2008, 2010). In another instance, no bone phenotype was observed in GPRC6A knockout mice (Wellendorph *et al.*, 2009).



[1] Compounds also acting as allosteric modulators of GPRC6A albeit with lower potency compared to CaR.
[2] It remains to be shown by mutagenesis if these compounds are ortho- or allosteric agonists.

Figure 5.4 Chemical structures and pharmacological activities of selected allosteric and orthosteric receptor ligands discussed in the text.

These discrepancies could relate to the actual design of the knockout construct or to the background of the mouse strains used. So far, the role of GPRC6A in the cells of the GI tract has not been investigated.

Closely related to the ligand preferences of CaR, GPRC6A responds to L-α-amino acids and is augmented by divalent cations Ca^{2+} and Mg^{2+} in physiologically relevant concentrations (Christiansen *et al.*, 2007; Kuang *et al.*, 2005; Wellendorph *et al.*, 2007). One report has even demonstrated a direct activation of GPRC6A by Ca^{2+} (Pi *et al.*, 2005). GPRC6A can thus be speculated to act in concert with CaR, supposedly in a reciprocal fashion, to sense a wide range of both L-amino acids and Ca^{2+} in plasma, in taste buds, or in the GI tract. Several synthetic L-α-amino acids carrying positively charged side chains, and coincidentally affecting the nitric oxide synthase and arginase isoenzymes, are also agonists at GPRC6A (Christiansen *et al.*, 2006b), whereas the tripeptide glutathione reported to modulate CaR, is devoid of activity at GPRC6A (Wang *et al.*, 2006b). Recently, it was reported that the known CaR negative modulator NPS 2143 and the CaR positive modulator Calindol (Fig. 5.4) inhibit the L-ornithine-induced responses in mouse GPRC6A overexpressed cells through interaction with the 7TM domain (Faure *et al.*, 2009). A selective GPRC6A antagonist still remains to be discovered and would constitute a very important tool compound in functional investigations. Based on homology modeling of hGPRC6A (Wellendorph *et al.*, 2005), two residues (S149 and T172), conserved across amino acid sensitive family C receptors for interaction with the α-amino acid moieties (Wellendorph and Bräuner-Osborne, 2009), have been demonstrated to interact with the α-amino acid function of L-lysine using mutagenesis studies (Wellendorph *et al.*, 2005). The presence of the archetypical family C 5-residue recognition motif has also been substantiated by inactivating mutations in the orthologous goldfish 5.24 receptor (Kuang *et al.*, 2003; Luu *et al.*, 2005). So far, the precise environment of the distal end of the GPRC6A-binding pocket is unaccounted for and the residue(s) responsible for binding the positively charged end of the basic amino acid agonists are not conserved from 5.24 (Wellendorph and Bräuner-Osborne, 2009). Neither has the Ca^{2+}-binding site been mapped. Thus, in contrast to the GPRC6A receptor, 5.24 appears to be less spatially restricted in the distal-binding pocket corresponding with the fact that it responds to a broader range of L-α-amino acids (Christiansen *et al.*, 2006a). The fact that GPRC6A is promiscuous makes this task somewhat more challenging, and the aid of synthetic orthosteric agonists would be an advantage.

D. L-Amino acid sensing by T1R1/T1R3

The T1R class of 7TM receptors consists of three subunits: T1R1, T1R2, and T1R3. In order to form functional receptors, the individual subunits T1R1 and T1R2 depend on coexpression and dimerization with T1R3

(Xu *et al.*, 2004; Zhao *et al.*, 2003). The heterodimer T1R1/T1R3 perceives L-amino acids and functions as an L-amino acid taste receptor in taste buds of the tongue and soft palate where it is highly expressed (Hoon *et al.*, 1999). T1R1/T1R3 is also called the umami taste receptor (umami is the savory taste of L-Glu and L-aspartate (L-Asp)) (Li *et al.*, 2002; Nelson *et al.*, 2002). However, there is biochemical and physiological evidence for at least one other umami receptor (Damak *et al.*, 2003), and one purported candidate is a truncated version of the $mGlu_4$ receptor, although this "umami" receptor is not potentiated by purinic ribonucleotides such as 5′-inosine monophosphate (IMP) (Chaudhari *et al.*, 2000), a hallmark of umami taste (Yamaguchi, 1991). Taste signal transduction is largely mediated by the G protein gustducin (Ruiz-Avila *et al.*, 2001), and the finding that both α-gustducin (Höfer *et al.*, 1996) and the different T1R subunits are expressed in the GI tract (Bezençon *et al.*, 2007; Dyer *et al.*, 2005; Nakamura *et al.*, 2010; Fig. 5.2), suggests the presence of taste-sensing mechanisms in the gut, more appropriately referred to as chemosensing (reviewed by (Rozengurt and Sternini, 2007)). Studies are emerging with reports on the functional role of T1R1/T1R3 in nutrient absorption including the ability of the receptor to downregulate expression of the oligopeptide transporter, PepT1, and upregulate expression of the glucose transporter GLUT2 and the L-Glu/L-Asp transporter EAAC1 (Mace *et al.*, 2009), Interestingly, the sweet sensing T1R2/T1R3 receptor is also able to decrease PepT1 expression and increase GLUT2 expression in the jejunum through activation of the same intracellular signaling molecule (PKC βII) (Mace *et al.*, 2009), in addition to several other functional roles. It is thus suggested that a taste receptor-coordinated transport network exists within the GI tract which cross-regulates expression of nutrient transporters (i.e., sugars regulate expression of amino acid/peptide transporters via T1R2/T1R3 and amino acids regulate glucose transport via T1R1/T1R3), which could well incorporate other nutrients as well (Mace *et al.*, 2009).

From *in vitro* studies conducted on both human and rodent T1R1/T1R3 receptors, there appears to be notable species differences (Li *et al.*, 2002; Nelson *et al.*, 2002). The human receptor is more than an order of magnitude more sensitive to L-Glu than to other amino acids, and is also sensitive to the synthetic L-Glu analogue L-AP4 in the presence of IMP, whereas the rodent orthologs have approximately equal sensitivity to L-Glu and the other L-amino acids in the presence of IMP but no sensitivity to L-AP4 (Li *et al.*, 2002; Nelson *et al.*, 2002). As CaR and GPRC6A do not exhibit any sensitivity to acidic L-amino acids, this ability of T1R1/T1R3 nicely complements the other amino acid sensors (Fig. 5.1).

Since L-Glu and IMP have no potentiating effect on the T1R2/T1R3 sweet taste receptor, it is inferred that these compounds bind to the VFT domain of T1R1 (Li *et al.*, 2002; Xu *et al.*, 2004), recently confirmed by mutagenesis studies (Zhang *et al.*, 2008). Hence, the differences in ligand

sensitivity may likely be assigned to the rather low percentage sequence identity (70% amino acid identity between rodent and human T1R1s (Nelson *et al.*, 2001)). The work by Zhang *et al.* presents a homology model for T1R1 and confirms by mutagenesis the involvement of four of the five key residues predicted to bind the α-amino acid moiety (Acher and Bertrand, 2005; Wellendorph and Bräuner-Osborne, 2009). Furthermore, they identify residues responsible for the binding of IMP and map the purinic ribonucleotide-binding site at an allosteric site adjacent to the L-Glu site (Fig. 5.3). Binding of IMP is proposed to stabilize VFT domain closure by electrostatic interactions between the charged phosphate group of IMP and a cluster of positive charges on the other lobe (Zhang *et al.*, 2008).

Other allosteric small molecules acting at the T1R1/T1R3 receptor include the allosteric enhancer cyclamate and the negative modulator lactisole (Fig. 5.4). Both these ligands bind in the 7TM domain at overlapping sites, similar in location to the CaR 7TM modulator site (Cui *et al.*, 2006) (Fig. 5.3).

E. Carbohydrate sensing by T1R2/T1R3

To date, the only described sweet sensing receptor is the heterodimeric receptor T1R2/T1R3 which displays an intriguingly broad sensitivity for naturally occurring sweet substances such as glucose, fructose, sucrose, and sweet-tasting D-amino acids but also for synthetic sweeteners like aspartame (NutraSweet®), cyclamate, saccharin, and acesulfam K (Li *et al.*, 2002; Nelson *et al.*, 2001). T1R2/T1R3 is expressed both in taste buds of the oral cavity (Nelson *et al.*, 2001), but also throughout the GI tract (Bezençon *et al.*, 2007; Dyer *et al.*, 2005; Hass *et al.*, 2010; Mace *et al.*, 2009) and in β cells of the pancreas (Nakagawa *et al.*, 2009), and within the last couple of years exciting links between dietary sugars and chemosensing have surfaced, notably the regulation of glucose absorption (Le Gall *et al.*, 2007; Mace *et al.*, 2007, 2009; Margolskee *et al.*, 2007; Stearns *et al.*, 2010), receptor-dependent insulin secretion (Nakagawa *et al.*, 2009), and enteroendocrine GLP-1 hormone secretion (Jang *et al.*, 2007; Kokrashvili *et al.*, 2009).

The fact that T1R2/T1R3 is so promiscuous for sweet-tasting substances suggests that the binding site is not very restricted. In order to localize the orthosteric-binding site in the T1R2/T1R3 heterodimer for the synthetic sweeteners aspartame and neotame, chimeric receptor studies have been particularly useful and clarified that the VFT domain of T1R2 is the subunit responsible for binding (Fig. 5.3). Alignments of T1R2 and $mGlu_1$ show that three of the five residues predicted to bind the α-amino acid moiety of the ligands are conserved (Li *et al.*, 2002). Correspondingly it has been verified by mutational analyses that neotame, aspartame, and sweet-tasting D-amino acids (for instance, D-Trp) bind via residues S144 and E302 of the human T1R2 (Xu *et al.*, 2004), corresponding to residues binding, respectively, the carboxylate group and amino group of the α-amino acid function of L-Glu in

$mGlu_1$ (Wellendorph and Bräuner-Osborne, 2009). Interestingly, the stereoselectivity for amino acids is reversed at T1R2/T1R3 compared to T1R1/T1R3, that is, only the D-forms but not the L-forms of Phe and Asn can activate T1R2/T1R3 (Nelson *et al.*, 2002).

In addition to compounds acting in the orthosteric site of the VFT domain, structurally diverse compounds acting at allosteric sites can stimulate or inhibit receptor signaling (Figs. 5.3 and 5.4). Compounds such as cyclamate and lactisole act at the common subunit T1R3 and are thus able to affect receptor signaling of both umami and sweet taste receptors but do this by different modes of interaction (Fig. 5.4). Cyclamate acts as an allosteric agonist at the T1R3 subunit of T1R2/T1R3, and notably, exclusively at the *human* T1R3 (Jiang *et al.*, 2005b). By contrast, cyclamate acts as an allosteric enhancer at the T1R3 subunit of T1R1/T1R3, thus requiring the presence of an orthosteric agonist such as Glu (Xu *et al.*, 2004). Also, the allosteric compound lactisole acts differently depending on the heterodimeric partner of T1R3, and appears to be a competitive antagonist at sweet-T1R3 but a negative allosteric modulator at umami-T1R3 (Xu *et al.*, 2004). Both cyclamate and lactisole act at defined allosteric sites in the 7TM region (Jiang *et al.*, 2005a, b). However, as these small molecules are not selective, there is a need for allosteric compounds acting selectively in the 7TM regions of T1R1 and T1R2. Recently, novel small molecule agonists and enhancers have been identified at T1R2/T1R3 from high-throughput screenings (Servant *et al.*, 2010; Zhang *et al.*, 2008). These compounds have been shown by mutagenesis studies to modulate sweet taste by a unique mechanism, involving binding near the opening of the pocket of hT1R2, hereby further stabilizing the closed/active conformation of the receptor (Zhang *et al.*, 2010). Sweet taste enhancers are interesting as pharmacological tool compounds and might have future relevance as artificial sweeteners or therapeutics, as their addition may permit lowering of the caloric content of food and beverage products while maintaining the desired taste (Servant *et al.*, 2010).

Finally, adding to the list of allosteric modulators and unique sites for family C 7TM receptors, the sweet-tasting protein brazzein is an allosteric agonist of T1R2/T1R3 with a novel-binding site residing in the CRD (Assadi-Porter *et al.*, 2010; Jiang *et al.*, 2004).

III. Family A Receptors as Promiscuous Sensors for Peptone and Free Fatty Acids (FFAs)

In addition to the family C 7TM receptors, several family A 7TM receptors are promiscuous in their ligand preferences, and several respond to organic nutrients from food, notably protein degradation products and FFAs (Table 5.1).

A. Peptone sensing by GPR93

The family A receptor GPR93 (IUPHAR name GPR92) has mainly been classified as a receptor for lysophosphatidic acid (Ishii *et al.*, 2009). However, a couple of studies have demonstrated that the receptor is also activated by peptone, a peptide mixture resembling proteolytic degradation products, either alone or in a synergistic fashion with lysophosphatidic acid (Choi *et al.*, 2007a, b). The receptor is highly expressed in the small intestine (Choi *et al.*, 2007a) and it has been shown that peptone stimulation of GPR93 in enterocytes and enteroendocrine cells leads to G protein-signaling cascades, ultimately promoting CCK gene transcription and CCK release (Choi *et al.*, 2007a, b). GPR93 might thus be the missing link for a previously described protein hydrolysate-mediated CCK expression and release in intestinal cells (Nemoz-Gaillard *et al.*, 1998; Nishi *et al.*, 2001), but additional studies using more specific ligands and/or knockout mice are needed to show this, or other physiological functions, explicitly.

B. Free fatty acid-sensing receptors

The physiological effects of FFAs are typically recognized as functions mediated by actions on cellular metabolism. However, not all biological effects can be ascribed to intracellular metabolism, but instead indicated to be mediated by cell surface receptors (Louet *et al.*, 2001; Sauer *et al.*, 2000). Thus, the recent deorphanization of several receptors activated by FFAs, belonging to the family A of 7TM receptors, has offered an alternative mechanism of action for FFAs. Moreover, the action of FFAs on cell surface receptors is known to play significant roles in the regulation of food intake.

A series of medium- and long-chain FFAs has been identified as ligands for the FFA1 (previously termed GPR40), GPR84, and GPR120 receptors (Briscoe *et al.*, 2003; Hirasawa *et al.*, 2005; Itoh *et al.*, 2003; Kotarsky *et al.*, 2003; Wang *et al.*, 2006a), whereas short-chain FFAs activate FFA2 and FFA3 (previously termed GPR43 and GPR41, respectively) (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). The short-chain FFAs are produced by anaerobic fermentation of dietary carbohydrate fibers, and FFA2 and FFA3 could therefore be classified as carbohydrate intake sensors rather than lipid intake sensors. FFA1, FFA2, and FFA3 are phylogenetically related, although the sequence similarity relatively limited (33–43% amino acid identity), and the GPR84 and GPR120 receptors are very distantly related to each other and other family A receptors (Gloriam *et al.*, 2007).

C. The FFA1 receptor

A variety of medium- and long-chain FFAs have been identified as agonists on the FFA1 receptor, with eicosatriynoic acid being the most potent example (Table 5.2) (Briscoe *et al.*, 2003; Itoh *et al.*, 2003; Kotarsky *et al.*, 2003). Interestingly, the carbon chain lengths of the saturated fatty acids correlate with the potency, with pentadecanoic acid (C15) and palmitic acid (C16) being the most potent. Contrarily, the chain length does not correlate with the potency of the unsaturated fatty acids (Briscoe *et al.*, 2003). The EC_{50} values in the micromolar range could be perceived as high; however, the blood plasma concentration of the medium- and long-chain FFAs is within this range (Swaminath, 2008). Consequently, the FFA1 receptor could be a nutrient sensor for FFAs consistent with the expression pattern of the receptor in taste buds, the islets of Langerhans from the pancreas, and the gut (Fig. 5.2 and Table 5.2) (Briscoe *et al.*, 2003; Cartoni *et al.*, 2010; Del Guerra *et al.*, 2010; Edfalk *et al.*, 2008; Kotarsky *et al.*, 2003; Ma *et al.*, 2008). This hypothesis has been substantiated by several studies using FFA1 knockout mice, RNA interference, and selective FFA1 small molecule agonists, showing that FFA1 regulates the FFA-mediated release of gastric inhibitory peptide (GIP) and GLP-1 from the gut and the FFA-mediated enhancement of insulin release from β-cells of the pancreas (Briscoe *et al.*, 2006; Christiansen *et al.*, 2008; Del Guerra *et al.*, 2010; Edfalk *et al.*, 2008; Itoh *et al.*, 2003; Pang *et al.*, 2010; Steneberg *et al.*, 2005; Tan *et al.*, 2008). Furthermore, an overexpression study of FFA1 demonstrated that FFA1 regulates glucose-stimulated insulin secretion (Nagasumi *et al.*, 2009). Moreover, FFA1 has been implicated in docosahexaenoic acid-induced neuronal differentiation of neural stem cells (Ma *et al.*, 2010). Recently, low nanomolar potent FFA1 agonists such as Compound C and TUG-424 (Fig. 5.4) have been described of which the former compound lowers glucose tolerance in wild-type mice but not in FFA1 knockout mice (Zhou *et al.*, 2010) and the latter compound increase glucose-stimulated insulin secretion in INS-1E cells (Christiansen *et al.*, 2008). Furthermore, FFA1 knockout mice display an impaired glucose-induced insulin secretion (Alquier *et al.*, 2009). In addition, sulfonamides, such as compound DC260126 (Fig. 5.4), have been found to be a new class of small organic antagonists for the FFA1 receptor that inhibit palmitic acid-induced glucose-stimulated insulin release in Min6 cells (Hu *et al.*, 2009). Recently, using knockout mice it has been shown that FFA1, along with GPR120 (see below), mediate taste detection of fatty acids (Cartoni *et al.*, 2010).

The molecular mechanism of the ligand interaction with the FFA1 receptor is not described in detail, although it seems that the carboxylic group of the FFA is required for receptor activation, since the methyl ester of linoleic acid is unable to activate FFA1, while linoleic acid is an activating ligand (Itoh *et al.*, 2003). In support of this notion, many potent small

Table 5.2 Overview of potencies of fatty acids activating human 7TM receptors along with their expression patterns and suggested physiological functions

Receptor	Major expression	Physiological function	Agonists	pEC_{50}
FFA1[a]	Pancreas, gastrointestinal tract and brain	Glucose-dependent insulin release	*Saturated fatty acids:*	
			Hexanoic acid (C6)	4.33
			Heptanoic acid (C7)	4.28
			Caprylic acid (C8)	4.42
			Nonanoic acid (C9)	4.40
			Capric acid (C10)	4.85
			Undecanoic acid (C11)	4.70
			Lauric acid (dodecanoic acid) (C12)	4.92
			Tridecanoic acid (C13)	4.93
			Myristic acid (C14)	4.84
			Pentadecanoic acid (C15)	5.18
			Palmitic acid (C16)	5.30
			Heptadecanoic acid (C17)	4.99
			Stearic acid (C18)	4.78
			Nonadecanoic acid (C19)	4.52
			Arachidic acid (C20)	4.21
			Heneicosanoic acid (C21)	4.49
			Behenic acid (docosanoic acid) (C22)	4.30
			Tricosanoic acid (C23)	4.31
			Unsaturated fatty acids:	
			Mead acid (C10:3)	5.60
			Palmitoleic acid (C16:1)	4.86
			α-Linolenic acid (C18:3)	4.90
			γ-Linolenic acid (C18:3)	5.05
			Linoleic acid (C18:2)	5.02

(*continued*)

Table 5.2 (*continued*)

Receptor	Major expression	Physiological function	Agonists	pEC_{50}
			Elaidic acid (C18:1)	5.16
			Oleic acid (C18:1)	4.39
			Petroselinic acid (C18:1)	5.00
			All-*trans*-retinal (vitamin A aldehyde) (C20:4)	4.16
			All-*trans*-Retinoic acid (vitamin A acid, tretinoin) (C20:4)	5.58
			cis-*9*-Retinoic acid (*cis*-9-tretinoin) (C20:4)	4.40
			Arachidonic acid (C20:4)	4.92
			(14*R*,15*S*)-Dihydroxyeicosatetraenoic acid (C20:4)	4.63
			Octadecynoic acid (C18:1)	5.12
			Eicosatriynoic acid (C20:3)	5.71
			cis-5,8-Eicosadienoic acid (C20:2)	5.11
			cis-11,14-Eicosadienoic acid (C20:2)	4.97
			cis-11,14,17-Eicosatrienoic acid (C20:3)	4.95
			cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5)	5.17
			Dihomo-γ linolenic acid (C20:3)	5.14
			cis-13,16,19-Docosatrienoic acid (C22:3)	5.17
			cis-7,10,13,16,19-Docosapentaenoic acid (C22:5)	5.33
			cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6)	5.37
			Adrenic acid (C22:4)	4.87

FFA2[b]	Adipose tissue, pancreas, spleen, lymph nodes, and bone marrow	Lipid accumulation, inhibition of lipolysis, and immune function	Formic acid (C1)	1.99–2.61
			Acetic acid (C2)	3.37–4.46
			Propionic acid (C3)	3.54–4.85
			Butyric acid (C4)	3.43–4.55
			Isobutyric acid (C4)	3.22–3.84
			Pivalic acid (C5)	2.34–2.59
			Pentanoic acid (C5)	2.72–3.06
			Isovaleric acid (C5)	2.51–2.67
			Hexanoic acid (C6)	2.86–2.88
FFA3[b]	Immune cells, adipose tissue	Leptin regulation and anti-inflammatory response	Formic acid (C1)	2.11
			Acetic acid (C2)	2.97–2.99
			Propionic acid (C3)	3.90–5.21
			Butyric acid (C4)	3.80–4.38
			Isobutyric acid (C4)	4.31–4.52
			Pivalic acid (C5)	3.19–3.63
			Pentanoic acid (C5)	3.85–4.38
			Isovaleric acid (C5)	3.91–4.24
			Hexanoic acid (C6)	3.87–3.99
GPR84[c]	Leukocytes and monocytes/ macrophages	Immune function	Nonanoic acid (C9)	4.15–4.28
			Capric acid (C10)	5.34–5.35
			Undecanoic acid (C11)	5.07–5.11
			Lauric acid (dodecanoic acid) (C12)	4.98–5.06
			Tridecanoic acid (C13)	4.61–4.67
			Myristic acid (C14)	4.03–4.84

(*continued*)

Table 5.2 (*continued*)

Receptor	Major expression	Physiological function	Agonists	pEC_{50}
GPR120[d]	Gastrointestinal tract, adipose tissue, and lung	GLP-1 secretion	Myristic acid (C14)	4.53
			Palmitic acid (C16)	4.28
			Palmitoleic acid (C16:1)	5.49
			Stearic acid (C18)	4.74
			Elaidic acid (C18:1)	4.48
			Oleic acid (C18:1)	4.51
			α-Linolenic acid (C18:3)	6.37
			γ-Linolenic acid (C18:3)	5.98
			cis-8,11,14-Eicosatrienoic acid (C20:3)	4.84
			cis-11,14,17-Eicosatrienoic acid (C20:3)	5.85
			cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5)	5.55
			cis-7,10,13,16-Docosatetraenoic acid (C22:4)	4.79
			cis-7,10,13,16,19-Docosapentaenoic acid (C22:5)	4.58
			cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6)	5.41

[a] Briscoe *et al.* (2003).
[b] Brown *et al.* (2003), Le Poul *et al.* (2003).
[c] Wang *et al.* (2006a).
[d] Hirasawa *et al.* (2005).

molecule FFA1 ligands with a carboxylic acid residue and only few other functional groups have been developed (Briscoe *et al.*, 2006; Christiansen *et al.*, 2008; Garrido *et al.*, 2006; Tikhonova *et al.*, 2008), suggesting that the FFAs and these small molecules share an overlapping binding site anchored by the carboxylic acid (Smith *et al.*, 2009; Sum *et al.*, 2007; Tikhonova *et al.*, 2007, 2008). Molecular modeling and mutagenesis studies have indicated three polar residues in the 7TM-binding cavity as the potential anchor point of the carboxylic head group of both linoleic acid and the small molecule agonists GW9508 (Fig. 5.4) and rosiglitazone (Smith *et al.*, 2009; Sum *et al.*, 2007; Tikhonova *et al.*, 2007). These amino acids (R183, N244, and R258) are located at the top of transmembrane segments 5, 6, and 7, respectively. Furthermore, a number of other residues have been shown to be involved in FFA1 receptor activation by long-chain FFAs, such as H86 and H137 that could be involved in forming aromatic contacts with GW9508 (Sum *et al.*, 2007; Tikhonova *et al.*, 2007). Additionally, the residue T91 has been implicated to be responsible for the superior potency of the synthetic agonist GW9508 compared to that of the endogenous linoleic acid, as this residue can make a hydrophilic interaction with GW9508 but not with linoleic acid (Sum *et al.*, 2007; Tikhonova *et al.*, 2007). The residues H137, R183, and R258 of the human FFA1 are conserved in FFA2 and FFA3, and the polarity of the residue corresponding to N244 in the human FFA1 is also retained in FFA2 and FFA3 (as H242 and H245, respectively). The conserved arginine residues in FFA2 and FFA3 have recently been identified by a mutational study to be important for the activation by FFAs of the receptors (Stoddart *et al.*, 2008a). Thus, it is feasible that the carboxylate group of the FFA interacts in a similar fashion with FFA2 and FFA3 as it does with FFA1.

D. FFA2 and FFA3 receptors

Several independent studies have identified short-chain FFAs as ligands for both FFA2 and FFA3 (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). There is some overlap in the activity of short-chain FFAs at FFA2 and FFA3 receptors, exemplified by formate, acetate, propionate, butyrate, and pentanonate that activate both FFA2 and FFA3 (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). However, pentanonate is more potent at FFA3 than acetate, in contrast to FFA2, where acetate is more potent than pentanonate (Table 5.2) (Brown *et al.*, 2003; Le Poul *et al.*, 2003). Recently, the first potent and selective FFA2 agonist, phenylacetamide 1 [(*S*)-2-(4-chlorophenyl)-3-methyl-*N*-(thiazol-2-yl)butanamide] (Fig. 5.4), was identified by high-throughput screening (Lee *et al.*, 2008). Interestingly, phenylacetamide 1 is an allosteric agonist as the compound is both able to activate FFA2 alone and to enhance the response of acetate and propionate. Molecular modeling and docking studies have suggested that

the carboxylate group of the FFAs bind to residues at the top of TM5 (Arg180), TM6 (His242), and TM7 (Arg255) as described for FFA1 above, whereas phenylacetamide 1, containing no carboxylate group, binds to an adjacent nonoverlapping pocket (Lee *et al.*, 2008). Characterization of a phenylacetamide 1 analogue performed in a FFA2 knockout mouse model showed a reduced plasma FFA level in wild-type mice, but not in knockout mice (Wang *et al.*, 2010).

The FFA2 receptor displays a widespread expression pattern with the highest levels in adipose tissue, pancreas, spleen, lymph nodes, and bone marrow (Fig. 5.2) (Brown *et al.*, 2003; Le Poul *et al.*, 2003). Several studies have indicated that FFA2 is involved in reduction of lipolysis and activation of adipogenesis (Ge *et al.*, 2008; Hong *et al.*, 2005). Furthermore, FFA2 is expressed in enteroendocrine cells that are expressing peptide YY and mucosal mast cells that contain 5-hydroxytryptamine (Karaki *et al.*, 2006). Interestingly, short-chain FFAs stimulate release of peptide YY (Cherbut *et al.*, 1998) and 5-hydroxytryptamine (Fukumoto *et al.*, 2003) from the ileum and colon (Table 5.2). Thus, it could be hypothesized that FFA2 is involved in this response.

Analysis of mRNA levels in tissues has revealed that FFA3 mRNA is primarily found in immune cells such as neutrophils, monocytes, and B-lymphocytes (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). Significant levels of FFA3 mRNA have been reported in immune tissues such as bone marrow and spleen, which could be due to the high concentration of immune cells in these tissues (Table 5.2) (Le Poul *et al.*, 2003). Additionally, FFA3 has also been described to be expressed in adipose tissue and to be involved in the production of leptin (Xiong *et al.*, 2004). This is contradicted by data showing no expression of FFA3 in adipose tissues and indications of the leptin response being mediated by FFA2 rather than FFA3 (Zaibi *et al.*, 2010). Furthermore, FFA3 expression has been reported in enteroendocrine cells in the gut epithelium (Samuel *et al.*, 2008). Investigation of a knockout mice model of FFA3 showed that depletion of FFA3 is associated with reduced expression of peptide YY, increased intestinal transit rate, and reduced extraction of energy from short-chain FFAs that are produced by the microbial fermentation of indigestible dietary polysaccharides (Samuel *et al.*, 2008). This suggests that FFA3 is involved in regulation of the energy balance through mechanisms that are dependent of the microbial fermentation (Samuel *et al.*, 2008).

Simultaneously with the cloning of the FFA1-3 receptors, a fourth gene, GPR42, was also cloned from the same gene cluster (Sawzdargo *et al.*, 1997). The GPR42 receptor shares 98% sequence identity with FFA3, but is not activated by FFA and is therefore hypothesized to be a pseudogene (Brown *et al.*, 2003). A mutagenesis analysis of the six amino acids that differ between the human FFA3 and GPR42, identified R174 in the second extracellular loop as an essential residue for activation (Brown *et al.*, 2003).

A mutant FFA3 receptor with the R174 residue mutated to the corresponding GPR42 residue, tryptophan, showed no activation by propionate. Conversely, introduction of R174 into GPR42 enables the receptor to be activated by propionate (Brown *et al.*, 2003). Presumably, this R174 residue forms a salt bridge with the carboxylate ligand (Brown *et al.*, 2003) even though the arginine residue is not conserved to FFA1 and FFA2 and thus not part of the previously discussed common anchor site.

E. GPR84 and GPR120

Two additional 7TM receptors, GPR84 and GPR120, have recently also been shown to be activated by FFAs, and not much is known about these receptors so far. The GPR84 receptor is activated by medium-chain FFA (C9-C14) and is expressed in leukocytes and monocytes/macrophages (Wang *et al.*, 2006a). The receptor has only been the subject of one pharmacological study and very little is thus known about the receptor at the moment. However, its expression pattern suggests it may be involved in linking fatty acid metabolism to immunological regulation (Wang *et al.*, 2006a). Like FFA1, GPR120 is a receptor for both saturated (C14–C18) and unsaturated (C16–C22) fatty acids (Table 5.2), and also require the carboxylate group in the ligand for activation (Briscoe *et al.*, 2006; Hirasawa *et al.*, 2005; Tanaka *et al.*, 2008). GPR120 mRNA and protein have been detected in the intestine, adipose tissue, and lung (Fig. 5.2; Hirasawa *et al.*, 2005; Miyauchi *et al.*, 2009; Tanaka *et al.*, 2008). Furthermore, GPR120 has been detected in taste tissue (Cartoni *et al.*, 2010; Matsumura *et al.*, 2007, 2009), suggesting that the receptor might function as a sensor for dietary fat. This has indeed been shown in GPR120 knockout mice which show a reduced preference for linolenic acid and oleic acid, and a reduced taste nerve response to several amino acids (Cartoni *et al.*, 2010). Interestingly, the FFA α-linolenic acid has been shown to stimulate the release of glucagon-like peptide-1 (GLP-1) in STC-1 intestinal endocrine cells and *in vivo* in mice and rats (Hirasawa *et al.*, 2005; Miyauchi *et al.*, 2009). However, α-linolenic acid is an agonist of both GPR120 and FFA1, which are both expressed in the intestine (Fig. 5.2, Table 5.2), and the latter has also been shown to mediate GLP-1 release as previously mentioned.

Recently, selective agonists to GPR120 have been described, which will help in the deduction of the role of GPR120 (Hara *et al.*, 2009; Suzuki *et al.*, 2008). Interestingly, the N/H, R, R residues at the top of TM5, TM6, and TM7, anchoring the carboxylate group in FFA1-3, are absent in GPR84 and GPR120 (Gloriam *et al.*, 2009), suggesting that the binding mode of FFAs in the GPR84 and GPR120 receptors is different from FFA1-3. This is in line with a recent study of small molecule GPR40/GPR120 agonists where modeling suggests that the carboxylate group of these agonists is

anchored by R99 at the top of TM2 (Suzuki *et al.*, 2008). It is thus plausible that the carboxylate group of FFAs is anchored in the same position, but the role of R99 remains to be validated by mutagenesis for both FFAs and the small molecule synthetic agonists.

IV. Therapeutic Perspectives

It is tempting to speculate that the metabolic syndrome, diabetes and/or obesity could be treated by activating the organic nutrient sensors thereby "tricking" the body to believe it has eaten. As shown for at least some of the receptors reviewed here, this could initiate physiological effects such as release of incretins from the gut or hormones from pancreas and fatty tissue. Such a view might be oversimplified, but as shown in Fig. 5.2, many of the promiscuous receptors discussed in the review are expressed in relevant organs and 7TM receptor-mediated signaling appear to exist for the different types of nutrients (Table 5.1). Selective ligands and/or genetically modified mice are now becoming available for some of the receptors, which is making it possible to address their physiological roles as organic nutrient sensors and their potential as drug targets. These tools are most advanced for FFA1 and have shown very interesting results in terms of potential treatment of diabetes/obesity via release of GLP-1 and insulin (Christiansen *et al.*, 2008; Stoddart *et al.*, 2008). However, the FFA1 receptor might also mediate the long-term toxicity of FFAs as shown in some but not all studies using FFA1 knockout mice (Brownlie *et al.*, 2008). More studies are thus needed to validate the FFA1 receptor as a drug target for long-term treatment of obesity/diabetes.

A second case showing that the road to novel drugs might not be that straightforward is the T1R2/T1R3 receptor, activated by sugars and artificial sweeteners, which was recently found to mediate GLP-1 release from the gut (Jang *et al.*, 2007; Kokrashvili *et al.*, 2009) and insulin release from pancreas (Nakagawa *et al.*, 2009). One would thus think that the artificial sweeteners could be used as drugs to treat type 2 diabetes via a dual GLP-1/insulin release. However, epidemiological studies have pointed to increased incidence of obesity and/or metabolic syndrome in diet soft-drink consumers (Dhingra *et al.*, 2007; Lutsey *et al.*, 2008). Additional studies are thus needed to sort out the therapeutic potential of the T1R2/T1R3 receptor.

It is still early days for the nutrient-sensing 7TM receptors discussed in the present review and there is still a lot of work to be done to establish them as validated drug targets. However, the field is evolving quickly as more and more selective pharmacological tools and genetically modified mice become available, which can then be applied in studies delineating the physiological role and therapeutic potential of the nutrient-sensing 7TM receptors.

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CHAPTER SIX

Central Regulation of Glucose-Dependent Insulinotropic Polypeptide Secretion

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Contents

Abstract

Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) are potent stimulators of glucose-dependent insulin secretion, a phenomenon known as incretin effect. After food ingestion, the circulating levels of GIP and GLP-1 rise more quickly than could be explained by the arrival of unabsorbed nutrients that have a direct stimulatory effect on K- and L-cells. Previous studies have thoroughly investigated the possible role of the autonomic nervous system on GIP secretion, demonstrating conflicting results. Recent data from intracerebroventricular infusions of different neuropeptides that participate in the overall regulation of energy homeostasis, suggest the possible existence of additional neuroendocrine mechanisms that may contribute to a central regulation of GIP secretion.

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I. Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino-acid hormone that is produced by enteroendocrine K-cells and released into the circulation in response to nutrient stimulation. Both GIP and glucagon-like peptide-1 (GLP-1) are potent stimulants of glucose-dependent insulin secretion and are classified as incretins (Asmar and Holst, 2010). The identification of the major role of incretins in postprandial insulin secretion and their implication in the pathogenesis of diabetes mellitus type 2, have rendered them pharmaceutical targets for the treatment of diabetes type 2 and obesity-induced diabetes (diabesity). GIP is considered to account for the 50–70% of the overall incretin response (Gault *et al.*, 2002) and, besides pancreatic β-cells, functional GIP receptors have been identified in a wide range of tissues.

Despite the extended research on the action of GIP, the mechanisms that regulate its secretion, apart from the well-documented role of nutrients, is far from being fully elucidated.

The enteroendocrine K- and L-cells are "open-type" endocrine cells, which are stimulated by direct contact with nutrients present in the intestinal lumen. The distribution of K-cells is mainly in the duodenum but they can also be found throughout the small intestine. Similarly, L-cells are found in highest density in jejunum and the distal ileum but they have also been identified in the colon.

However, the concentrations of GIP and GLP-1 rise more quickly than could be explained by the arrival of unabsorbed nutrients having a direct stimulatory effect on these distally located cells (Deacon, 2005). This rapid increase in GIP and GLP-1 concentrations in the first 3–5 min after food ingestion has pointed to a neural regulation of incretin secretion.

II. Structure and Action of GIP

Structure-activity studies on GIP and GIP analogs have shown that the N-terminus and central region of the GIP molecule is critical for its biological activity (Hinke *et al.*, 2003). Truncated forms of GIP, such as GIP 1–39 (Sandberg *et al.*, 1986) and GIP 1–30 (Wheeler *et al.*, 1995), preserve high biological activity, while the GIP metabolite (GIP 3–42), produced after cleavage by the enzyme dipeptidyl-peptidase IV (McIntosh, 2008), is lacking insulinotropic activity (Schmidt *et al.*, 1987).

Although GIP was originally identified on the basis of its effects on gastric function, its insulinotropic activity is now considered to be of major

importance. The most well-known action of GIP, at least until now, is the stimulation of insulin release from pancreatic β-cells via glucose-dependent mechanisms (Schauder *et al.*, 1975). Studies in GIP-R knockout and double knockout (GIP-R−/− and GLP-1 R−/−, DIRKO) mice have confirmed the importance of GIP action on glucose homeostasis (Hansotia *et al.*, 2004, 2007; Preitner *et al.*, 2004). In addition, *in vivo* and *in vitro* data have shown that GIP also potentiates leucine- and arginine-induced insulin secretion (Mazzaferri *et al.*, 1983).

GIP receptors have also been identified in pancreatic α-cells, although its physiological action is still controversial. However, recent data demonstrated that GIP administration rapidly increases glucagon levels under fasting conditions (Morgan, 1996; Nauck, 2003), suggesting a potential physiological role for GIP in maintaining normoglycemia in the absence of nutrients.

Apart from the regulation of insulin secretion, GIP has also been shown to protect pancreatic islets from glucotoxicity- and lipotoxicity-induced apoptosis, in rodents (Kim *et al.*, 2005).

Furthermore, a number of studies have been focused on a direct action of GIP in adipocytes. GIP was found to enhance the lipolytic activity of lipoprotein lipase (LPL) in cultured preadipocytes and mature adipocytes (Kim *et al.*, 2007; Knapper *et al.*, 1995), in the presence of high concentrations of free fatty acids (FFAs). On the other hand, GIP also stimulates lipolysis (McIntosh *et al.*, 1999; Yip *et al.*, 1998) during fasting conditions. GIP's lipolytic action on differentiated adipocytic cell line (3T3-L1) is inhibited by insulin (McIntosh *et al.*, 1999), and thus it is suggested that GIP may play an important role in maintaining circulating FFAs at appropriate levels during fasting, when insulin levels are low.

Recent data have also demonstrated a potential anabolic action of GIP in bone tissue. Expression of GIP-R was identified in osteoblastic cell lines (SaOs2 and MG63) in osteoclasts and in bone marrow stromal cells (Bollag *et al.*, 2000, 2001; Ding *et al.*, 2008; Zhong *et al.*, 2007). GIP increases alkaline phosphatase activity and expression of collagen type 1 from osteoblasts, while it decreases PTH-induced osteoclastic bone resorption (Zhong *et al.*, 2007). Thus, although previous studies had excluded the role of GIP (Henriksen *et al.*, 2003) in postprandial bone response, it is now suggested that GIP may also play a role in the postprandial reduction of osteoclastic bone resorption. Furthermore, expression of GIP-R in bone marrow stromal cells was related to prevention of age-induced loss of bone mass and bone strength (Ding *et al.*, 2008). In line with the *in vitro* data, *in vivo* studies with GIP-R knockout or GIP-R overexpressing mice, demonstrated osteopenia or increased bone density, respectively (Xie *et al.*, 2005, 2007).

III. Regulation of GIP Secretion

In humans, total GIP levels increase up to sixfold in response to nutrients (Creutzfeldt and Ebert, 1977; Krarup, 1988), especially fat.

Triglycerides have been found to strongly stimulate GIP release in both human and animal studies (Creutzfeldt and Ebert, 1977; Krarup, *et al.*, 1985; Pederson *et al.*, 1975). However, it has been shown that its response to oral or intraduodenal fat is slower and much more prolonged than it is with glucose (Krarup *et al.*, 1985), partly because of delayed gastric emptying. Oral glucose intake (Andersen *et al.*, 1978; Creutzfeldt and Ebert, 1977; Krarup *et al.*, 1985) results in rapid release of GIP, with levels reaching a peak within 15–30 min and returning to fasting values after approximately 3 h, with a similar time course to circulating levels of glucose and insulin.

In general, response of GIP to nutrients is mainly dependent on the meal size and their rate of absorption from the small intestine (Chaikomin *et al.*, 2005). Increased content of carbohydrate or fat (Murphy *et al.*, 1995) in a meal results in greater GIP responses, while reduced nutrient absorption, as seen in inflammatory bowel diseases, results in lower GIP responses (Besterman *et al.*, 1979).

IV. Neural Regulation of GIP Secretion

Several studies have suggested a nervous regulation of GLP-1 secretion, mainly through the autonomic nervous system.

V. The Role of Autonomic Nervous System

Autonomic and endocrine responses to food ingestion, which are triggered by sensory mechanisms and not by the absorbed nutrients, are called cephalic phase responses.

The cephalic response of insulin secretion consists of a rapid, within the first 3–4 min, increase in circulating insulin levels after oral glucose, before any increase in circulating glucose (Teff *et al.*, 1993). Even though the contribution of the cephalic phase is only $1 \pm 3\%$ of the total insulin secretion after glucose intake (Teff and Engelman, 1996), it is of major importance for postprandial glucose levels and glucose tolerance. Studies in humans and rodents have shown that inhibition of the preabsorptive insulin response by somatostatin or the ganglionic antagonist, trimetophane, resulted in impaired glucose tolerance and increased glucose concentrations

at 45 and 60 min after food intake (Calles-Escandon and Robbins, 1987). On the other hand, short-term treatment with insulin during the first 15 min after food ingestion improved glucose tolerance in type 2 diabetic patients (Bruttomesso *et al.*, 1999).

The rich innervation of pancreatic islets by parasympathetic, sympathetic, and sensory nerves (Ahren, 2000), and the complete inhibition of cephalic phase of insulin secretion after vagotomy, ganglionic blockade, or muscarinic antagonism (Berthoud *et al.*, 1981; Strubbe 1992; Strubbe and Steffens, 1975), suggested a possible role of the autonomic nervous system in the neural regulation of the cephalic phase of insulin secretion. Further research revealed that the central integrative circuit of the cephalic insulin response is localized in the ventro-medial hypothalamus and the dorsal motor nucleus of the vagus nerve (Berthoud and Powley, 1990; Berthoud *et al.*, 1981), and the efferent neural pathway is mediated through both cholinergic and noncholinergic mechanisms.

Despite the major contribution of incretins GIP and GLP-1 in the postprandial insulin secretion after oral glucose, the preabsorptive insulin response was not found to be related to the circulating levels of GLP-1 and GIP (Ahrén and Holst, 2001). However, the role of the autonomic nervous system in the neural regulation of the incretin hormones secretion has also been thoroughly investigated.

A. Parasympathetic nervous system

Older studies had demonstrated that apart from the well-documented role of fat, glucose, and amino acids in GIP secretion, gastric acid is also a potent stimulant of GIP release (LeRoith *et al.*, 1980). A positive correlation was found between maximum acid output in response to tetragastrin and integrated incremental response of GIP after oral glucose loading (Imamura *et al.*, 1984). In accordance with these findings, other researchers had reported that both fasting and nutrient-stimulated GIP and insulin levels were significantly higher in duodenal ulcer patients (Arnold *et al.*, 1978). However, clinical studies with duodenal ulcer patients had also shown that this hyperacidity might be related to vagal hyperactivity (Jaup, 1988).

Reports from patients with vagotomy and pyloroplasty, demonstrated controversial results, showing either enhancement of GIP response to nutrients or no effect at all (Gayle and Ludewig, 1978; Sirinek *et al.*, 1974; Thomford *et al.*, 1974). Further investigation on the role of vagus nerve in neural regulation of GIP secretion (Imamura *et al.*, 1984) suggested that the participation of the celiac branch of the vagal nerve was mainly involved in the release of GIP. Thus, the integrated incremental responses of GIP after oral glucose loading was greater, after selective vagotomy with pyloroplasty, where both celiac and hepatic branches were preserved, than after truncal vagotomy and pyloroplasty. In addition, Yoshiya *et al.* (1985)

had reported a differential effect of vagotomy on postprandial GIP response with regard to nutrients. In this study (Yoshiya *et al.*, 1985), truncal vagotomy increased significantly glucose-induced GIP release but had no effect on fat-induced GIP release.

On the contrary, administration of the muscarinic acetylocholine receptor antagonist, atropine, in humans and animal models did not show any significant modification of GIP response to glucose load (Nelson *et al.*, 1986; Greenberg and Pokol-Daniel, 1994). Moreover, when interruption of vagal conduction was performed by reversible blockade by cooling, to avoid adaptive changes, and the nutrient load was given to conscious dogs intraduodenally, so as to avoid variations in gastric emptying, there was no effect in the postprandial plasma GIP levels (Greenberg and Pokol-Daniel, 1994).

This discrepancy of the results on the potential role of the parasympathetic nervous system in GIP may be attributed to the diversity of experimental procedures that were used, including, diet format, size of vagotomy, and oral or intraduodenal administration of nutrient load. It should also be emphasized that alteration in gastric emptying and gastric acid secretion that usually accompanies altered parasympathetic discharge is an important factor that could mediate the effect of the parasympathetic nervous system in the regulation of GIP secretion. Furthermore, patients with vagotomy and autonomic neuropathy are frequently presented with impairment of gastric emptying. Therefore, even though insulin response to nutrient is regulated by vagal and nonvagal muscarinic pathways, the parasympathetic nervous system does not seem to play a direct major role in the neural regulation of GIP secretion.

B. Sympathetic nervous system

Intravenous infusion of β-adrenergic agonists in healthy volunteers increased both insulin and plasma GIP levels in fasting conditions and enhanced glucose-induced secretion of both peptides (Flaten *et al.*, 1982; Kogire *et al.*, 1990). On the other hand, α-adrenergic stimulation reduced plasma GIP response and completely inhibited insulin response to oral glucose load, while no alteration was reported in fasting GIP levels (Salera *et al.*, 1982). One study, however, failed to detect any significant modifications of GIP secretion after beta or alpha adrenergic blockade during glucose load (Nelson *et al.*, 1986).

C. The role of neuropeptides

Apart from being richly innervated by cholinergic and adrenergic nerves, other nerve fibers have also been identified in the enteric nervous system.

Neuropeptide Y (NPY) is a 36-amino-acid neurotransmitter, which is present in both the central, mainly in the neurons of the hypothalamic

arcuate nucleus, and the enteric nervous system. NPY-positive nerve cell bodies and fibers have been found in the myenteric and the submucosal nerve plexus, and also in enteroendocrine cells of the gastrointestinal tract (Goumain *et al.*, 1998; Wang *et al.*, 1997). Along with other neuropeptides, such as cholecystokinin (CCK), calcitonin gene-related peptide (CGRP), and gastrin-related peptide (GRP), NPY is cosecreted with norepinephrine in sympathetic nerves (Swart *et al.*, 2001; Taylor and Bywater, 1988). It is regarded to be one of the most potent orexigenic neuropeptides of the brain, with a preference in carbohydrate intake (Levine *et al.*, 2003; Stanley *et al.*, 1992).

Infusion of NPY into the hypothalamus and cerebral ventricles stimulated insulin secretion in rats, independent of the presence of nutrients in the gastrointestinal tract (Marks and Waite, 1996). In turn, insulin was found to inhibit NPY expression in the arcuate nucleus of the hypothalamus through GABAergic systems, in a negative feedback regulation of appetite (Sato *et al.*, 2005). However, studies with NPY-deficient mice did not confirm the suggested positive regulation of insulin secretion by NPY (Imai *et al.*, 2007).

Neural regulation of GIP secretion by NPY was studied in conscious dogs in our laboratory, using a prototype epicranial apparatus surgically placed in the skull, which allowed easy and exact localization of the third ventricle for infusions or sampling. We demonstrated that intracerebroventricular (icv) infusion of NPY (5, 10, and 25 μg) dose-dependently increased the circulating levels of GIP in the absence of nutrients (Fig. 6.1) (Yavropoulou *et al.*, 2008). Consistent with a previous study (Marks and

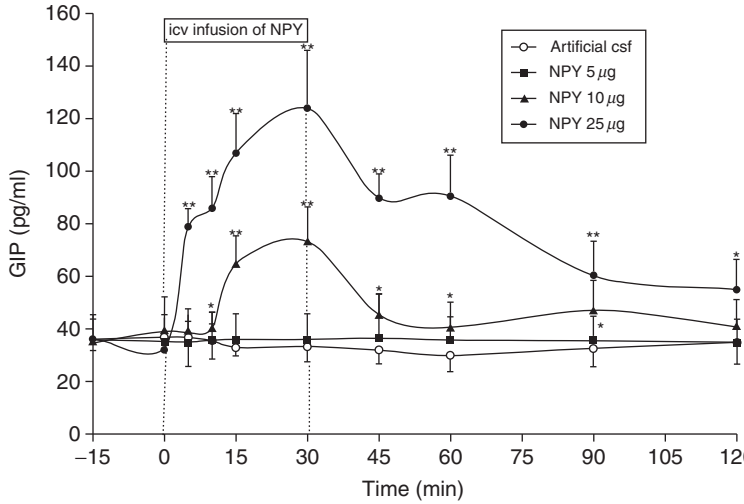

Figure 6.1 Change in plasma GIP levels after icv infusion of NPY. All values are expressed as means ± SEM. $*p < 0.05$ and $**p < 0.001$. csf; cerebrospinal fluid.

Waite, 1996), icv infusion of NPY also produced a smaller increase in the circulating levels of insulin without any significant effect on glucose levels. Thus, increased hypothalamic levels of NPY exerted a positive effect in GIP and insulin secretion, independent of the ambient glucose concentrations and without significantly facilitating the incretin effect of the former.

A role for GIP in the fasting state has been demonstrated in *in vitro* studies with the differentiated adipocytic cell line 3T3-L1, where it exerted a direct lipolytic effect, which was inhibited by the presence of insulin (McIntosh, *et al.*, 1999). On the other hand, NPY has an anabolic role in adipose tissue, increasing LPL activity, and insulin sensitivity of adipocytes (Billington *et al.*, 1994). We hypothesize that a possible neurally mediated interaction of NPY and GIP can coordinate more than one component of energy homeostasis, integrating a regulatory network that involves the central nervous system and peripheral organs such as the gastrointestinal tract, the adipose tissue, and the pancreas.

Neurotensin levels are known to increase rapidly into the circulation after meal ingestion (Gullo *et al.*, 1998; Shaw and Buchanan, 1983). Apart from direct stimulation of neurotensin secreting cells (N-cells) in the intestinal mucosal in the presence of nutrients, it has been reported that the anorexigenic hormones leptin, insulin, and a-melanocyte-stimulating hormone are also capable of directly inducing neurotensin gene expression (Cui *et al.*, 2005) in neurotensin-expressing hypothalamic cell lines. In addition, central injection of neurotensin reduces food intake through neurotensin receptor 1 (Ntsr1) and is also postulated to mediate, in part, the anorectic effects of leptin in the hypothalamus (Kim *et al.*, 2008). Intracerebroventricular administration of neurotensin (NT), also produced significant changes in the glucostatic parameters and in GIP secretion independently of the presence of nutrients in the gastrointestinal tract (Fig. 6.2) (Yavropoulou *et al.*, 2010a). Bolus and continuous (over a 3-h period) icv infusion of neurotensin demonstrated approximately the same results in blood glucose, insulin, and GIP levels, suggesting a possible saturation of the hypothalamic NT receptors after the first minutes of the infusion. Expanding our study, we also concurrently administered glucose intraduodenally through a Mann–Bollman fistula, to simulate for post-prandial conditions. The Mann–Bollman fistula was established using an isolated ileum loop, the distal end of which was connected laterally to the duodenum, and the open proximal (oral) end was sutured to the abdominal wall.

When icv infusion of neurotensin was accompanied by intraduodenal administration of glucose, it produced a more significant increase of insulin and glucose levels, compared to infusion of artificial cerebrospinal fluid (aCSF). Interestingly, however, GIP response to glucose load was blunted after icv infusion of NT compared to infusion of aCSF (Yavropoulou *et al.*, 2010a) (Fig. 6.3).

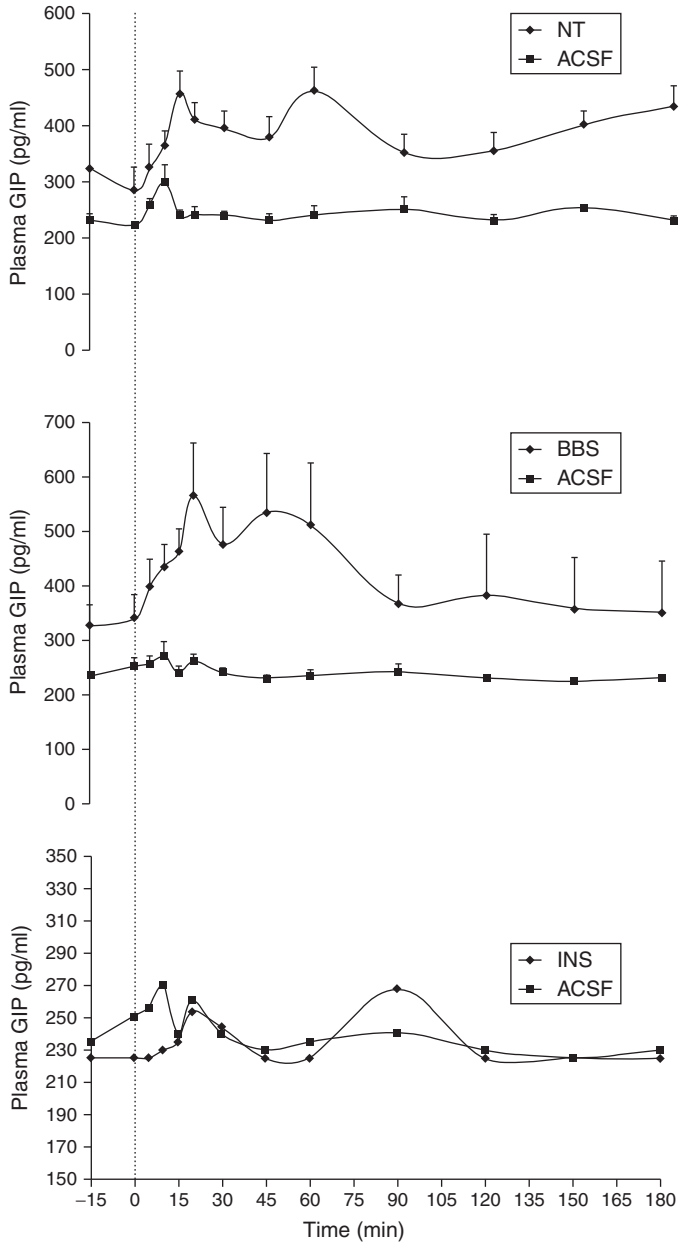

Figure 6.2 Change in plasma GIP levels after bolus icv infusion of 50 μg neurotensin (NT), 200 ng bombesin (BBS), and 500 μU crystal insulin (INS) or aCSF (artificial cerebrospinal fluid) over a 3-h period. All values are expressed as means ± SEM. $^{\star}p < 0.05$.

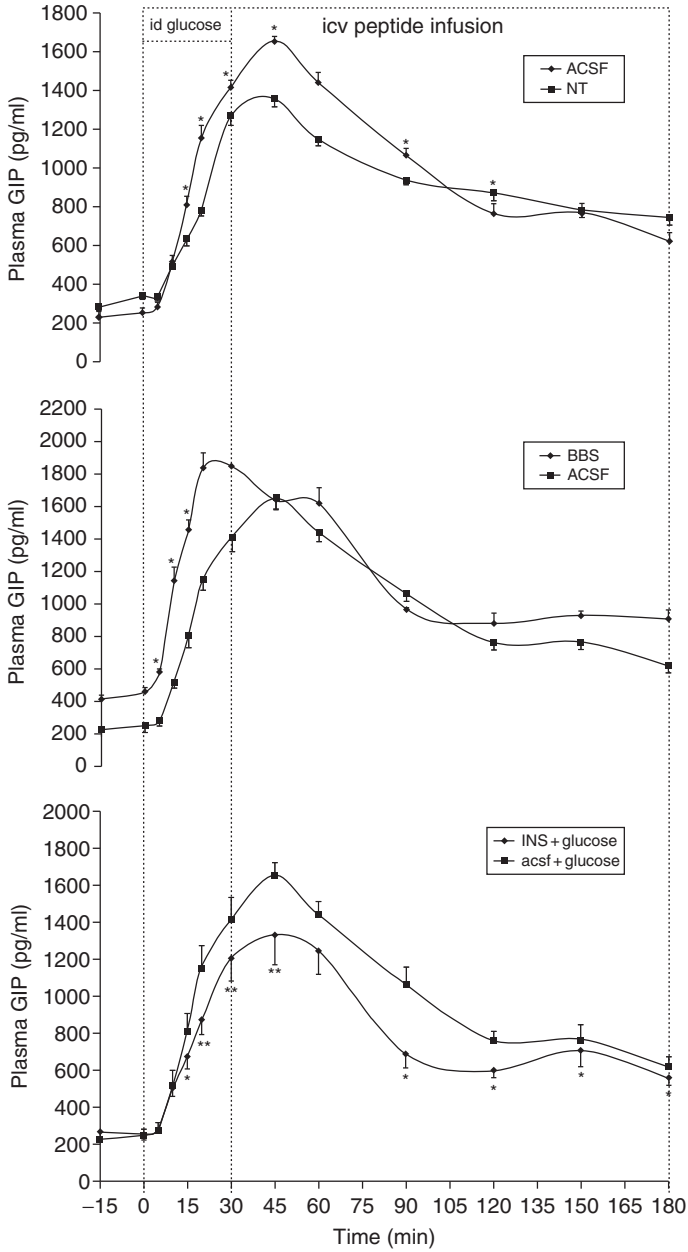

Figure 6.3 Change in plasma GIP levels after continuous icv infusion of regular insulin (INS) (250 μU/kg/h), neurotensin (NT) (1.0 μg/kg/h), and bombesin (BBS) (5.4 ng/kg/h) or aCSF (artificial cerebrospinal fluid) over a 3-h period with a simultaneous intraduodenal infusion of a glucose load for 30 min. All values are expressed as means ± SEM. $\star p < 0.05$ and $\star\star p < 0.02$.

Although very few data refer to the possible negative feedback regulation of GIP secretion by peripheral levels of insulin (Bryer-Ash *et al.*, 1994), our study cannot exclude the contribution of opposing influences between plasma insulin and hypothalamic neurotensin in GIP secretion after a glucose load. Nevertheless, it could be that the central levels of neurotensin exert a differential effect on GIP secretion dependent on the energy load provided.

We have no data concerning the pathway that mediates this neural modulation of GIP secretion by neurotensin. Previous older studies have demonstrated that neurotensin-expressing neurons are also involved in the regulation of efferent sympathetic nerve discharge (Kenney *et al.*, 2003), and the central effect of neurotensin was abolished after administration of the antihistamine agent diphenhydramine (Nagai and Frohman, 1978). Thus, regulation of the autonomic nervous system or histaminergic receptors could have a role in the neural regulation of GIP secretion by neurotensin.

In vitro studies in isolated intestinal cells and *in vivo* studies in rats and dogs have demonstrated that noncholinergic, nonadrenergic nerves releasing GRP can also stimulate GIP secretion in a dose-dependent manner (Greenberg *et al.*, 1985; Kieffer *et al.*, 1994; Varga *et al.*, 1994).

Gastrin-releasing peptide is a 27-amino-acid peptide, which presents a strong homology with the carboxyl terminus of bombesin (McDonald *et al.*, 1979). Even though GRP has been originally considered to be the mammalian counterpart of the amphibian bombesin (Reeve *et al.*, 1983), the existence of two different genes encoding bombesin and GRP in amphibians raised the possibility that there is a gene encoding a true mammalian bombesin yet to be characterized (Nagalla *et al.*, 1992). Moreover, it had been reported that bombesin can act through activation of GRP preferring receptors, located on the surface of the pancreatic β-cells and intestinal cells (Kieffer *et al.*, 1994; Kloss *et al.*, 1999, Varga *et al.*, 1994), and bombesin-like peptides produced by enteric neurons are well-known secretagogues of GIP release (Kieffer *et al.*, 1994). We have shown that icv administration of bombesin in conscious dogs produces a significant increase in plasma GIP levels, a few minutes after the beginning of both bolus and continuous infusions, in the absence of nutrients in the gastrointestinal tract (Fig. 6.2), accompanied by a strong clinical reaction in the animals indicative of a robust sympathetic stimulation (Yavropoulou *et al.*, 2010b). Plasma bombesin levels failed to increase after icv administration of this neuropeptide, pointing to a possible neural-mediated effect.

Even though the centrally administered bombesin has been reported to increase epinephrine and norepinephrine levels by activating the cells of adrenal medulla (Usui *et al.*, 2009), epinephrine alone did not seem to influence basal GIP levels in the absence of nutrients (Salera *et al.*, 1982). Thus, the increase in GIP secretion after bolus and continuous infusion of bombesin is only partly, if it is at all, attributed to adrenal stimulation. In this experiment, simultaneous

intraduodenal infusion of glucose produced a more pronounced increase in GIP levels after icv infusion of bombesin (Fig. 6.3).

Finally, when we administered insulin intracerebroventricularly (Yavropoulou *et al.*, 2009), there was no effect on GIP secretion in the absence of nutrients (Fig. 6.2), although plasma insulin levels were increased, correlating with the increased levels in the cerebrospinal fluid (Wallum *et al.*, 1987). Simultaneous intraduodenal glucose produced a partial inhibition of glucose-stimulated GIP secretion, suggesting that hypothalamic insulin levels could participate, at least in part, in the inhibition of GIP secretion that is observed after glucose load (Bryer-Ash *et al.*, 1994).

VI. Concluding Remarks

In the last few years, there has been particular interest in understanding the molecular mechanisms that regulate release of GIP from K-cells. The wide tissue distribution of GIP receptors have pointed to a more general contribution of GIP in energy homeostasis in both fasting and postprandial state, except for the well-documented insulinotropic effect. Data presented here suggest a tight regulation of neural GIP secretion, independent of the presence of nutrients, by neuropeptides that also exert a significant influence on feeding behavior and energy disposal in peripheral tissues and provide further support to the critical role of the central nervous system in the regulation of energy management. Future research is warranted to elucidate the still unknown aspects of the mechanisms that regulate GIP release from K-cells and its functional role regardless of the presence of food in the gastrointestinal system, as a first step in the development of potential drugs in the treatment of diet-induced obesity and diabetes type 2.

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CHAPTER SEVEN

Incretin Hormone Secretion Over the Day

Bo Ahrén,* Richard D. Carr,† *and* Carolyn F. Deacon‡

Contents

Abstract

The two incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are key factors in the regulation of islet function and glucose metabolism, and incretin-based therapy for type 2 diabetes has gained considerable interest during recent years. Regulation of incretin

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hormone secretion is less well characterized. The main stimulus for incretin hormone secretion is presence of nutrients in the intestinal lumen, and carbohydrate, fat as well as protein all have the capacity to stimulate GIP and GLP-1 secretion. More recently, it has been established that a diurnal regulation exists with incretin hormone secretion to an identical meal being greater when the meal is served in the morning compared to in the afternoon. Finally, whether incretin hormone secretion is altered in disease states is an area with, so far, controversial results in different studies, although some studies have demonstrated reduced incretin hormone secretion in type 2 diabetes. This review summarizes our knowledge on regulation of incretin hormone secretion and its potential changes in disease states.

I. Introduction

The incretin hormones are of vital importance for a normal insulin secretion and glucose tolerance. They are released from the gut after meal ingestion or a glucose load, and stimulate insulin secretion (Creutzfeldt, 1979). By comparing the insulin response to oral glucose ingestion with isoglycaemic intravenous glucose infusion (i.e., resulting in matched blood glucose levels), it has been estimated that the incretin effect can account for more than 70% of the insulin response to an oral glucose load (depending on the amount of glucose administered) in humans (Nauck *et al.*, 1986a). A recent study in mice showed that a similar importance of the incretin hormones also exists in rodents (Ahrén *et al.*, 2008).

In daily life, incretins are released throughout the day (even in the overnight fasting period) with increases in relation to meal and snack ingestion. Therefore, full appreciation of the physiological impact of incretin hormones for islet function and glucose homeostasis requires consideration of incretin hormone secretion in relation not only to oral glucose, but also to ingestion of other macronutrients, together with meal (and snack) ingestion at different times of the day. This review summarizes the present knowledge on incretin hormone secretion with emphases on humans studies.

II. GIP and GLP-1 Secretion After Meal Ingestion

The two most important incretin hormones are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Kim and Egan, 2008). GIP is secreted from the intestinal K-cells, located predominantly in the duodenum, while GLP-1 is produced by the more distally located (ileum and colon) L-cells. However, detailed immunohistological studies revealed that GIP- and GLP-1 positive cells are actually

found throughout the entire small intestine, with a subset of cells staining positively for both incretin hormones (Mortensen *et al.*, 2003). Thus, the incretin producing cells are not restricted to the duodenum (K-cells) or distal ileum (L-cells) but occur throughout the intestine (Deacon, 2005; Holst, 2007).

The main stimulus for incretin hormone secretion is the presence of nutrients in the intestinal lumen. Consequently, their release throughout a 24-h period largely coincides with meal ingestion, as has been reported in several studies (Elliott *et al.*, 1993; Mari *et al.*, 2005a; Ørskov *et al.*, 1996). After meal ingestion, the initial response of the two incretins is rapid and the increased levels above baseline persist for several hours. The dynamics of incretin hormone secretion after meal ingestion are shown in Fig. 7.1. A mixed meal (560 kcal) was served to 12 healthy lean male volunteers (age 20–30 years, BMI 20–25 kg/m^2) over 5 min and plasma levels of GIP and GLP-1 were determined using antibodies directed to the C-terminal end of each of them. These antibodies, therefore, detect both the intact forms of the incretins, as well as their primary metabolites, which lack insulinotropic activity. This is important when estimating incretin hormone secretion, because of the rapid cleavage of the N-terminal end of the peptide after their secretion (halftime 1–2 min for intact GLP-1 and 7 min for intact GIP) by the enzyme dipeptidyl peptidase-4 (Deacon, 2005; Holst, 2007). Figure 7.1 shows that both GIP and GLP-1 are secreted after meal ingestion

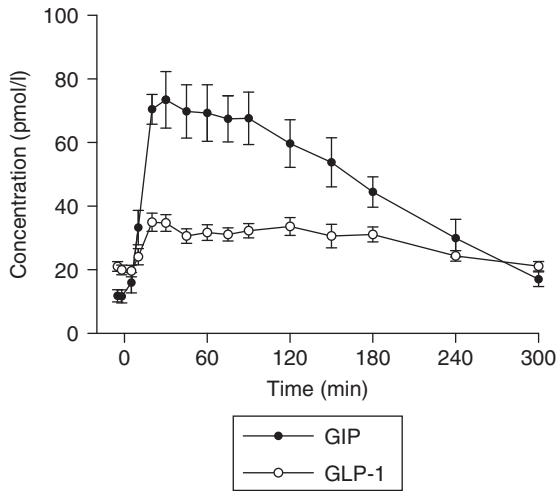

Figure 7.1 Plasma levels of GIP and GLP-1 (determined by C-terminally directed assay, that is, measuring total incretin hormone concentrations) after ingestion of a standardized mixed meal (560 kcal) in healthy male volunteers, aged 20–30 years ($n = 12$). Results are presented as mean ± SEM.

and that on a molar basis, more GIP than GLP-1 is released. Levels of GIP increase significantly from baseline values of around 12 pmol/l to ~16 pmol/l already after 5 min. This illustrates the rapidity of GIP secretion after meal ingestion, which probably reflects the proximity of the bulk of GIP cells in the duodenum. Notably, GLP-1 levels also increase rapidly, albeit not quite as quickly as GIP; hence, while the 5 min level is not significantly different from baseline, after 10 min, GLP-1 levels have significantly increased from the baseline of ~20–24 pmol/l. This suggests that the GLP-1 rapidly secreted after meal ingestion emanates from L-cells which are located more proximally in the small intestine, and is consistent with a finding that the early and rapid GLP-1 response to oral glucose is not altered by intestinal resection (Nauck *et al.*, 1996). It should be emphasized, however, that the early GLP-1 response to meal ingestion is delayed when compared with GIP (see Fig. 7.1).

After the early incretin hormone secretion after meal ingestion, GIP levels continue to increase over the first half hour after meal ingestion to reach a maximum after 30 min; thereafter, a slow decline is observed and the levels do not reach baseline until after 240 min. Also GLP-1 levels reach maximum after 30 min and then return to baseline which is reached after 240 min. Therefore, following meal ingestion, incretin hormone secretion is stimulated rapidly and also persistently for 3–4 h.

III. Regulation of GIP and GLP-1 Secretion

A. Nutrients

The main stimulus for incretin hormone secretion after meal ingestion is the nutrients derived from food. These nutrients pass along the intestine and reach the K- and L-cells from the intestinal luminal side. Both cell types are located in the gut epithelia and are "open-type" endocrine cells, which means that they have apical surfaces which open into the gut lumen (Buchan *et al.*, 1978; Eissele *et al.*, 1992). Nutrients can therefore come in to contact directly with the plasma membrane of the cells, initiating a series of intracellular events which ultimately results in stimulation of secretion (Deacon, 2005). A study comparing the levels of total GIP and GLP-1 after test meals comprising of 375 kcal of carbohydrate, fat, or protein in healthy subjects reported that carbohydrate and fat but not protein stimulated a substantial GIP secretion, whereas all three types of macronutrients stimulated GLP-1 secretion (Elliott *et al.*, 1993). We recently reexamined whether or not all macronutrients stimulate GIP and GLP-1 secretion in more detail. We thereby administered equicaloric amounts (8 kcal/kg body weight) of pure glucose, pure fat (olive oil), or a protein mixture (Promax protein 85) consisting of milk and egg protein to 12 healthy lean male

volunteers (age 20–30 years, BMI 20–25 kg/m^2) over 5 min and determined the concentrations of GIP and GLP-1 in samples taken the following 5 h. We observed that all three types of nutrients stimulate secretion of both incretins (Carr *et al.*, 2008, 2010). Hence, our data suggest that all types of nutrients stimulate incretin hormone secretion, and that the rapid and robust release of the incretins after meal ingestion is explained by the nutrients reaching the K- and L-cells rapidly after ingestion.

B. Meal size

It has also been shown that secretion of the incretin hormones is related to meal size (Vilsbøll *et al.*, 2003a,b). Thus, the 3 h GLP-1 and GIP secretion, as determined by the area under the curve of the total GLP-1 and GIP concentrations, were 75% and 60% higher, respectively, after a 560 kcal meal compared to a 265 kcal meal (Vilsbøll *et al.*, 2003a,b). Furthermore, the 20 min peak GLP-1 levels were ~100% higher after ingestion of 100 g glucose compared to 50 g glucose in healthy subjects (Schirra *et al.*, 1996).

C. Hormones and autonomic nerves

Nutrients may, however, not be the only stimuli for incretin hormone secretion after meal ingestion, and involvement of both hormonal and neural mechanisms have been suggested from studies in animal models (Deacon, 2005; Dubé and Brubaker, 2004). However, whether hormones play a role in the stimulation of incretin hormone secretion in humans is still controversial, since administration of the known gastrointestinal hormones has not been shown to increase GIP or GLP-1 secretion in humans (Deacon, 2005). Conversely, it appears that somatostatin exerts a tonic suppressive effect to restrain L-cell secretion, as evident in the perfused porcine ileum (Hansen *et al.*, 2004) probably via a local paracrine action. Following meal ingestion, autonomic nerves are activated and for insulin secretion, a rapid and early so-called cephalic response of insulin secretion is seen after meal ingestion which is dependent on autonomic nerves (Ahrén, 2000). This has also been shown for incretin hormone secretion in rats (Vahl *et al.*, 2010). Furthermore, muscarinic and β2-adrenoceptor agonists have been shown to stimulate incretin hormone secretion *in vitro*, suggesting that autonomic nerves might be involved (Dumoulin *et al.*, 1995). However, a cephalic phase does not seem to be operative for incretin hormone secretion in humans. This was evident from a study showing that disruption of the autonomic signal in humans using the ganglionic antagonist, trimetophane, which inhibits the early insulin response to meal ingestion, does not affect the GIP or GLP-1 response to meal ingestion in humans (Ahrén and Holst, 2001).

D. Gastric emptying

In addition, it has been shown that GLP-1 secretion after a test meal or oral glucose correlates to the rate of gastric emptying (Miholic *et al.*, 1991). In fact, the rate at which nutrients are delivered into the duodenum from the stomach seems to be a controlling mechanism for GLP-1 secretion (Schirra *et al.*, 1996). In contrast, GIP secretion does not seem to be as dependent on rate of gastric emptying as GLP-1 secretion, but rather the rate of nutrient absorption from the duodenum (Schirra *et al.*, 1996).

E. Gastric distension

Another mechanism underlying incretin hormone release following meal ingestion could be the increased gastric volume which occurs after meal ingestion. Increases in gastric volume *per se* are known to affect gut hormone profiles independent from nutrient entry in the gut (Vazzquez Roque *et al.*, 2006). Whether this contributes to incretin hormone secretion after meal ingestion was estimated by administering a load of water holding the same volume as a mixed meal (400 ml) to 12 healthy volunteers. Determination of the subsequent plasma levels of the incretin hormones over the following 5 h revealed that the water load did indeed increase both GIP and GLP-1 secretion (Fig. 7.2). Plasma GIP levels were elevated for 45 min, whereas the effect of the water load on GLP-1 secretion was more prolonged, with increased plasma GLP-1 levels being sustained for 150 min. From a quantitative point of view, however, this water effect is marginal compared to the effect of the meal itself; suprabasal AUC for GIP after water ingestion corresponds to ≈2% of that after meal ingestion, and the corresponding figure for GLP-1 is ≈4.5%. Nevertheless, this gastric distension phase might contribute to a certain degree.

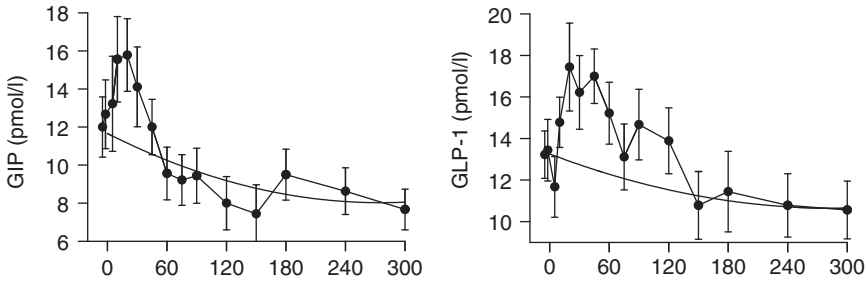

Figure 7.2 Plasma levels of GIP and GLP-1 (determined by C-terminally directed assay, that is, measuring total incretin hormone concentrations) after ingestion of plain water (400 ml) in healthy male volunteers, aged 20–30 years ($n = 12$). Results are presented as mean ± SEM. The line indicates correlation between time and concentration for baseline values and values from min 60 (GIP) and 120 (GLP-1).

IV. Mechanisms of GIP and GLP-1 Secretion

The cellular basis for incretin hormone secretion is less well established, although some information exists. A mechanism for glucose-induced incretin hormone secretion has been proposed involving the sodium glucose cotransporter 1 (SGLT-1). Thus, the SGLT-1 inhibitor, phloridzin has been shown to inhibit glucose-stimulated GIP secretion in rodent intestine (Sykes *et al.*, 1980) and GLP-1 secretion from the isolated perfused canine ileum (Sugiyama *et al.*, 1994). It was recently also proposed that the mechanism involves co-uptake of each glucose molecule with one or two sodium ions, which in turn generates an inward current causing the cell to depolarize, thereby triggering action potentials with subsequent opening of voltage dependent calcium channels (Gribble *et al.*, 2003). However, other mechanisms, including closure of ATP-dependent potassium channels (Gribble *et al.*, 2003; Wang *et al.*, 2003) and elevation of cyclic AMP (Parker *et al.*, 2009), have also been suggested to be involved. There is also some evidence that the sweet taste-specific G protein, gustducin, may be involved in glucose-stimulated GLP-1 secretion. Thus, L-cells have been shown to express gustducin (Rozengurt *et al.*, 2006), and glucose-stimulated GLP-1 secretion can be blocked by a gustducin antagonist (Jang *et al.*, 2007). Moreover, α-gustducin null mice show impaired GLP-1 responses (and deficiencies in the regulation of plasma insulin and glucose) following glucose ingestion (Jang *et al.*, 2007). The incretin responses to other nutrients are likely to involve yet other mechanisms. It has, for example, been demonstrated that lipid amides activate the G protein coupled receptor GPR119 in intestinal cells thereby stimulating GLP-1 secretion (Chu *et al.*, 2008) and, furthermore, that fatty acids activate the G protein couple receptors GPR120 (Hirasawa *et al.*, 2005) and GPR40 (Edfalk *et al.*, 2008), which, likewise, are followed by stimulation of GLP-1 secretion, suggesting potential mechanisms mediating the incretin hormone responses to fat ingestion. However, this is still an area that needs to be explored in more detail.

V. GIP and GLP-1 Secretion Over the Day

A. Dynamic response to each meal

During the course of a 24-h period, secretion of incretin hormones shows a dynamic pattern, with increases observed after each meal (Elliott *et al.*, 1993; Mari *et al.*, 2005a; Ørskov *et al.*, 1996). The incretins are elevated for 3–4 h after intake of a regular meal (see Fig. 7.1). Assuming a three meal pattern

over the course of the day, this would indicate that secretion of the incretins is stimulated over baseline for 9–12 h per 24-h period. Increases in response to snacks would add to this, showing that incretin hormone secretion is stimulated for a considerable part of the day.

When further analyzing the incretin hormone secretion over the day, it is important to acknowledge that meal composition and meal size vary between breakfast, lunch, and dinner. For example, ingestion of a 520 kcal meal versus a 260 kcal meal results in a considerably higher GIP and GLP-1 response (Vilsbøll *et al.*, 2003a,b). Whether this is a linear relationship holding true not only for maximal levels but also for duration, is not known. However, it may be speculated that incretin hormones are elevated at least 3–4 h after each meal and perhaps 1–2 h after snack ingestion, suggesting elevated levels during 75% of the 24-h period.

B. Diurnal variation

A further complexity when estimating the 24-h incretin hormone profile is a potential diurnal pattern in their secretion. A diurnal variation exists for the insulin responses to oral glucose or meal ingestion, since it was demonstrated several decades ago that these responses are significantly higher in the morning than in the evening (Jarrett *et al.*, 1972; Zimmet *et al.*, 1974). A potential explanation for this may be corresponding diurnal variations in the release (and/or action) of GIP and GLP-1. Earlier studies showed, however, no clear diurnal pattern of the incretin hormones (Elliott *et al.*, 1993; Jones *et al.*, 1985; Mari *et al.*, 2005a,b; Ørskov *et al.*, 1996), but the possibility that this exists cannot be fully excluded since these earlier studies did not compare the incretin responses to precisely the same stimulus given at different times during the day. To circumvent this, we recently examined the incretin responses to identical meals in the morning versus in the afternoon in healthy men (Lindgren *et al.*, 2009). The results are shown in Fig. 7.3. As can be seen, the increase in GIP and GLP-1 secretion over the first 30 min after meal ingestion was markedly (~80%) higher in the morning than in the afternoon. Interestingly, since the area under curve for both GIP and GLP-1 during the first 30 min after meal ingestion correlated to the corresponding area under curve for insulin, it is likely that this faster and more profound incretin hormone secretion in the morning contributes to the higher insulin secretion in the morning. The difference in the early and rapid incretin hormone responses to meal in the morning versus the afternoon contrasted with the later response, where the concentration curves after the initial 30 min were almost identical. Hence, this study shows that a diurnal variation in incretin hormone secretion to identical challenges does seem to exist, but that this is restricted to the early (first 30 min) responses. The mechanism behind this diurnal pattern in the rapid incretin hormone secretion is not known. It may be

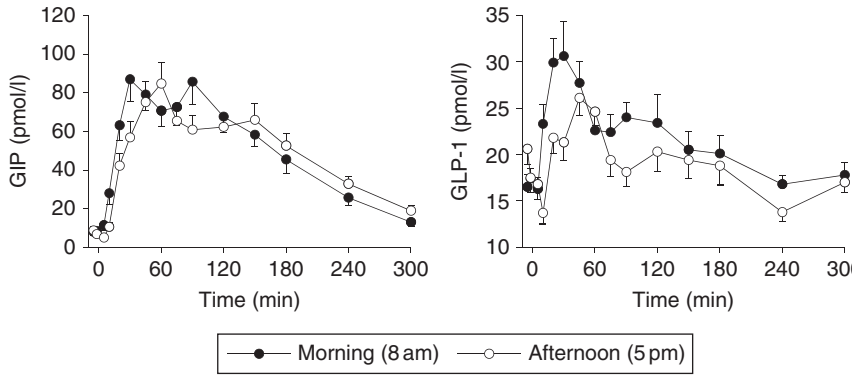

Figure 7.3 Plasma levels of GIP and GLP-1 (determined by C-terminally directed assay, that is, measuring total incretin hormone concentrations) after ingestion of a standardized mixed meal (524 kcal) in the morning (0800 h) and afternoon (1700 h) in healthy male volunteers, aged 20–30 years ($n = 12$). Results are presented as mean ± SEM. Reproduced from Lindgren *et al.* (2009). Copyright 2009. The Endocrine Society.

related to diurnal variability in activity of autonomic nerves or gastrointestinal motility but may also be related to diurnal variation in levels of growth hormone and cortisol. Further studies are required to investigate these possibilities.

VI. Incretin Hormone Secretion in Glucose Intolerance and Disease States

A. Type 2 diabetes

It was proposed as long ago as 1986 that the incretin effect is severely impaired in subjects with type 2 diabetes (Nauck *et al.*, 1986b). An impaired incretin effect has also been demonstrated in subjects with impaired glucose tolerance (IGT) (Muscelli *et al.*, 2006) and in obese subjects with normal glucose tolerance (Knop *et al.*, 2008). These observations could be interpreted to suggest a pathophysiological role of incretin hormones during the various phases of development of the disease, with the reduced incretin effect in type 2 diabetes being explained by a poor effect of GIP and/or GLP-1 to stimulate insulin secretion. Indeed, it has been shown that the insulinotropic effect of GIP is severely suppressed in type 2 diabetes (Nauck *et al.*, 1993). The insulinotropic action of GLP-1 is also reduced in type 2 diabetes (Højberg *et al.*, 2008), although supraphysiological doses clearly

and markedly stimulate insulin secretion, which is the basis for incretin-based therapy. However, although current evidence appears to suggest that while impairments in incretin action may contribute to the worsening of glycaemic control, they do not seem to be the underlying cause of type 2 diabetes, since similar impairments in the action of the incretins are seen in diabetic subjects regardless of etiology or phenotype (Knop *et al.*, 2007; Vilsbøll *et al.*, 2003a,b). Whether in addition to impairments in action, a reduction in incretin hormone secretion also contributes to the deficient incretin effect in type 2 diabetes has been the subject of several studies. It has, thus, been demonstrated that the responses of both GIP and GLP-1 after mixed meal ingestion are reduced in type 2 diabetes (Toft-Nielsen *et al.*, 2001, Vilsbøll *et al.*, 2001). Similarly, after oral glucose, the GIP but not GLP-1 responses are impaired in type 2 diabetes (Nauck *et al.*, 1993). However, there are also reports that incretin hormone secretion is not reduced after oral glucose or mixed meal in type 2 diabetes (Ryskjaer *et al.*, 2006; Vollmer *et al.*, 2008). On the contrary, an exaggerated GIP response to mixed meal, but not to oral glucose, has been reported in type 2 diabetes (Vollmer *et al.*, 2008). In fact, exaggerated GIP response to oral glucose in type 2 diabetes was reported already in 1977 (Ross *et al.*, 1977). These controversial data may probably be explained by the different study populations that have been included, mainly in regard to severity of diabetes. Thus, in studies examining diabetics with a higher HbA_{1c}, there is a more marked reduction in incretin hormone responses. For example, the patients with type 2 diabetes who were studied by Toft-Nielsen *et al.* (2001) and who had reduced incretin hormone secretion had a mean HbA_{1c} value of 8.4%, whereas those studied by Vollmer *et al.* (2008) and who had a normal incretin hormone secretion had a mean HbA_{1c} of only 6.8%. Additionally, other factors may contribute to the observed differences between studies, such as technical differences in sample handling and analysis, the use of different types of meal or glucose challenge, and differences in the degree of insulin resistance in the different populations under study. A most important difference between studies might be the length of time during which any antihyperglycemic treatment was withdrawn prior to the study, that is, the so-called wash-out period. If this period is too short, any carry over effect of pharmacological agents (of the agents themselves directly or indirectly via their effects on glucose control) might mask a reduction in incretin hormone secretion. Notable in this context is the study showing impaired incretin hormone secretion, where the wash-out period was longer (3 days; Toft-Nielsen *et al.*, 2001) than in the studies showing normal incretin hormone secretion (2 days, Vollmer *et al.*, 2008; overnight only, Ryskjaer *et al.*, 2006). More studies are, therefore, required to establish whether incretin hormone secretion is altered in type 2 diabetes, and these studies should also include different severity of the disease as well as studies undertaken with different macronutrients during different times of the day.

B. Subjects at increased risk of developing type 2 diabetes

Whether incretin hormone secretion is reduced in individuals at increased risk of developing type 2 diabetes has also been the subjects of several studies. One study examined identical twins that were discordant for type 2 diabetes and found meal-induced GLP-1 secretion was reduced only in the diabetic twin (Vaag *et al.*, 1996). Other studies examined first degree relatives of patients with type 2 diabetes and showed that there were no reduction in GIP or GLP-1 secretion throughout a 24-h study period (Nyholm *et al.*, 1999) or after oral glucose (Nauck *et al.*, 2004). Furthermore, another study showed that incretin hormone secretion after oral glucose in normal glucose tolerant women with a history of gestational diabetes mellitus was not reduced (Meier *et al.*, 2005). These results again suggest that the impaired incretin hormone secretion in type 2 diabetes is not a primary event in relation to development of diabetes, but rather a consequence of the disease. On the other hand, studies in subjects with IGT, which precedes the onset of diabetes, have shown defective incretin hormone secretion, although there are controversies in the results. Thus, one study showed reduced GIP but normal GLP-1 response to oral glucose in subjects with IGT (Ahrén *et al.*, 1997), whereas another study showed a defective early (30 min) GLP-1 response but a normal GIP response to oral glucose in IGT (Rask *et al.*, 2004). Furthermore, a third study showed impairments of both GIP and GLP-1 responses to mixed meal in IGT (Toft-Nielsen *et al.*, 2001). Hence, more studies are required regarding incretin hormone secretion in subjects with IGT.

C. Obesity

Toft-Nielsen *et al.* (2001) showed that the reduction in incretin hormone secretion in type 2 diabetes correlated to BMI, that is, was more pronounced in subjects with higher BMI, and Vollmer *et al.* (2008) found a negative correlation between GLP-1 secretion and BMI. Reduced GLP-1 responses have also been observed in obesity (Muscelli *et al.*, 2008; Näslund and Hellström, 1998; Ranganath *et al.*, 1996). We recently examined in detail the incretin hormone responses to mixed meal and oral glucose in subjects with obesity. We found a dissociated regulation of secretion in that GLP-1 secretion, but not GIP secretion, was reduced after mixed meal ingestion or oral glucose in obese subjects compared to lean subjects (Carr *et al.*, 2010). These results taken together would suggest that obesity by some mechanism inhibits GLP-1 secretion. One mechanism potentially contributing to this might be insulin resistance, perhaps through increased insulin levels. This hypothesis is supported by studies demonstrating reduced GLP-1 responses to meal ingestion in subjects with insulin resistance and normal glucose tolerance (Rask *et al.*, 2001). A potential

mechanism might be related to abnormally reduced leptin signaling, which is associated with obesity, since leptin has been demonstrated to stimulate GLP-1 secretion in rodents (Anini and Brubaker, 2003).

D. Antidiabetic agents

Several of the antidiabetic treatment regimens approved for treatment of diabetes today affect the secretion and metabolism of the incretin hormones. It has thus been demonstrated that α-glucosidase inhibitors and metformin increase the secretion of GLP-1 (Lee *et al.*, 2002; Qualmann *et al.*, 1995; Yasuda *et al.*, 2002). The mechanism for this is not known but may possibly be explained by increased exposure of nutrients to the distal small intestines, where the bulk of the GLP-1 cells are located, due to increased gut transit time and/or impaired absorption of nutrients in the proximal portion of the small intestines. This would be in agreement with reports of increased GLP-1 secretion after accelerated gastric emptying (Miholic *et al.*, 1991). Conversely, reduced exposure to nutrients of distally located L-cells might tentatively also contribute to impaired GLP-1 secretion observed in obese subjects, since obesity is associated with an increased proximal absorption rate (Wisén and Johansson, 1992). Moreover, there is a preliminary preclinical report suggesting that metformin may upregulate proglucagon expression in the intestinal L-cells (Sinha Roy *et al.*, 2007). Furthermore, DPP-4 inhibitors increase the concentrations of the intact forms of both incretin hormones two- to threefold by inhibiting the enzyme which degrades both GLP-1 and GIP (Ahrén, 2009). However, whether DPP-4 inhibitors will also affect incretin hormone secretion is less clear. There is some evidence that DPP-4 inhibitor administration is associated with a modest reduction in overall incretin hormone secretion (Herman *et al.*, 2006), possibly mediated via a feedback loop, whereby the increased levels of intact peptides suppress K- and L-cell secretion (Deacon *et al.*, 2002).

VII. GIP and GLP-1 Secretion in Fasting State

Plasma levels of GIP and GLP-1 are low after an overnight fast, but not immeasurable. Moreover, it has been demonstrated that even these low levels can be further reduced by somatostatin (Toft-Nielsen *et al.*, 1996) and that the concentrations of intact GLP-1 and GIP are increased by DPP-4 inhibition both during fasting conditions and between meals (Mari *et al.*, 2005b). These findings suggest that, although of relatively low magnitude, there is an active baseline secretion of the incretin hormones.

Whereas the role of the incretin hormones in augmenting insulin secretion after meal ingestion or oral glucose is well established, the potential

contribution of baseline (fasting) incretin hormone levels for glucose homeostasis is not known. One approach to examine a potential contribution of fasting incretin hormone secretion would be to cross-sectionally analyze the correlation between fasting GIP or GLP-1 levels on one hand and fasting insulin on the other. By doing this in a data base with 108 lean healthy volunteers, aged 20–30 years, it was found that basal levels of GIP were correlated to fasting insulin ($r = 0.24$, $P = 0.024$ as determined by the C-terminal assay, and $r = 0.30$, $P \geq 0.004$ as determined by the N-terminal assay), whereas no such significant correlation was found for GLP-1 levels. This could imply that secretion of GIP in the fasting state might contribute to fasting insulin secretion. On the other hand, a contribution by GLP-1 cannot be excluded, based upon results from a recent study which showed that a slight increase in fasting levels of both GIP and GLP-1 by the DPP-4 inhibitor vildagliptin was associated with improved insulin response to intravenous glucose in the fasting condition in subjects with type 2 diabetes (D'Alessio *et al.*, 2009). Collectively, therefore, these results suggest that, in addition to their well-characterized prandial and postprandial effects, the incretin hormones are also likely to have physiological impact in the fasting state.

VIII. Conclusion and Perspective

Incretin hormones are important for metabolism, and an understanding of the factors regulating their secretion is, therefore, fundamental for full appreciation of the complex regulation of islet function and metabolism. As reviewed here, incretin hormone secretion is influenced by nutrient ingestion, and meal size and composition, in addition to gastric emptying and gastric distension, whereas the roles of the autonomic nerves and other hormones are less clear. Furthermore, there is a diurnal variation with a more rapid incretin response to meal ingestion in the morning than in the afternoon. Incretin hormone secretion is reduced in type 2 diabetes in some, but not all studies, and may also be perturbed in obesity. Finally, to add to the complexity, antidiabetic compounds may have the capacity to affect incretin hormone secretion, either directly or indirectly, which has been demonstrated for metformin and the α-glucosidase inhibitors. It is, therefore, apparent that the full picture of the regulation of incretin hormone secretion is far from complete, suggesting that more studies are required. In particular, the issue whether incretin hormone secretion is reduced in type 2 diabetes needs to be examined in larger studies with control of the washout period and using subjects with different degrees of glucose dysregulation. Furthermore, the potential increase in incretin hormone secretion by metformin (and, possibly, other antihypeglycemic agents) requires further study. Finally, because of the pleiotropic effects of the incretin hormones on

the β-cell and the inhibition of glucagon secretion by GLP-1, enhancement of incretin hormone secretion is emerging as a potential new target for treatment of type 2 diabetes, which should be explored in more detail.

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CHAPTER EIGHT

Using the Lymph Fistula Rat Model to Study Incretin Secretion

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Abstract

The past several decades have witnessed a flourish of interest in the field of incretin biology. The importance of the two incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), in health and disease is becoming more apparent as the prevalence of type 2 diabetes and other metabolic disorders escalates. Rodent models have become indispensable in the study of the physiological function of GIP and GLP-1; however, investigators have run into several roadblocks when untangling the regulation of incretin secretion in these systems. The low circulating levels of the incretin hormones combined with sensitivity of the currently available assays require substantial amounts of blood to be removed from an animal if the hormones are to be analyzed over a period of time. Because of these limitations, continuous monitoring of GIP and GLP-1 secretion becomes difficult. A more effective means of studying incretin secretion in small animal models is therefore desirable. This chapter evaluates the use of the lymph fistula rat as a model to study the secretion of incretins. Lymph fistula models, in a variety of animals, have been used for decades to study the absorption and transport of lipid and lipophilic compounds; however, only recently has the value of this model been appreciated as a tool to explore incretin secretion.

I. Introduction

As the prevalence of type 2 diabetes and other metabolic disorders rises, much attention has been focused on the role of the two incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) in human health and disease. The incretin hormones are involved in a number of physiologic processes, many centering around the maintenance of glucose homeostasis. To fully appreciate the complex system interactions involved in these processes, rodent models have become a fundamental tool. Investigators have often been limited by the low circulating concentrations of both incretin hormones in the blood. The following chapter evaluates the use of the lymph fistula rat model as an alternative *in vivo* system to continuously monitor the secretion of the incretin hormones. As will be discussed, the concentration of GIP and GLP-1 is significantly higher in lymph than in plasma, making lymph an ideal medium to study the regulation of incretin secretion. The chapter begins with a review of the incretin system that is not meant to be exhaustive; for further information, the authors refer the reader to several excellent reviews (Baggio and Drucker, 2007; Doyle and Egan, 2007; Fehmann *et al.*, 1995; Hansotia and Drucker, 2005; Kieffer and Habener, 1999; Meier and Nauck, 2005; Nauck, 2009) and the additional chapters included in this volume. Following an account of the lymph fistula model in different animal models, the chapter focuses on the use and benefits of the

lymph fistula rat model to study incretin secretion. The chapter is then concluded with a discussion of recently published studies and future questions that can be addressed with the lymph fistula rat model.

II. The Incretin Hormones

A. Discovery

Although initially introduced in the early 1900s (Bayliss and Starling, 1902; Moore *et al.*, 1906), the concept of intestinal factors stimulating the secretion of pancreatic substances did not become prevalent until the 1960s. Around this time, several investigators observed that the plasma insulin response of individuals to intravenous glucose was significantly lower than that seen after an oral glucose load (Elrick *et al.*, 1964; McIntryre *et al.*, 1964). This suggested that an alimentary mechanism, in addition to elevated blood glucose levels, regulated insulin release. The postprandial enhancement of insulin secretion by gut factors was termed the *incretin effect*. Over the next 25 years, gastric inhibitory polypeptide (GIP), later named glucose-dependent insulinotropic polypeptide, and GLP-1 were discovered to be the incretin hormones involved (Brown *et al.*, 1970; Schmidt *et al.*, 1985). GIP and GLP-1 are the only known incretin hormones, and together they fully account for the incretin effect in humans (Vilsbøll *et al.*, 2003).

B. Action

GIP is released from enteroendocrine K cells, which are primarily located in the duodenum and proximal jejunum, and is secreted in response to nutrient absorption. The basal circulating levels range from 60 to 100 pM in humans and increase to 200–500 pM after a meal (Vilsbøll *et al.*, 2001). The main function of GIP is to enhance glucose-dependent insulin secretion from the pancreatic β-cell in response to increased blood glucose concentrations. GIP upregulates β-cell insulin gene transcription and biosynthesis, stimulates β-cell proliferation, and reduces β-cell apoptosis (Kim *et al.*, 2005; Wang *et al.*, 1996). Additionally, GIP enhances lipogenesis by stimulating lipoprotein lipase activity, enhancing fatty acid synthesis and incorporation into triglycerides, and downregulating glucagon-stimulated lipolysis, all of which promote fat deposition rather than mobilization (Getty-Kaushik *et al.*, 2006). In fact, mice lacking the receptor for GIP are resistant to diet-induced obesity (Miyawaki *et al.*, 2002; Zhou *et al.*, 2005), making inhibition of signaling through the GIP receptor a potentially attractive target for antiobesity therapy.

GLP-1 is secreted from enteroendocrine L cells located mainly in the distal jejunum, ileum, and proximal colon. GLP-1 results from a

tissue-specific posttranslational proteolytic cleavage of the proglucagon gene, and secretion is stimulated by a variety of nutrient, neural, and endocrine factors (Deacon, 2005). Fasting plasma levels range between 5 and 10 pM and increase approximately two- to threefold after a meal (Vilsbøll *et al.*, 2001). Similar to GIP, GLP-1 enhances glucose-dependent insulin secretion, stimulates β-cell proliferation, and decreases β-cell apoptosis, and in the presence of high glucose concentrations, GLP-1 has been shown to reduce glucagon secretion. GLP-1 additionally improves glycemic control by decreasing gastric emptying via the ileal brake reflex, thereby reducing the delivery of absorbed nutrients to the circulation over time (Layer *et al.*, 1990; Maljaars *et al.*, 2008; Nauck *et al.*, 1997; Spiller *et al.*, 1988). GLP-1 provides another level of glycemic control by regulating food intake (Gutzwiller *et al.*, 1999; Larsen *et al.*, 2001; Näslund *et al.*, 1999; Turton *et al.*, 1996).

Impairments in the incretin response have been well documented in individuals with type 2 diabetes. Several studies (Nauck *et al.*, 1993; Vaag *et al.*, 1996; Vilsbøll *et al.*, 2001) have reported decreased postprandial GLP-1 secretion. In contrast, GIP secretion from enteroendocrine cells in type 2 diabetic patients is normal or slightly elevated. These patients do, however, present with impaired insulinotropic effects of GIP at the pancreatic β-cells (Deacon *et al.*, 2000; Nakanome *et al.*, 1983; Nauck *et al.*, 1993). The attenuated GLP-1 secretion and compromised GIP function contribute to the pathology of type 2 diabetes. Because the glucoregulatory properties of GLP-1 are still functional in insulin-resistant individuals unlike that of GIP, therapeutic strategies have focused on the development of GLP-1 receptor agonists. Exenatide, the only approved GLP-1 receptor agonist, is a synthetic form of exendin-4, a 39 amino acid peptide originally isolated from the venom of the *Heloderma suspectum* lizard (Gila monster) (Eng *et al.*, 1992). Exendin-4 is a potent agonist of the GLP-1 receptor and is not susceptible to dipeptidyl-peptidase IV (DPP-IV) degradation (see discussion below).

C. Secretion

As previously mentioned, the primary stimulus for incretin secretion is the ingestion of nutrients. Carbohydrate, fat, and protein alone, as well as mixed meals, have all been documented to induce the release of GIP and GLP-1 from enteroendocrine cells. Based on observations from patients with intestinal malabsorption (Besterman *et al.*, 1979) and studies using pharmacological agents that impede nutrient uptake (Fukase *et al.*, 1992; Fushiki *et al.*, 1992), it is thought that GIP secretion is dependent on nutrient absorption rather than the mere presence of nutrients in the intestinal lumen. Whether or not absorption is essential for GLP-1 secretion appears to be nutrient-specific.

Studies aimed at deciphering the mechanism underlying carbohydrate-induced GLP-1 secretion found that glucose and other metabolizable sugars act through glucose metabolism, closure of K_{ATP} channels, and action potential production (Reimann and Gribble, 2002), analogous to the process in pancreatic β-cells. More recently, Parker *et al.* (2009) and Reimann *et al.* (2008) generated transgenic mice with fluorescently labeled GIP-secreting K cells and GLP-1-secreting L cells, respectively; studies from these transgenic mice have also implicated K_{ATP} channels. Furthermore, it has been suggested that sweet taste receptor pathways are actively involved in glucose-sensing by the K and L cells (Jang *et al.*, 2007; Margolskee *et al.*, 2007).

Several investigators have observed that glucose-induced incretin release is differentially modulated by the ingestion of protein. Supplementing meals with protein increases the levels of the incretin hormones, markedly augments the plasma insulin response, and enhances glucose disposal in mice and humans (Frid *et al.*, 2005; Gunnarsson *et al.*, 2006; Karamanlis *et al.*, 2007). The underlying mechanism behind protein-stimulated GLP-1 secretion is still under investigation. However, it has been suggested that glutamine triggers membrane depolarization and increased intracellular calcium concentrations, resulting in the release of GLP-1 (Reimann *et al.*, 2004).

Lipid-induced incretin secretion has been well documented. Investigations looking at the potential of individual fatty acids have found that monounsaturated fatty acids are more potent stimulators of incretin secretion than both saturated and polyunsaturated fatty acids (Beysen *et al.*, 2002; Rocca *et al.*, 2001; Thomsen *et al.*, 1999). Additionally, only those fatty acids with a chain length greater than 14 carbons are capable of stimulating GLP-1 secretion from fetal rat intestinal cells (FRICs) (Rocca and Brubaker, 1995). Recently, Hirasawa and colleagues (2005) have implicated the G-protein-coupled receptor, GPR120, in mediating lipid-induced GLP-1 secretion. In accordance with these findings, expression of *GPR40*, *GPR119*, and *GPR120* (all documented fatty acid GPCRs) has been detected in primary K (Parker *et al.*, 2009) and L cells (Reimann *et al.*, 2008).

In addition to direct nutrient stimuli, GLP-1 secretion is induced by a variety of neural and endocrine factors (Deacon, 2005). Rocca and Brubaker (1999) demonstrated the importance of the vagus nerve in stimulating GLP-1 secretion. Bilateral subdiaphragmatic vagotomy completely abolished fat-induced GLP-1 secretion, and direct stimulation of the celiac branch of the vagus nerve resulted in significant GLP-1 release. Furthermore, atropine, a nonselective muscarinic receptor blocker, inhibited GLP-1 secretion in humans. GIP has also been implicated in regulating GLP-1 secretion via the vagus nerve (Roberge and Brubaker, 1993; Rocca and Brubaker, 1999). Additionally, murine, human, and fetal rat L cells express the leptin receptor, and when exposed to leptin, all the three cell lines secreted GLP-1 (Anini and Brubaker, 2003). More recently, Katsuma

et al. (2005) and Thomas *et al.* (2009) described a role for the bile acid GPCR, TGR5, in GLP-1 secretion and glucose homeostasis.

D. Degradation

Once secreted from the intestine into the circulation, both GIP and GLP-1 are rapidly degraded by DPP-IV (Mentlein *et al.*, 1993). DPP-IV cleaves dipeptides from the amino terminus of proteins that contain a penultimate alanine or proline; the GIP and GLP-1 metabolites produced by DPP-IV are inactive. DPP-IV is ubiquitously expressed and found in multiple tissues and cell types. Most notably, DPP-IV is located on the surface of endothelial cells, particularly the cells lining the blood vessels of the intestinal mucosa. Hansen and colleagues (1999) estimated that greater than 50% of GLP-1 released from the L cells is degraded in the capillaries draining the intestinal mucosa. Furthermore, after a single pass through the liver, approximately 40% of the remaining bioactive GLP-1 is degraded (Deacon *et al.*, 1996). Consequently, the half-life for both incretin hormones is quite short, lasting only 2–3 min.

E. Measurement of incretin secretion

Traditionally, *in vivo* study of the incretin hormones requires measurement of the circulating levels in the peripheral blood, and occasionally the portal blood. Due to the low concentration of the incretin hormones in plasma, investigators studying GIP and GLP-1 secretion in small animal models are limited by the amount of blood that can be removed from an animal during the course of a study. Because of these limitations, continuous monitoring of GIP and GLP-1 secretion becomes difficult. A more effective means of studying incretin secretion in small animal models is therefore desirable. This chapter evaluates the use of the lymph fistula rat as a model to study the secretion of incretins. Since lymph collects fluid drained from the intestinal lamina propria, it is conceivable that the concentrations of incretin hormones would be higher in the intestinal lymph than in plasma. Additionally, since lymph has a lower flow rate than blood, the hormones would be less diluted, thereby raising the concentration of GIP and GLP-1.

Indeed, the presence of incretin hormones in lymph had been reported in the early 1980s. Although no measurements of plasma levels were reported, Adrian *et al.* (1983) successfully demonstrated the presence of GIP in lymph sampled from the intestinal lymphatic duct of conscious calves. Using a conscious pig model, Manolas *et al.* (1985) compared GIP concentrations following consumption of a standard meal (13.01 kJ/kg: 50% carbohydrate, 14% protein, 6% fat, 30% nonnutrient residual material) in plasma and lymph collected from the cisterna chyli. The test meal stimulated the release of GIP, which was observable in both plasma and

lymph; however, both the integrated and total incremental responses (postprandial peptide response above basal levels and total postprandial peptide response, respectively) were greater in plasma than in lymph. Regardless, the lymph–plasma ratio was higher for GIP compared to the pancreatic hormones measured in the study. The authors suggested that the close proximity of the intestinal secretory cells to the lymphatic system may account for this difference.

III. Anatomy and Physiology of the Gastrointestinal and Lymphatic Systems

To facilitate a better understanding of the rationale behind using the lymph fistula model to measure incretin secretion, a brief overview of the anatomy and physiology of the gastrointestinal and lymphatic systems will be presented.

A. The gastrointestinal system

The gastrointestinal system consists of the gastrointestinal tract and the gastrointestinal glands. The gastrointestinal tract is essentially a tube divided into several segments: the oral cavity, esophagus, stomach, small and large intestine, rectum, and anus. The small intestine is further divided into three parts: the duodenum, jejunum, and ileum. The small intestine is the site of terminal food digestion, nutrient absorption, and endocrine secretion. The lining of the small intestine consists of a series of permanent spiral or circular folds, termed the plicae circulares, which amplify the organ's surface area, promoting efficient nutrient absorption. The mucosal surface area is increased further by finger-like projections and depressions, called villi and crypts, respectively. The villi and crypts are covered by a continuous sheet of epithelial cells. The predominant epithelial cell type is the absorptive cell, the enterocyte; however, a variety of other cell types, such as enteroendocrine cells, can be found on the intestinal villus (Junqueira and Carneiro, 2005). The enteroendocrine cells are specialized cells of the gastrointestinal system that produce and secrete hormones.

The enterocytes lining the villi are the cells responsible for nutrient absorption. Once absorbed, nutrients are either transported to the circulation via the portal blood or, in the case of lipid digestive products, to the lymph via chylomicrons (triglyceride-rich lipid particles). The structure of the villi (Fig. 8.1) facilitates this divergent nutrient transport. Directly below the epithelial lining of the villi lies the intestinal lamina propria. The lamina propria is composed of loose connective tissue with nerve fibers and smooth

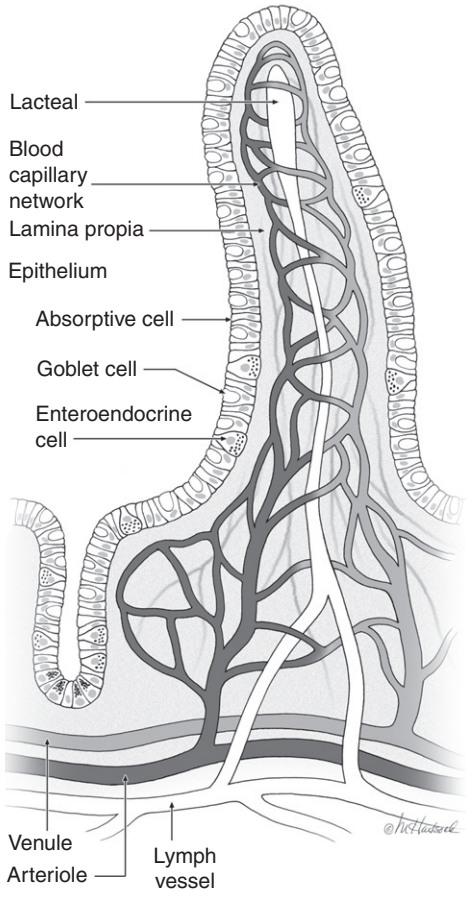

Figure 8.1 Structure of the intestinal villus. The mucosal surface of the small intestine is comprised of finger-like projections and depressions, called villi and crypts. The villi and crypts are covered by a continuous sheet of epithelial cells. The primary epithelial cell is the absorptive enterocyte; however, a variety of other cells, such as enteroendocrine cells, can also be found on the villus. The enteroendocrine cells are sparsely distributed throughout the intestinal; in general, there is 1 enteroendocrine cell for every 1000 enterocytes. The GIP-secreting K cells and GLP-1-secreting L cells are two different types of enteroendocrine cell. Directly below the epithelial lining of the villi lies the intestinal lamina propria. The lamina propria is composed of loose connective tissue with nerve fibers and smooth muscle cells. Within each villi is a specialized lymphatic capillary, called a lacteal, which is surrounded by a blood capillary network. Lipid and lipophilic compounds are transported from the enterocyte into the lacteal. The lymph in the lacteal also contains material drained from the lamina propria, such as hormones secreted from the enteroendocrine cells. (© 2010 Marcia Hartsock, MA, CMI.)

muscle cells. Additionally, located within each villus is a specialized lymphatic capillary, called a lacteal, which is surrounded by a blood capillary network (Granger *et al.*, 1985).

B. The lymphatic system

The lymphatic system is comprised of a series of lymphatic vessels and lymph nodes. Lymph is formed when fluid and proteins from the interstitial space that are not reabsorbed by the blood capillaries enter the lymphatic capillaries. Following formation, lymph drains from the lymphatic capillaries into afferent (prenodal) lymphatic ducts, which transport lymph to regional lymph nodes. Efferent (postnodal) lymphatic ducts transport the lymph through a series of successive lymph nodes and progressively larger lymphatic vessels. Lymph drained from the upper right half of the body culminates in the right lymphatic duct, whereas lymph drained from the remainder of the body terminates in the thoracic duct, before first collecting in the cisterna chyli. Both the right lymphatic duct and the thoracic duct drain into the circulatory system at the right and left subclavian vein, respectively (Swartz, 2001).

Beyond draining excess fluid and proteins, the lymphatics of the gastrointestinal tract are also responsible for the transport of lipoproteins and lipophilic compounds to the circulatory system. After being absorbed by the lacteals in the intestinal villi, these particles are transported via first the intestinal (mesenteric) lymphatic duct and then the thoracic duct before being drained into the circulation through the subclavian vein (Cueni and Detmar, 2008).

IV. The Lymph Fistula Model

Molecules transported by the lymph after being absorbed by the gastrointestinal system can be studied using lymph cannulation in a variety of animal models. Two general lymph cannulation techniques have been described in the literature: cannulation of the intestinal lymphatic duct or cannulation of the thoracic duct. Intestinal lymphatic cannulation allows for the collection of lymph from the stomach, intestine, pancreas, spleen, and portions of the liver. Cannulation of the thoracic duct, on the other hand, allows for the collection of lymph not only from the intestinal lymphatic duct, but also from the remainder of the body, excluding the lymph drained from the upper right quadrant of the body. Numerous lymph fistula animal models have been developed over the past few decades. The most widely used animal is the rat; however, several larger animal models are also used to study the transport of lipids and lipophilic compounds. A few of these models will be discussed.

A. The lymph fistula rat model

The lymph fistula rat model has been described and utilized by several investigators. Variations in methodology involve differences in cannulation sites, use of anesthesia, and pre- and postoperative care. Bollman and colleagues (1948) provided one of the first reports of thoracic and intestinal lymphatic duct cannulation in the anesthetized rat. In this model, the rats are initially anesthetized with ether and then injected with 0.1 ml of a 0.5% solution of Evans blue dye to facilitate visualization of the lymphatic vessel. To assist locating the intestinal lymphatics, Bollman and colleagues suggest providing the rats a lipid-based meal prior to surgery; the intestinal lymphatic duct will appear a milky-white color rather than clear. After the duct (thoracic or intestinal) has been located and incised, plastic tubing is inserted into the duct and ligated into place. The tubing is then exteriorized and placed into a graduated centrifuge tube below the plane of the animal to allow continuous lymph collection by gravity. Following the operation, the rats are positioned in restraint cages for the duration of the lymph collection period to prevent the animals from removing the lymphatic cannula. Although the cages prevent the animals from turning around, the cages do allow some forward and backward movements (Bollman, 1948). One of the limitations of the Bollman model is the absence of rehydration. Subsequent lymph fistula rat models describe the addition of either a duodenal infusion tube or jugular vein cannula for saline rehydration.

Porter and Charman (1996) describe an updated anesthetized lymph fistula rat model. In this model, the investigators use a triple-cannulation method, in which the intestinal lymphatic duct, jugular vein, and duodenum are cannulated. Prior to the operation and every 2 h, thereafter, the animals were kept anesthetized with sodium pentobarbitone. Following the procedure, the animals remained anesthetized during the entirety of the study, while lymph is continuously collected from the intestinal lymphatic duct cannula, plasma is sampled via the jugular vein cannula, and nutrients and saline (for rehydration) are provided through the duodenal cannula.

While the use of anesthetized models has many advantages, specifically elimination of any problems associated with animal movement during lymph collection, the lymph flow rate is affected by the use of anesthesia. In conscious animals, the fasting lymph flow rate ranges from 2 to 3 ml/h; however, in anesthetized rats, the fasting lymph flow rate drops dramatically to 0.1–0.6 ml/h (Dahan *et al.*, 2007; Edwards *et al.*, 2001; Polderman *et al.*, 1944). This reduction in lymph flow may be attributed to changes in gastrointestinal motility, altered capillary permeability, and reduced interstitial fluid formation. Additionally, the continuous use of anesthesia poses several economical and logistical problems. Not only does the cost of the study increase, but also the animals require constant observation to monitor the effects of anesthesia on respiration and heart rate.

Due to alterations in lymph flow rate from anesthesia, the use of conscious rat lymph fistula models to study the transport of lipids and lipophilic compounds is increasing. Tso and Simmonds (1984) provide an excellent detailed description of the lymph fistula procedure and collection in unanesthetized animals. Similar to the procedure described by Bollman and colleagues (1948), once duct has been cannulated, the cannula is ligated into place at two locations; however, unlike Bollman, the duct is further secured using a drop of methyl cyanoacrylate glue. Following surgery, the animals are allowed to recover from anesthesia and are then placed in restraint cages. Rehydration occurs via a duodenal cannula; however, a jugular vein cannula can be used instead. Beyond presenting a meticulous description of the procedure, Tso and Simmonds (1984) additionally provide several hints for the successful completion of the procedure and discuss the advantages and disadvantages of cannulating the intestinal lymphatic duct over the thoracic lymphatic duct. The procedure described by Tso and Simmonds is economical and has a high success rate (~90%, when performed by a trained surgical technician). This model has been extensively used in lipid absorption and transport studies.

B. The lymph fistula model for large animals

In addition to the described rat models, the lymph fistula procedure has been adapted for use in larger animal models, such as the dog and the pig. Collection of lymph from the dog typically occurs through the thoracic duct. For short-term lymph collection, an external thoracic duct fistula, which continually drains the lymphatic fluid, is suitable. In these procedures, once the duct has been cannulated, the tube is exteriorized at the neck and attached to a collecting flask (Grindlay *et al.*, 1950). The dogs used by Grindlay and colleagues were trained to remain still for extended periods of time, thus not requiring the use of a restraining device. Several years later, Rampone (1959) designed a leather harness that could be used to hold the collecting flask in place and still permit movement in the animals. For long-term lymph studies, complete drainage and collection have been argued to alter the physiological state of the animal by altering the dynamics of the thoracic duct circulation and depleting the animal of fluid, salts, and proteins, despite the use of rehydrating fluids. To circumvent this concern, thoracic duct lymph can be sampled from a side-fistula, in which the cannula is still exteriorized but occluded with a plastic clamp and secured around the neck of the dog (Girardet and Benninghoff, 1973). As an alternative to the side-fistula, a thoracic duct-to-duct (Girardet and Benninghoff, 1973) or thoracic duct-venous (Doemling and Steggerda, 1960) shunt can be employed to sample lymph during long-term studies. In both of these shunt procedures, lymph is diverted from the thoracic duct into the cannula and returned to the circulation

(via the thoracic duct for the thoracic duct-to-duct shunt or the jugular vein for the thoracic duct-venous shunt).

Intestinal lymphatic duct and thoracic duct cannulations can also be performed in anesthetized or conscious pigs. To collect lymph from the gastrointestinal system, either the cisterna chyli (Manolas *et al.*, 1985) or a branch of the intestinal lymphatics (White *et al.*, 1991) can be cannulated. The intestinal lymphatics of the pig are highly branched; thus, cannulation of a duct that allows complete collection of the intestinal lymph is challenging. Because of this, only qualitative measurements of the collected lymph can be obtained. Collection of lymph from the thoracic duct, however, allows for quantitative analyses since drainage of the entire lymph pool is possible. As with the dog, long-term lymph drainage has been argued to affect the physiological state of the animal; a thoracic duct-venous shunt model can be used for long-term lymph sampling to avoid these potential issues (Jensen *et al.*, 1990). In this model, both the left external jugular vein and thoracic duct are cannulated; the catheters are then connected by two 3-way valves connected in series. Although lymph cannot be continuously collected, the shunts allow the animals to move freely and allow lymph sampling for several days (shunt patency averaged 5.5 days), postoperatively.

Descriptions of intestinal lymphatic cannulations in other large animal models, such as the cat, cow, and sheep, can be found elsewhere (Lascelles and Morris, 1961; Romsos and McGilliard, 1970; Stepanov, 1958, respectively).

V. Using the Lymph Fistula Rat Model to Study Incretin Secretion

A. Fasting and postprandial concentrations of GIP and GLP-1 are higher in intestinal lymph than in peripheral or portal plasma

Traditional methods for measuring incretin secretion have sampled circulating levels in the peripheral, and occasionally, the portal blood. Unfortunately, investigators are often hampered by the low concentration of both GIP and GLP-1 in the blood. Not only are the hormones rapidly degraded by DPP-IV once appearing in the circulation, but also they are quickly diluted by the portal blood. Furthermore, investigators using small animal models to study the incretin secretion are limited by the amount of blood that can be removed from an animal during the course of a study. Therefore, a more efficient means of studying incretin secretion is needed.

As previously discussed, the lymph fistula model is commonly used to study the transport of lipid and lipophilic compounds in a variety of animal models. However, the collected lymph from these models also contains

material drained from the lamina propria, in addition to the lipids and lipophilic compounds. Because of the close proximity of the incretin-secreting enteroendocrine cells to the lacteals (Fig. 8.1) and because the lymph has a lower flow rate than blood (lymph flow rate = 2–3 ml/h; portal blood flow rate = 8–20 ml/min) (Sherman *et al.*, 1996; Tsuchiya *et al.*, 1978), it is plausible that the concentration of the incretin hormones may be higher in intestinal lymph than in peripheral or portal plasma.

To investigate this hypothesis, D'Alessio and colleagues (2007) measured the concentration of several gastrointestinal hormones in Sprague–Dawley rats outfitted with an intestinal lymphatic duct cannula and a portal or jugular vein catheter. Following an intragastric mixed meal bolus [Ensure Plus (Abbott Nutrition): 5 ml, 7 kcal], lymph was continuously collected and portal or jugular venous blood was sampled for 4 h. Plasma and lymph samples were analyzed for insulin, GLP-1, and peptide tyrosine–tyrosine (PYY) using commercially available radioimmunoassay (RIA) kits. (The surgery, recovery, and lymph collection protocol for the measurement of incretin secretion is described at the end of this chapter.).

The results demonstrated that intestinal lymph contains measurable amounts of insulin, GLP-1, and PYY and that the lymphatic secretion profile of these hormones parallels to that of plasma. However, D'Alessio and colleagues (2007) found that the relative concentrations in lymph and plasma vary for the three hormones. Thirty minutes following the mixed meal bolus, insulin levels peaked (293 ± 60 pM) and remained elevated for the entirety of the experiment. On the other hand, insulin levels in the intestinal lymph peaked later (60 min after the nutrient challenge; 160 ± 40 pM) and returned to near basal levels by 120 min. At the time of peak secretion, insulin levels were substantially lower in lymph than in portal plasma (Fig. 8.2). The data suggest that insulin is not preferentially secreted into the lymph but rather enters the lymphatic system via capillary filtration.

Interestingly, D'Alessio and colleagues (2007) found that both the fasting and postprandial concentrations of the incretin hormone GLP-1 were higher in the intestinal lymph than in either peripheral (jugular) or portal plasma. Following the mixed meal challenge, GLP-1 levels peaked at 13.2 ± 3.1 pM by 15 min in peripheral plasma, at approximately 50 pM by 30 min in portal plasma, and at 308 ± 73 pM by 30 min in intestinal lymph. At the time of peak secretion, GLP-1 was six times higher in lymph than in portal plasma and 23 times higher in lymph than in peripheral plasma (Fig. 8.3). Compared to insulin, GLP-1 has a higher lymph–plasma ratio, which indicates that GLP-1 is highly concentrated in the lymph compartment. Moreover, the data indicated that GLP-1 enters the lymphatic system directly rather than by capillary filtration. If the latter were correct, then the lymphatic concentration would be lower and peak later than the plasma concentration, similar to the insulin data. Concurrent with their rationale,

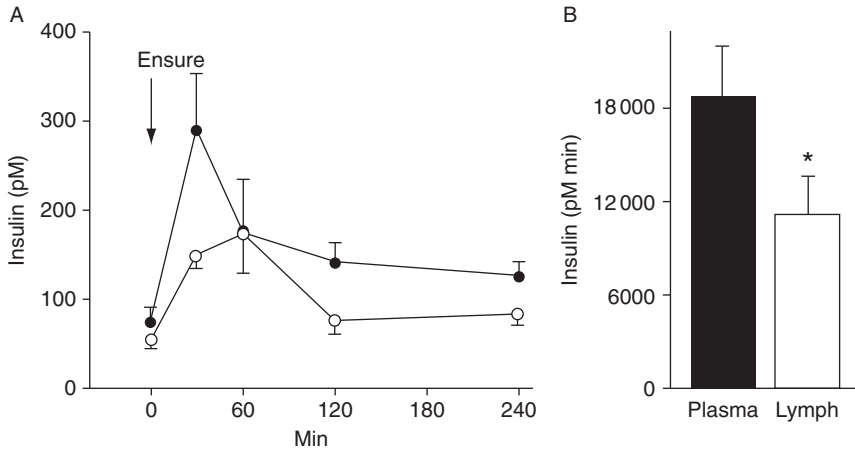

Figure 8.2 Concentrations of insulin in samples of intestinal lymph (○) and portal plasma (●) taken before and after an intragastric bolus of Ensure Plus. Data are presented as means ± SE. *Denotes $P < 0.05$ versus plasma. (A) Hourly insulin concentration. (B) Insulin AUC as determined by the trapezoidal method. (Used with permission from D'Alessio *et al.*, 2007.)

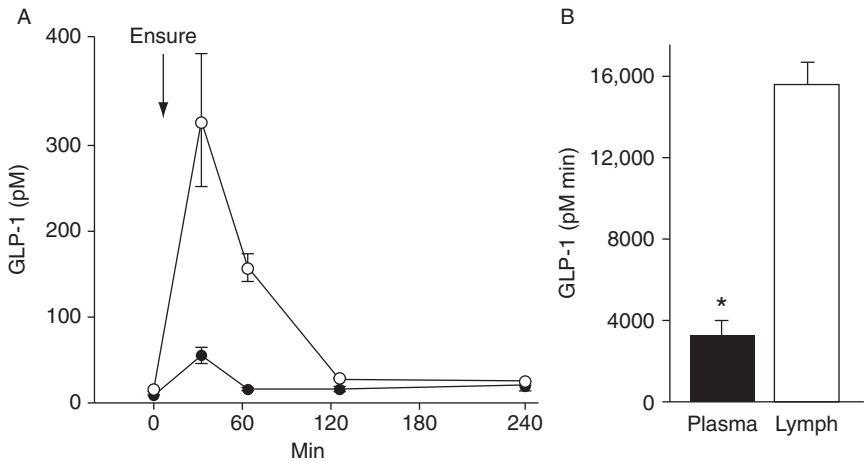

Figure 8.3 Concentrations of GLP-1 in samples of intestinal lymph (○) and portal plasma (●) taken before and after an intragastric bolus of Ensure Plus. Data are presented as means ± SE. *Denotes $P < 0.05$ versus plasma. Note that the concentration of GLP-1 is higher in lymph than in plasma for the entire 240 min collection period. (A) Hourly GLP-1 concentration. (B) GLP-1 AUC as determined by the trapezoidal method. (Used with permission from D'Alessio *et al.*, 2007.)

the data suggested that the high lymph–plasma ratio may be due to the close proximity of the lacteal to the basal end of the GLP-1-secreting L cells. To further test this idea, D'Alessio and colleagues (2007) measured the concentration of PYY in intestinal lymph and peripheral plasma.

PYY is a member of the PP-fold family, which includes pancreatic polypeptide (PP) and neuropeptide Y (NPY). Like GLP-1, PYY is also secreted from the enteroendocrine L cells (predominately located in the distal jejunum, ileum, and proximal colon) following meal consumption (Wren and Bloom, 2007). If the elevated lymph–plasma ratio for GLP-1 is only due to the proximity of the endocrine cell to the lymphatic capillary, then the concentration of PYY should also be higher in intestinal lymph than in peripheral plasma. Although postprandial lymphatic PYY concentrations were higher than plasma levels, the lymphatic concentrations were generally less than twice as large (D'Alessio *et al.*, 2007; Fig. 8.4). The secretion profile of PYY is in between that of insulin and GLP-1, suggesting that the anatomical location of secretory cells to the lymphatic capillaries is not the sole reason for the elevated lymph–plasma GLP-1 ratio. Rather, D'Alessio and colleagues (2007) argue that the higher lymphatic concentration of GLP-1 indicates targeted secretion of the hormone into the intestinal lymphatic system. Although the lymphatic system may not be the major route of transport for GLP-1 into the circulation, the higher lymphatic concentrations raise the possibility that GLP-1 has specific physiological effects mediated in this compartment.

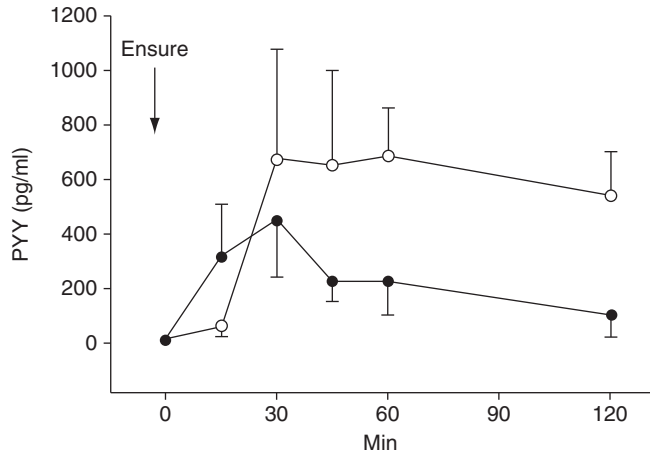

Figure 8.4 Concentrations of PYY in samples of intestinal lymph (○) and peripheral plasma (●) taken before and after an intragastric bolus of Ensure Plus. Data are presented as means ± SE. *Denotes $P < 0.05$ versus plasma. (Used with permission from D'Alessio *et al.*, 2007.)

In a similar study to D'Alessio *et al.* (2007), Lu and colleagues (2008) compared the intestinal lymph and portal plasma concentrations of GIP following a duodenal mixed nutrient challenge [Ensure (Abbott Nutrition): 3 ml, 3 kcal]. Both lymphatic and plasma GIP concentrations increased after the duodenal nutrient bolus, peaking at 60 min before returning to basal levels; however, similar to GLP-1, the lymphatic GIP concentration was significantly higher than the portal plasma concentration. Plasma GIP peaked at 603 ± 244 pg/ml, whereas lymphatic GIP peaked at 1733 ± 257 pg/ml, approximately three times greater than the plasma GIP concentration (Fig. 8.5). The dynamics of lymphatic and plasma GIP secretion were similar; however, GIP was more concentrated in the intestinal lymphatics than in the hepatic portal vein, which is presumably the area of circulation where the levels of GIP are the highest.

Less dilution by the fluid compartment and the apparent targeting of GIP and GLP-1 to the intestinal lymphatics results in the higher lymphatic concentration of the incretin hormones. The hormones also undergo less degradation by DPP-IV in the lymph compartment. The activity of the incretin degrading enzyme DPP-IV was assessed in lymph and plasma following either an intraduodenal continuous lipid [10% Liposyn II (Hospira)] infusion (D'Alessio *et al.*, 2007) or an intraduodenal mixed nutrient [Ensure (Abbott Nutrition)] bolus (Lu *et al.*, 2007). In both studies, the DPP-IV activity was significantly higher in plasma than in lymph. Prior to the lipid infusion, the plasma DPP-IV activity was approximately 20 times

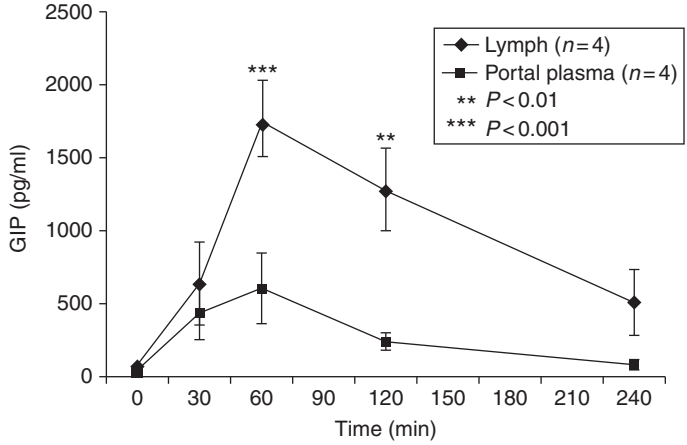

Figure 8.5 Concentrations of GIP in samples of intestinal lymph (◆) and portal plasma (■) taken before and after an intraduodenal bolus of Ensure. Data are presented as means ± SE. **Denotes $P < 0.01$; ***denotes $P < 0.001$. Note that the concentration of GIP is higher in lymph than in plasma for the entire 240 min collection period. (Used with permission from Lu *et al.*, 2008.)

greater than the lymph DPP-IV activity, and this difference remained constant during the entirety of the lipid infusion (D'Alessio *et al.*, 2007; Fig. 8.6). Although the differences in DPP-IV activity between lymph and plasma were not as large those reported by D'Alessio *et al.* (2007), Lu and colleagues (2007) observed the same trend following the mixed nutrient challenge. The data add another piece to the puzzle: the concentration of the incretin hormones is higher in lymph not only because of less dilution and apparent targeting to the intestinal lacteals but also because of less degradation by DPP-IV.

Interestingly, DPP-IV is not the only proteolytic enzyme that has reduced levels in lymph compared to plasma. Lo and colleagues (2009) recently found that the activity of aminopeptidase, the protease involved in degradation of cholecystokinin, is also significantly lower in intestinal lymph than in plasma.

In summary, the benefits of using the lymph fistula rat model to measure the secretion of GIP and GLP-1 are numerous. The concentration of both GIP and GLP-1 is higher in intestinal lymph than in portal or peripheral plasma due to less degradation by DPP-IV and less dilution by the smaller circulating fluid compartment. Additionally, this model allows the continuous collection of lymph from conscious animals, eliminating any potential side effects on lymph flow and gastrointestinal function due to anesthesia.

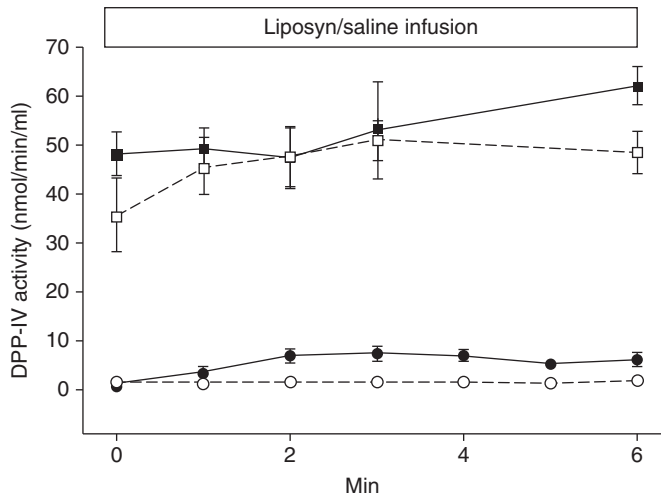

Figure 8.6 Comparison of DPP-IV activity in intestinal lymph (circles) and peripheral plasma (squares) following a constant intraduodenal infusion of lipid emulsion (closed symbols, solid lines) or saline (open symbols, dashed lines). Data are presented as means ± SE. Note that the levels of DPP-IV activity are augmented in plasma compared to lymph. (Used with permission from D'Alessio *et al.*, 2007.)

Furthermore, the volume of fluid collected from the animals is sufficient to perform a number of assays beyond the measurement of GIP and GLP-1, thus removing the restrictive factor of blood volume.

B. Using the lymph fistula rat model to study the secretion of GLP-1 and GIP to lipid and carbohydrate

From the data, both D'Alessio *et al.* (2007) and Lu *et al.* (2008) concluded that the conscious lymph fistula rat model is a novel, alternative method to study the secretion of both GIP and GLP-1. Because the concentration of both hormones is higher in lymph than in peripheral and portal plasma, the intestinal lymphatic concentration provides a more accurate representation of the local milieu within the intestinal mucosa where the hormones may interact with other enteroendocrine cells and signal through the dendritic cells and enteric or autonomic neurons. Although it is known that both GIP and GLP-1 signal directly through their respective receptors located on peripheral organs, because of the rapid degradation of the hormones and consequent low plasma concentrations, more emphasis is being placed on the local signaling via incretin receptors on neurons innervating the intestinal mucosa and hepatic portal vein.

Utilization of the lymph fistula rat model provides increased sensitivity for the detection of changes in response to various stimuli, for example, nutrients. Accordingly, Lu and colleagues (2007, 2008) used the model to characterize the secretion of GLP-1 and GIP to individual and combination of nutrients. In both studies, lymph fistula rats were provided with isocaloric and isovolumetric boluses of either lipid [20% Intralipid (Hospira): 3 ml, 4.4 kcal] or carbohydrate [Dextrin (Sigma–Aldrich): 3 ml, 4.4 kcal]. A third group of animals was provided an isocaloric, isovolumetric combined nutrient bolus (20% Intralipid + Dextrin: 3 ml, 4.4 kcal).

The individual nutrient doses, as well as the combined nutrient dose, stimulated intestinal lymphatic secretion of GLP-1 (Lu *et al.*, 2007). Lipid-induced GLP-1 secretion peaked 30 min following the nutrient bolus, whereas GLP-1 levels peaked later at 60 min following the carbohydrate bolus. Despite peaking at different times, lipid and carbohydrate induced comparable increases in GLP-1 secretion (~650 fmol/h). The authors state that the carbohydrate bolus was thicker and more viscous than the lipid bolus, and thus argue that the delay in dextrin-induced GLP-1 secretion was due to the differences in the physical form of the two nutrient boluses. The combination of nutrients induced a peak in GLP-1 secretion at 60 min that was additive, compared to the individual lipid- and carbohydrate-induced peaks.

Similar to GLP-1, the individual and the combined nutrient doses stimulated GIP release (Lu *et al.*, 2008). Lipid-induced GIP secretion produced a peak of 1159 ± pg/h at 30 min but then remained elevated for the following 120 min. GIP peaked at 2410 ± 566 pg/h 60 min

following the carbohydrate bolus. Interestingly, and unlike GLP-1, the combination of nutrients elicited a peak in GIP secretion at 60 min (8027 ± 1057 pg/h) that was significantly larger than the sum of the lipid and carbohydrate peak, suggesting that products of lipid and carbohydrate digestion are synergistic in stimulating GIP secretion from the enteroendocrine K cells.

C. Further studies using the lymph fistula rat model to characterize GIP and GLP-1 secretion

Several studies have adapted the methods developed by D'Alessio *et al.* (2007) and Lu *et al.* (2007, 2008) to further characterize the stimulation of the incretin hormones to an assortment of nutrient challenges. Yoder *et al.* (2009) used the lymph fistula rat model to determine the GIP and GLP-1 responses to increasing caloric doses of intraduodenal lipid. Five isovolumetric doses of increasing caloric amount of lipid [20% Liposyn II (Hospira): 3 ml; 0.275, 0.55, 1.1, 2.2, 4.4 kcal] were given as a bolus via the surgically inserted duodenal feeding tube. In response to increasing lipid calories, lymphatic GIP and GLP-1 increased dose-dependently; however, the distally located GLP-1-secreting L cells were more sensitive to changes in the intraluminal lipid content. The authors conclude that the increased sensitivity of the GLP-1-secreting cells to changes in intestinal lipid content reflects the hormone's role in the ileal brake reflex; as more lipid (nutrients) reach the distal gut, GLP-1 is secreted in a dose-dependent manner to reduce intestinal motility and enhance proximal fat absorption. Whether or not similar trends will be observed with increasing doses of the other macronutrients (carbohydrate and protein) is a question that can be easily addressed with the lymph fistula rat model.

Kindel *et al.* (2008) used the lymph fistula model to monitor the nutrient-induced incretin response in a widely used lean rodent model of type 2 diabetes, the Goto-Kakizaki (GK) rat. Although previous studies had demonstrated defects in pancreatic β-cell mass and insulin secretion (Giroix *et al.*, 1993; Ostenson and Efendic, 2007), prior to this report, little was known regarding the incretin-secretory capacity of these animals. Following the lymph fistula procedure, the animals were provided isocaloric and isovolumetric boluses of either lipid [20% Liposyn II (Hospira): 3 ml, 4.4 kcal], carbohydrate [Dextrin (Sigma–Aldrich): 3 ml, 4.4 kcal], or a combined nutrient bolus (20% Lipoysn II + Dextrin: 3 ml, 4.4 kcal). The GK rats maintained normal lipid-induced incretin secretion, compared to Wistar control rats; however, the GK rats mounted a diminished incretin response to the carbohydrate-only and mixed nutrient challenge. The data suggested that the GK rats possessed a defect in carbohydrate-induced incretin secretion that was shared by both the enteroendocrine K and L cells. Without the use of the lymph fistula model, the authors would have

been limited by the amount of blood able to be removed from these animals, thus rendering the study difficult to execute.

D. Lymph fistula surgical procedure, recovery protocol, and lymph collection methodology for the study of incretin secretion

The lymph fistula rat model utilized to study incretin secretion in the above studies is adapted from that described by both Bollman and colleagues (1948) and Tso and Simmonds (1984). The use of various cannulae, in addition to the intestinal lymph duct cannula, can be used to sample plasma, bile, pancreatic secretions, and/or infuse saline, nutrients, and test compounds. Furthermore, the animals are allowed to recover overnight following the surgical procedure and are conscious during the lymph collection, thus negating any side effects from the use of anesthesia on lymph flow and gastrointestinal function. The following section describes the surgical and recovery protocol, as well as the lymph collection procedure, used for the study of incretin secretion.

Prior to surgery, the rats are fasted overnight but allowed free access to water. The following morning, anesthesia is induced and maintained with a 2–3% isoflurane solution mixed with oxygen. After the animal is properly anesthetized and prior to the start of surgery, preemptive analgesic (0.1 mg/kg buprenorphine) is provided subcutaneously and ophthalmic ointment is applied to the eyes. Once the animal has been prepped for surgery, the abdominal cavity is exposed via a ventral midline incision. To aid visualization of the lymphatic duct, the stomach, small intestine, and colon are retracted and gently pushed under the abdominal muscle walls. The inferior vena cava and the left renal vein will now be exposed, and the intestinal lymphatic duct can be seen as a clear vessel running parallel to and lying directly above the superior mesenteric artery (Fig. 8.7). Both the intestinal lymphatic duct and the superior mesenteric artery are attached by connective tissue. Prior to cannulation, the connective tissue surrounding the lymphatic duct is cleared using a cotton swap; insufficient removal of the connective tissue will impede successful isolation of the duct. A small incision is made in the duct using iridectomy scissors, being careful not to sever the vessel. Once cut, white lymph will escape from the duct. The beveled end of the lymph cannula (polyvinyl chloride tubing; 0.5-mm inner diameter, 0.8-mm outer diameter), previously filled with a heparin–saline solution (20 units heparin/ml), is then inserted into the incision and advanced approximately 3–5 mm. If drops of the heparin–saline solution are produced at the opposite end of the cannula, successful cannulation of the lymphatic duct has occurred. The cannula is secured with a drop of cyanoacrylate glue, positioned beneath the liver, and then exteriorized through the right flank (Fig. 8.7).

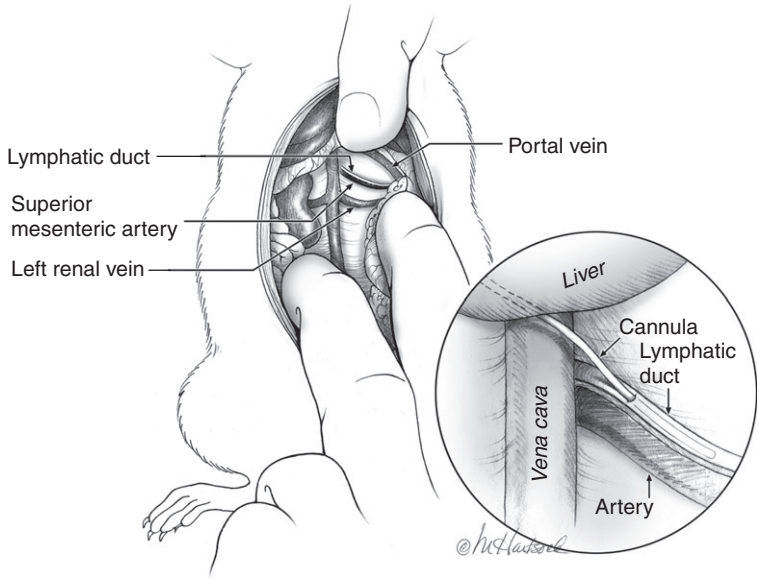

Figure 8.7 Lymph fistula procedure. After opening the abdominal cavity of the rat, the stomach, small intestine, and colon are retracted and gently pushed under the abdominal muscle walls. The surgeon's fingers can be used to keep the liver out of the surgical plane. The inferior vena cava and left renal vein will now be exposed and the intestinal lymphatic duct can be seen as a clear vessel running parallel to and lying directly above the superior mesenteric artery. With proper anatomical references, locating the intestinal lymphatic becomes less challenging. The inset shows the site of the cannula placement. The incision should be made proximal to the vena cava, allowing insertion of the cannula and advancement approximately 3–5 mm. Once secured by a drop of cyanoacrylate glue, the cannula is positioned beneath the liver and exteriorized through the right flank of the animal. (© 2010 Marcia Hartsock, MA, CMI.)

For purposes of fluid and electrolyte replenishment and nutrient infusion, a duodenal cannulation is also typically performed. The cannula (silicone; 1.02-mm inner diameter, 2.16-mm outer diameter) is introduced into the stomach via a small gastrotomy and advanced 1–2 cm beyond the pylorus into the duodenum. The cannula is secured with a purse-string suture in the stomach and then exteriorized through the right flank, caudal to the lymph cannula. If desired, additional cannulations (e.g., bile duct, pancreatic duct, jugular vein, portal vein, etc.) can be performed and are described elsewhere (Waynforth and Flecknell, 1992). The abdomen is then irrigated and closed in two layers.

Following surgery, the animals are placed in Bollman restraint cages (Bollman, 1948) and allowed to recover overnight (18–22 h); the animals are kept in a temperature-regulated chamber (24 °C). It is important to keep

the animals warm during the procedure, as they are unable to curl up and may suffer hypothermia. To compensate for fluid and electrolyte loss due to lymphatic drainage, a 5% glucose–saline solution is infused into the duodenum at 3 ml/h for 6–7 h, followed by an overnight infusion of saline at 3 ml/h. Since the fasting lymph flow rate is 2–3 ml/h, the duodenal infusion of 3 ml/h is sufficient to replace lost fluids and electrolytes. The lymph cannula is placed in a graduated centrifuge tube for continuous collection; the end of the tube should be below the animal's body to aid flow by gravity. Postoperative analgesia (0.1-mg/kg buprenorphine) is provided subcutaneously every 8–12 h, as needed.

The lymph collection procedure can begin after an overnight recovery. Nutrients or test compounds can be either continuously infused or given as a bolus dose through the duodenal tube; for the study of incretin secretion, nutrients are generally provided as a 3-ml bolus dose. Lymph is continuously collected on ice for 1 h prior and 0.5, 1, 2, and 3 h following the nutrient bolus. Shorter time periods for lymph collection are also possible, as long as the animal produces sufficient volumes of lymph for the incretin assays. Lymph flow is determined gravimetrically, assuming a density of 1 g/ml. To prevent hormone degradation, 10% by volume of an antiproteolytic cocktail (0.25 M EDTA, 0.80-mg/ml aprotinin, 80-U/ml heparin) should be added to the lymph samples. The samples can be stored at $-20\,^{\circ}C$ until analysis.

VI. Concluding Remarks and Future Directions

As the prevalence of type 2 diabetes and other metabolic disorders escalates, the interest in the field of incretin biology has expanded. Defects in the incretin system have been implicated in T2DM and may have an important role in the development and perpetuation of obesity. Although cell culture systems are important in deciphering the cellular mechanisms behind incretin synthesis and secretion, to truly understand the complex whole body interactions of these metabolic disturbances, rodent models have become a crucial arm of the investigator's arsenal.

In vivo study of the incretin hormones traditionally involves sampling of the peripheral blood for determining the circulating concentrations. However, the animals need to be continuously monitored to have an accurate depiction of the postprandial incretin dynamics. The low circulating levels of the incretin hormones combined with the sensitivity of the currently available assays require substantial amounts of blood to be removed from an animal. Because of their low blood volume ($\sim$20 ml for the rat), rodent models are limited by the amount of blood that can be removed during the course of a given study.

The lymph fistula rat is an alternative model to study the regulation and the secretion of GIP and GLP-1. The concentrations of both hormones are higher in lymph than in peripheral or portal plasma. The elevated concentrations are due in part to the reduced degradation by DPP-IV and also due to less dilution by the circulating fluid; not only does lymph have a smaller fluid pool size than blood but the flow rate of lymph is also slower. Additionally, this model allows the continuous collection of lymph from conscious animals, eliminating any potential side effects on lymph flow and gastrointestinal function due to anesthesia. The volume of fluid collected from the animals is sufficient to perform a number of assays beyond the measurement of GIP and GLP-1, thus removing the restrictive factor of blood volume. Furthermore, additional collecting cannulae can be surgically inserted to simultaneously sample pancreatic juices, bile, and portal/peripheral blood. Most importantly, the concentration in the intestinal lymph provides a more accurate representation of the local milieu within the intestinal mucosa where the hormones may interact with other enteroendocrine and/or dendritic cells and signal through the enteric or autonomic neurons. As the majority of both incretin hormones have been degraded (up to 80% of the initial secreted output) before entering the peripheral circulation, it is plausible to suspect that the greater part of GIP and GLP-1 action is not effected via traditional endocrine signaling but rather through stimulation of neurons innervating the hepatic portal vein and the intestinal mucosa. Indeed, recent findings have demonstrated that the presence of the GLP-1 receptor on enteric neurons and signaling through these neurons regulates intestinal motility (Amato *et al.*, 2010); therefore, it is possible that signaling through the GLP-1 receptors on these enteric neurons may play important roles in food intake and glucose-sensing.

Several recent studies utilizing the lymph fistula rat model to investigate the regulation of the incretin hormones in response to various acute nutrient challenges have been presented in this chapter. Nevertheless, the potential of the lymph fistula rat model is only beginning to be tapped. Many questions remain unanswered: Is there specific targeting of the incretin hormones to intestinal lymph, as suggested by D'Alessio and colleagues (2007)? Does this differential transport play a physiological role in the action of GIP and/or GLP-1? Will other gastrointestinal hormones, such as gastrin, secretin, and xenin, have elevated lymph–plasma ratios? Interestingly, lymphatic concentrations of ghrelin, an orexigenic hormone secreted by the stomach, have recently been reported to be higher than those documented in the plasma (Tong *et al.*, 2010). The lymph fistula model can also be used to further explore the mechanism underlying nutrient-induced incretin secretion. What is the role of nutrient absorption in regulating incretin release? Is nutrient uptake sufficient to stimulate secretion? What proportion of the effect of the incretin hormones is mediated through innervation of the intestinal mucosa?

As the interest in incretin biology matures, not only for health and disease but also for the development of putative therapeutic agents, advances in methodologies will need to follow suit; the improvement in current protocols, creation of new technologies, and adaptations of knowledge from outside fields will constantly be needed. The lymph fistula rat model is an excellent example of the latter. It is a tool that has been around for the past 60 years; however, only recently has it been exploited for its significance in the study of incretin hormones.

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CHAPTER NINE

Structural Basis for Ligand Recognition of Incretin Receptors

Christina Rye Underwood,* Christoph Parthier,† *and* Steffen Reedtz-Runge‡

Contents

Abstract

The glucose-dependent insulinotropic polypeptide (GIP) receptor and the glucagon-like peptide-1 (GLP-1) receptor are homologous G-protein-coupled receptors (GPCRs). Incretin receptor agonists stimulate the synthesis and secretion of insulin from pancreatic β-cells and are therefore promising agents for the treatment of type 2 diabetes. It is well established that the N-terminal extracellular domain (ECD) of incretin receptors is important for ligand binding and ligand specificity, whereas the transmembrane domain is involved in receptor activation. Structures of the ligand-bound ECD of incretin receptors have been solved recently by X-ray crystallography. The crystal structures reveal a similar fold of the ECD and a similar mechanism of ligand binding, where the ligand adopts an α-helical conformation. Residues in the C-terminal part of the ligand interact directly with the ECD and hydrophobic interactions appear to be the main driving force for ligand binding to the ECD of incretin receptors.

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Obviously, the—still missing—structures of full-length incretin receptors are required to construct a complete picture of receptor function at the molecular level. However, the progress made recently in structural analysis of the ECDs of incretin receptors and related GPCRs has shed new light on the process of ligand recognition and binding and provided a basis to disclose some of the mechanisms underlying receptor activation at high resolution.

I. G-Protein-Coupled Receptors

The super family of G-protein-coupled receptors (GPCRs), also known as seven-transmembrane (7TM) receptors, constitutes one of the largest families of proteins in the human genome (Venter *et al.*, 2001). GPCRs consist of an extracellular N-terminus, seven membrane-spanning α-helices connected by three extracellular and three intracellular loops, and an intracellular C-terminus (Fredriksson *et al.*, 2003). It is believed that all GPCRs share the same characteristic 7TM topology, as the receptors interact with a common intracellular repertoire of heterotrimeric guanine nucleotide-binding proteins (G-proteins). The structural variety among GPCRs is related primarily to a tremendous diversity of ligands (ions, nucleosides, peptide hormones, glycoproteins, and amino acids, etc.). Ligand binding via the extracellular part of a GPCR leads to conformational changes in the interior part of the transmembrane domain, resulting in activation of G-proteins. Based on protein sequence similarity, GPCRs are divided into at least three main families, class A, the rhodopsin-like receptors; class B, the secretin receptor-like receptors; and class C, the metabotropic glutamate receptor-like receptors (Foord *et al.*, 2005).

Class A is by far the largest and best characterized GPCR subfamily. Until recently, the only known structure of a GPCR was that of bovine rhodopsin covalently bound to its ligand—the chromophore retinal—in the "dark" inactive state (Palczewski *et al.*, 2000). The crystal structure of rhodopsin completed decades of biochemical and biophysical work upon the interrelationship of the seven α-helices in GPCRs (Fig. 9.1A; Baldwin *et al.*, 1988; Unger and Schertler, 1995; Unwin and Henderson, 1975). However, although representing class A GPCRs, bovine rhodopsin shows little sequence homology with other class A receptors. The first crystal structure of a typical class A GPCR was published recently: the β_2-adrenoreceptor bound to the inverse agonist carazolol (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007). These structures were soon succeeded by crystal structures of other ligand-bound class A GPCRs, all of which represented inactive conformations of the receptor in question (Jaakola *et al.*, 2008; Warne *et al.*, 2008).

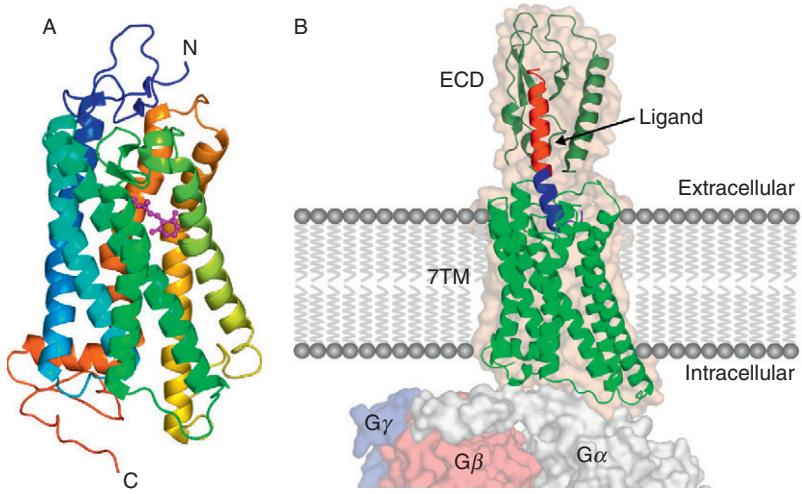

Figure 9.1 Structural features of GPCRs: (A) The crystal structure of chromophore-bound bovine Rhodopsin, a class A GPCR. Cartoon representation showing the arrangement of the seven transmembrane α-helices; the bound chromophore retinal is colored in magenta (PDB code: 1U19). (B) A model for ligand binding in class B GPCRs. Peptide ligands are believed to bind to class B receptors according to the "two-domain" model. First, the C-terminal part of the ligand (colored in red) binds the ECD of the receptor (green), followed by binding of the N-terminal part of the ligand (blue) to the transmembrane (7TM) receptor domain. Structural rearrangements in the receptor activate the heterotrimeric G_s-protein, leading to dissociation of the Gα subunit, and transduce receptor signaling which results in formation of the second messenger cAMP.

The incretin receptors, that is, the glucose-dependent insulinotropic polypeptide (GIP) receptor and the glucagon-like peptide-1 (GLP-1) receptor, belong to class B, which was originally defined in 1991 by the cloning and expression of the rat secretin receptor (Ishihara *et al.*, 1991). Subsequently, other class B receptors were cloned including the parathyroid hormone (PTH) receptor (Jüppner *et al.*, 1991), the calcitonin receptor (Lin *et al.*, 1991), the corticotrophin-releasing factor (CRF) receptor (Chen *et al.*, 1993), the vasoactive intestinal polypeptide (VIP) receptor (Sreedharan *et al.*, 1991), the pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptor (Pisegna and Wank, 1993), and the glucagon receptor (Jelinek *et al.*, 1993).

The peptide hormone receptors of class B are characterized by a relatively large extracellular domain (ECD) of about 120–150 amino acid residues. The ECD is essential for ligand binding and the structural integrity of the ECD relies on three conserved disulfide bonds and several conserved residues as described in detail below. It is assumed that peptide ligands bind

to class B receptors according to the "two-domain" model (Fig. 9.1B). This model suggests that the C-terminal part of the peptide binds the ECD of the receptor while the N-terminal part of the peptide binds to the TM domain and extracellular loops (ECLs).

Ligand binding induces a conformational change that enables the intracellular segments of the receptor to trigger signaling events (reviewed in Hoare, 2005).

The binding orientation of class B ligands was first acknowledged in studies of chimeric peptides and receptors (Bergwitz *et al.*, 1996; Holtmann *et al.*, 1995; Laburthe *et al.*, 2002; Runge *et al.*, 2003a,b; Stroop *et al.*, 1995). The two-domain model is also generally consistent with photoaffinity cross-linking studies of several class B receptors. With a few exceptions, photoreactive side chains in the C-terminal part of the peptide ligand interact with residues in the ECD of the receptor, whereas photoreactive side chains in the N-terminal part of the ligand bind to the TM receptor domain (Assil-Kishawi and Abou-Samra, 2002; Dong *et al.*, 2002, 2004; Gensure *et al.*, 2001). Most recently, substantial evidence for the two-domain model has resulted from the structural characterization of the isolated ECDs of several class B GPCRs. The first structure of an isolated ECD of a class B GPCR was solved by NMR spectroscopy (Grace *et al.*, 2004); subsequently, structures of ligand-bound ECDs were solved including the ECD of the human type-1 CRF receptor (CRFR1) (Pioszak *et al.*, 2008), the human type-1 PACAP receptor (PAC1-R) (Sun *et al.*, 2007), the human GIP receptor (GIP-R) (Parthier *et al.*, 2007), the human GLP-1 receptor (GLP-1R) (Runge *et al.*, 2008), and the human type-1 PTH receptor (PTH-1R) (Pioszak and Xu, 2008). The ECDs of class B receptors all share a common molecular architecture, termed the secretin family recognition fold (Parthier *et al.*, 2009), which is described in detail in the following paragraph.

II. The GLP-1 Receptor

In the 1980s, it was suggested that GLP-1 acts through specific receptors located on the surface of pancreatic β-cells, as high-affinity binding sites of GLP-1 (K_d = 200 pM) and activation of cAMP signal transduction (EC_{50} = 250 pM) were identified in insulinoma β-cell lines (Göke and Conlon, 1988). The rat GLP-1R was cloned in 1992 from a rat pancreatic islet cDNA library (Thorens, 1992) followed by cloning of the human GLP-1R in 1993 (Thorens *et al.*, 1993). The GLP-1R consists of a predicted signal sequence, the ECD of approximately 120 residues, seven membrane-spanning α-helices connected by three extracellular and three intracellular loops (the TM domain), and a C-terminal intracellular domain.

The ECD contains three disulfide bridges and three potential asparagine (*N*)-linked glycosylation sites (Thorens, 1992). Inhibition of the glycosylation process with tunicamycin reduced the number of GLP-1 binding sites, indicating that glycosylation of the GLP-1R ECD is important for receptor expression at the cell surface but not for ligand binding (Göke *et al.*, 1994).

A. The N-terminal extracellular domain (ECD)

The isolated ECD of the GLP-1R can be expressed recombinantly in *Escherichia coli* inclusion bodies and refolded to form a functional receptor domain (Bazarsuren *et al.*, 2002; López de *et al.*, 2003; Runge *et al.*, 2007). GLP-1 binds to the isolated ECD with reduced affinity ($IC_{50} > 500$ nM), compared to the full-length receptor ($IC_{50} \sim 0.5$ nM) (López de *et al.*, 2003; Runge *et al.*, 2007), suggesting that additional binding determinants of GLP-1 are present in the TM domain in agreement with the two-domain binding model.

Two crystal structures of the ECD were solved recently (Runge *et al.*, 2008; Underwood *et al.*, 2010). The first structure showed the ECD in complex with the antagonist exendin-4 (9–39) (Runge *et al.*, 2008) and the second structure showed a complex with the natural ligand GLP-1 (Underwood *et al.*, 2010). The tertiary structure of the ECD in the two ligand-bound forms is almost identical and it is described below. The molecular details of GLP-1 and exendin-4 (9–39) binding are presented in later paragraphs.

The core structure of the GLP-1R ECD represents the typical "secretin family recognition fold" of class B GPCRs (Fig. 9.2A) with an N-terminal α-helix (residues Leu^{32}-Glu^{52}) linked by a disulfide bridge to a central core consisting of two regions of antiparallel β-sheets (β-strand β_1–β_4). Residues Thr^{65}-Phe^{66} (β_1) and Cys^{71}-Trp^{72} (β_2) constitute the first region of antiparallel β-sheets, and the second region is comprised of residues Gly^{78}-Ser^{84} (β_3) and His^{99}-Thr^{105} (β_4) (Fig. 9.2A and B; Runge *et al.*, 2008). Six loop regions (L1–L6) are present between the α-helix and β-strands. The tertiary structure is stabilized by the three conserved disulfide bonds and by several intramolecular interactions (Runge *et al.*, 2008). The α-helix is connected to β_2 through a disulfide bridge between Cys^{46} and Cys^{71} (Fig. 9.3A). The α-helix also interacts with β_1, as the side chain of Arg^{64} (β_1) forms hydrogen bonds with the backbone of Leu^{50}, Asp^{53}, and Pro^{54} (Fig. 9.3A). The two regions of antiparallel β-sheets are joined by the disulfide bridge between Cys^{62} (before β_1) and Cys^{104} (β_4). The last disulfide bridge connects the end of β_3 (Cys^{85}) to a small α-helical segment in the C-terminal part of the ECD (Cys^{126}) (Runge *et al.*, 2008).

In addition to the three disulfide bridges, six residues are conserved in the ECD of all class B GPCRs (Asp^{67}, Trp^{72}, Pro^{86}, Arg^{102}, Gly^{108}, and Trp^{110} in GLP-1R) and they are important for the tertiary structure (Runge

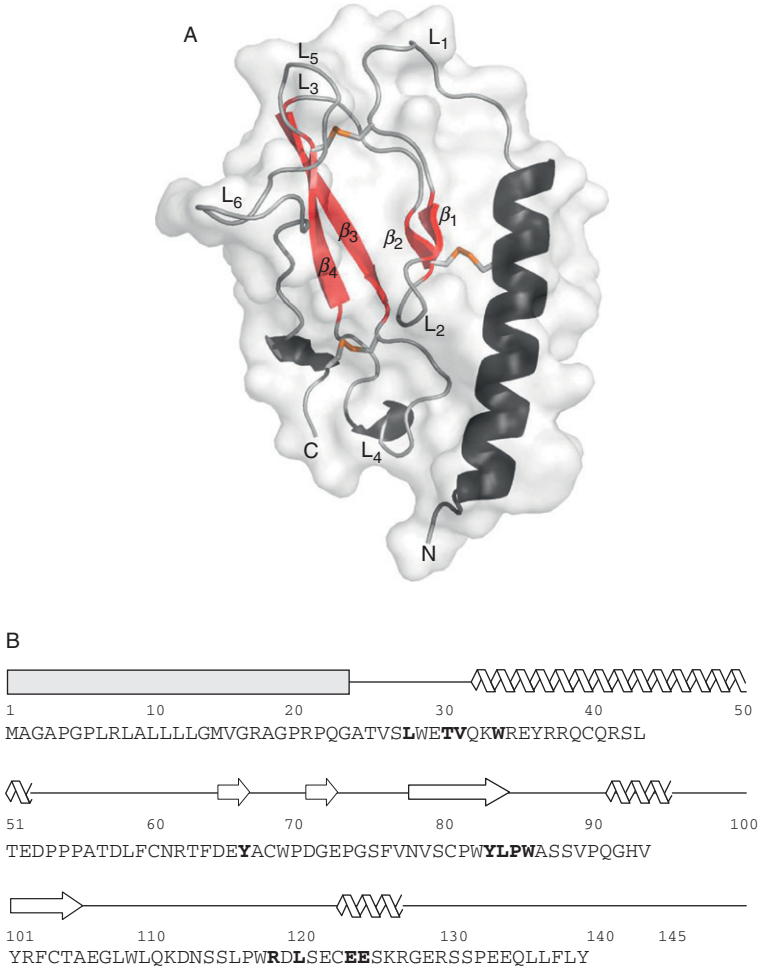

Figure 9.2 The ECD of the GLP-1R: (A) Ribbon representation of the structure of GLP-1R ECD (α-helix in black, β-strands in red, and loops in gray). Disulfide bridges are shown as orange sticks, and loop regions L1–L6 are indicated. Residues Thr^{65}-Phe^{66} (β_1) and Cys^{71}-Trp^{72} (β_2) constitute the first region of antiparallel β-sheets, and the second region is comprised of residues Gly^{78}-Ser^{84} (β_3) and His^{99}-Thr^{105} (β_4). (B) Sequence of the GLP-1R ECD (residues 1–145). Residues shown in bold form the ligand binding site. The secondary structure is depicted in the panel above the sequence. The predicted signal sequence is indicated by a gray rectangle, α-helical segments are shown as helixes, and β-strands are shown as arrows.

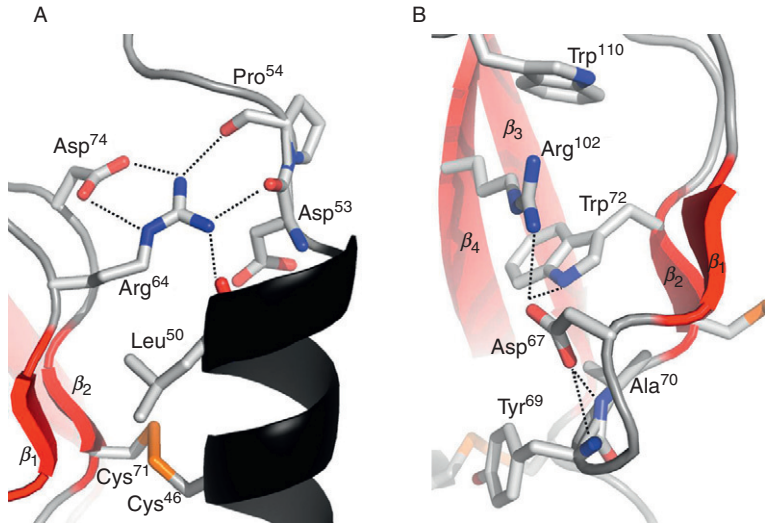

Figure 9.3 Intramolecular interactions of the ECD: (A) Stick representation of the disulfide bridge between Cys^{46} on the α-helix (black) and Cys^{71} on β_2 (red) and the interactions of the guanidine group of Arg^{64} with Leu^{50}, Asp^{53}, and Pro^{54}. (B) Stick representation of the residues Asp^{67}, Tyr^{69}, Ala^{70}, Trp^{72}, Arg^{102}, and Trp^{110}.

et al., 2008). The carboxyl group of Asp^{67} stabilizes the turn between β_1 and β_2 through hydrogen bonds with the backbone nitrogens of Tyr^{69} and Ala^{70} (Fig. 9.3B). In addition, the carboxyl group of Asp^{67} forms hydrogen bonds with the sidechain nitrogens of Arg^{102} on β_4 and Trp^{72} on β_2. Arg^{102} is aligned between the side chains of Trp^{72} and Trp^{110} forming a central cluster, which is also observed in other structures of class B ECDs (Grace *et al.*, 2004; Parthier *et al.*, 2007).

B. Ligand binding to the N-terminal extracellular domain

The ligand binding site of the ECD is formed by residues of the α-helix (Leu^{32}, Thr^{35}, Val^{36}, and Trp^{39}), loop regions (Tyr^{69} of L2 and Tyr^{88}, Leu^{89}, Pro^{90}, Trp^{91} of L4), and the C-terminal part of the ECD (Arg^{121}, Leu^{123}, Glu^{127}, and Glu^{128}). The binding site is the same for GLP-1 and exendin-4 (9–39), which is not surprising given the competitive binding of GLP-1 and exendin-4 (9–39) at both the ECD and the full-length GLP-1R.

1. GLP-1 binding to the ECD

The crystal structures of the ligand-bound ECD revealed that the ligands bind to the ECD in accordance with the two-domain binding model, as only residues in the C-terminal part interact with the ECD. Throughout the text GLP-1 residues are designated with ⋆.

When bound to the ECD, GLP-1 is a continuous α-helix from $Thr^{13\star}$ to $Val^{33\star}$, with a kink around $Gly^{22\star}$ (Fig. 9.4). Only residues between $Ala^{24\star}$ and $Val^{33\star}$ interact with the ECD. The α-helical segment of GLP-1 is amphiphilic, allowing hydrophilic and hydrophobic interactions through opposite faces of the α-helix (Underwood *et al.*, 2010).

The hydrophilic face of GLP-1 comprises residues $Gln^{23\star}$, $Lys^{26\star}$, $Glu^{27\star}$, and $Lys^{34\star}$, of which only $Lys^{26\star}$ interacts directly with the ECD by forming a hydrogen bond with the side chain of Glu^{128} (Fig. 9.5A). $Ala^{24\star}$, $Ala^{25\star}$, $Phe^{28\star}$, $Ile^{29\star}$, $Leu^{32\star}$, and $Val^{33\star}$ define the hydrophobic face of GLP-1, which interacts with the ECD. The hydrophobic residues are exposed toward the complementary hydrophobic binding pocket in the

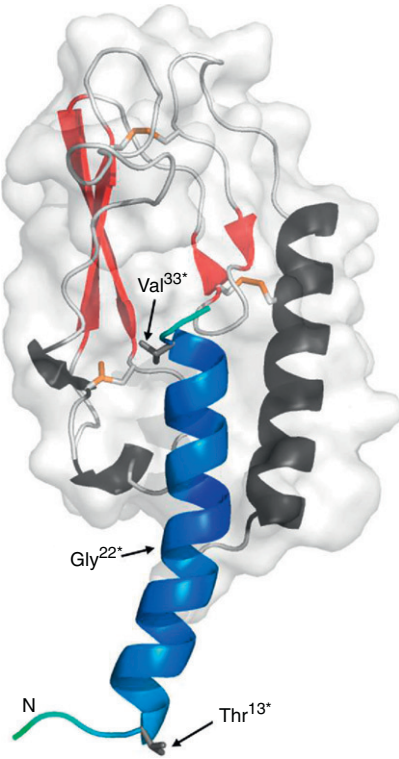

Figure 9.4 Structure of the GLP-1-bound ECD of the GLP-1R, GLP-1 (color gradient according to B-factors; blue: low B-factors; red: high B-factors) bound to the ECD of the GLP-1R (α-helix in black, β-strands in red, loops in gray, and surface in white). Disulfide bridges are shown as orange sticks. GLP-1 is a continuous α-helix from $Thr^{13\star}$ to $Val^{33\star}$ with a kink around $Gly^{22\star}$. $Thr^{13\star}$ and $Val^{33\star}$ are shown as sticks.

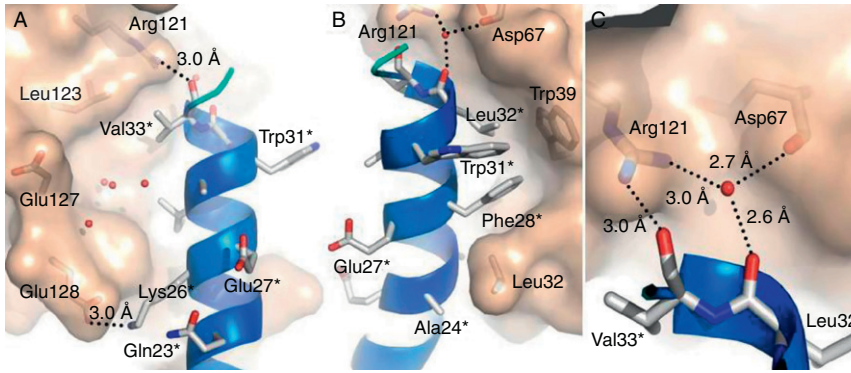

Figure 9.5 Interactions between GLP-1 and GLP-1R ECD: (A) Ribbon representation of GLP-1 (color gradient according to B-factors; blue: low B-factors; red: high B-factors) and its hydrophilic interactions with the GLP-1R ECD (gray surface). GLP-1 residues Gln^{23*}, Lys^{26*}, Glu^{27*}, Trp^{31*}, and Val^{33*} are illustrated as sticks, and receptor residues Arg^{121}, Leu^{123}, Glu^{127}, and Glu^{128} are shown as sticks. Water molecules are shown as red spheres. (B) GLP-1 and its hydrophobic interactions with GLP-1R ECD. GLP-1 residues Ala^{24*}, Glu^{27*}, Phe^{28*}, Trp^{31*}, and Leu^{32*} are illustrated as sticks, and so are ECD residues Leu^{32}, Trp^{39}, Asp^{67}, and Arg^{121}. (C) Ribbon diagram illustrating a common motif found in the GLP-1R ECD and in the GIP-R ECD. The side chain of Arg^{121} interacts with the backbone carbonyls of Asp^{67} and Leu^{32*} through a water molecule. GLP-1 residues Leu^{32*} and Val^{33*} are illustrated as sticks, and so are ECD residues Asp^{67} and Arg^{121}.

ECD (Fig. 9.5B). The importance of particularly Phe^{28*} in GLP-1 binding was previously demonstrated by Ala-scanning (Adelhorst *et al.*, 1994). Substitution of Phe^{28*} with Ala had the most severe effect on GLP-1 affinity in the Ala-scan (IC_{50} value increased by 1300-fold). The Ile^{29*}-Ala and Leu^{32*}-Ala substitutions also reduced GLP-1 affinity significantly (IC_{50} value increased by 93- and 17-fold, respectively) (Adelhorst *et al.*, 1994). Phe^{28*} of GLP-1 is uniquely conserved in the glucagon peptide family (GLP-1, GLP-2, GIP, and glucagon), and it interacts directly with Leu^{32}, Thr^{35}, Val^{36}, and Trp^{39} on the α-helix of ECD (Fig. 9.5B). The hydrophobic surface of GLP-1 is directly facing Trp^{39} and Trp^{91} of ECD, and the importance of Trp^{39} and Trp^{91} in GLP-1 binding to the full-length receptor has previously been established by site-directed mutagenesis (Wilmen *et al.*, 1997). Val^{33*} is the final residue in the α-helix of GLP-1, which interacts with the ECD (Underwood *et al.*, 2010). The side chain of Val^{33*} makes hydrophobic contacts with Tyr^{69} and Leu^{123} and the backbone carbonyl of Val^{33*} interacts through a hydrogen bond with one of the terminal nitrogens of Arg^{121} (Fig. 9.5C). The other terminal nitrogen of Arg^{121} interacts with a water molecule, which is also coordinated by the backbone carbonyl groups of Asp^{67} and Leu^{32*} (Fig. 9.5C). A similar motif is found in the

GIP-R ECD (Parthier *et al.*, 2007). $Trp^{31\star}$ is also on the hydrophobic face of GLP-1, but is rather solvent exposed and does not interact with the ECD. $Trp^{31\star}$ is conserved in the glucagon peptide family, which implies a unique role of this residue. However, substitution of $Trp^{31\star}$ with Ala only reduced the binding affinity of GLP-1 slightly for the full-length GLP-1R (Adelhorst *et al.*, 1994), so the role of $Trp^{31\star}$ in receptor binding is unclear.

2. Exendin-4 (9–39) binding to the ECD

Exendin-4 is a 39 amino acid peptide originally isolated from the venom of the lizard *Heloderma suspectum*, also known as the Gila monster (Eng *et al.*, 1992). GLP-1 and exendin-4 are approximately 50% identical (Fig. 9.6), and exendin-4 classifies as a member of the glucagon peptide family. Throughout the text, exendin-4 residues are designated with $\star\star$.

Soon after the discovery of exendin-4, it was recognized that both exendin-4 and the N-terminally truncated form exendin-4 (9–39) bind with high affinity to the full-length GLP-1R ($K_d = 0.14$ nM and 3 nM, respectively) (Göke *et al.*, 1993). It was also demonstrated that exendin-4 activates GLP-1R with similar potency compared to GLP-1, whereas exendin-4 (9–39) is an antagonist of GLP-1R (Göke *et al.*, 1993).

The crystal structure of the exendin-4 (9–39)-bound ECD showed that exendin-4 (9–39) is a well-defined α-helix from $Leu^{10\star\star}$ to $Asn^{28\star\star}$ (Runge *et al.*, 2008), and the residues that interact with the ECD lie within $Glu^{15\star\star}$ and $Ser^{32\star\star}$ (Fig. 9.7; Runge *et al.*, 2008).

The α-helical segment of exendin-4 (9–39) is defined by $Glu^{16\star\star}$, $Glu^{17\star\star}$, $Arg^{20\star\star}$, $Glu^{24\star\star}$, and $Lys^{27\star\star}$ of which $Arg^{20\star\star}$ and $Lys^{27\star\star}$ interact directly with ECD (Fig. 9.8A). The side chain of $Arg^{20\star\star}$ binds to the side chain of Glu^{128} of ECD through two hydrogen bonds and the side chain of $Lys^{27\star\star}$ interacts with the side chain of Glu^{127} (Fig. 9.8A).

Like $Val^{33\star}$ of GLP-1, the backbone carbonyl of $Lys^{27\star\star}$ of exendin-4 (9–39) forms a hydrogen bond with one of the terminal nitrogens of Arg^{121} (Fig. 9.8A). The other terminal nitrogen of Arg^{121} interacts with a water molecule, which is coordinated by the backbone carbonyl groups of Asp^{67} and $Leu^{26\star\star}$ in a manner similar to the GLP-1-bound form (Fig. 9.5C). $Val^{19\star\star}$, $Phe^{22\star\star}$, $Ile^{23\star\star}$, and $Leu^{26\star\star}$ define the hydrophobic face of

```
                7  10   15   20   25   30   35
GLP-1           HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
Exendin-4       HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS
                1   5    10   15   20   25   30   35
```

Figure 9.6 Sequence alignment of GLP-1 and exendin-4: Conserved residues are highlighted in gray. The highest degree of identity is found in the N-terminal part, where 9 out of 11 residues are conserved. Residue number 1 of exendin-4 corresponds to residue number 7 of GLP-1.

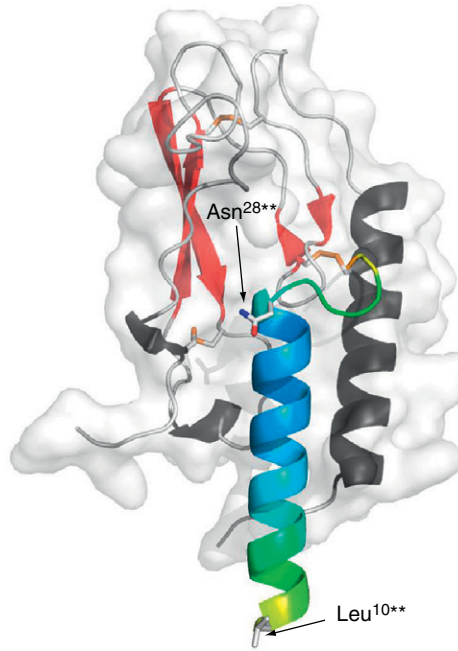

Figure 9.7 Structure of the exendin-4 (9–39) bound to the ECD of the GLP-1R. Exendin-4 is colored according to B-factors; blue: low B-factors; red: high B-factors. The α-helix of the ECD is shown in black, the β-strands in red, the loops in gray, and the surface in white. Disulfide bridges are shown as orange sticks. Exendin-4 (9–39) is a continuous α-helix from Leu^{10**} to Asn^{28**}.

exendin-4 (9–39), which interacts with the ECD. As for GLP-1, the hydrophobic residues are exposed toward the hydrophobic binding pocket of ECD (Fig. 9.8B). Trp^{25**} is also on the hydrophobic face of exendin-4 (9–39), but like Trp^{31*} of GLP-1, Trp^{25**} does not bind directly to ECD (Fig. 9.8B).

3. Binding modes of GLP-1 and exendin-4

Binding studies with the isolated ECD revealed that exendin-4 and exendin-4 (9–39) maintain a surprisingly high affinity for the ECD ($IC_{50} = 6$ nM), whereas GLP-1 binds to the ECD with reduced affinity ($IC_{50} > 500$ nM), compared to the full-length receptor ($IC_{50} \sim 0.5$ nM for both GLP-1 and exendin-4) (López de *et al.*, 2003; Runge *et al.*, 2007). Both GLP-1 and exendin-4 (9–39) are mainly in α-helical conformation when bound to the ECD (Figs. 9.4 and 9.7), and the C-terminal part of the ligands binds the ECD in agreement with the two-domain binding model. The crystal structures of the ligand-bound ECD show that important

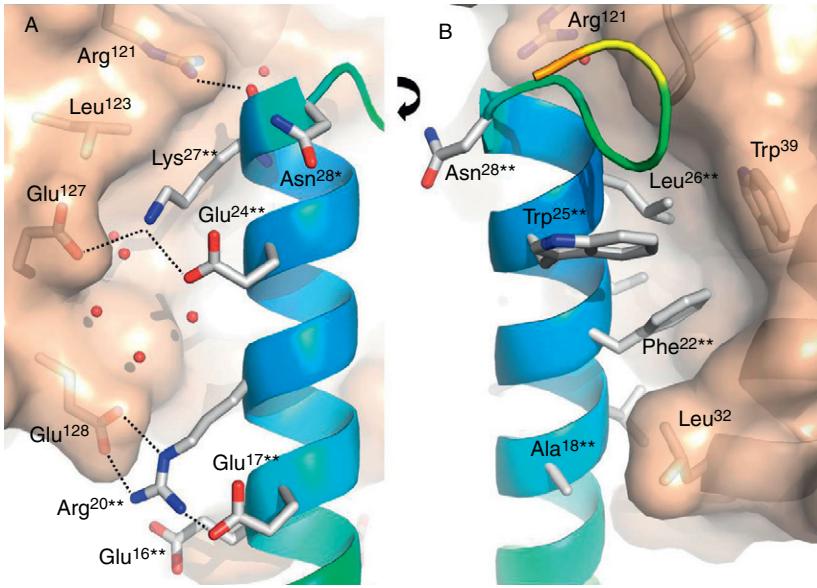

Figure 9.8 Interactions between exendin-4 (9–39) and GLP-1R ECD: (A) Ribbon representation of exendin-4 (9–39) (color gradient according to B-factors; blue: low B-factors; red: high B-factors) and its hydrophilic interactions with GLP-1R ECD (gray surface). Exendin-4 (9–39) residues Glu^{16**}, Glu^{17**}, Arg^{20**}, Glu^{24**}, Lys^{27**}, and Asn^{28**} are illustrated as sticks, and receptor residues Arg^{121}, Leu^{123}, Glu^{127}, and Glu^{128} are shown as sticks below the surface. (B) Ribbon representation of exendin-4 (9–39) (color gradient according to B-factors; blue: low B-factors; red: high B-factors) and its hydrophobic interactions with GLP-1R ECD (gray surface). Exendin-4 (9–39) residues Ala^{18**}, Val^{19**}, Phe^{22**}, Ile^{23**}, Trp^{25**}, Leu^{26**}, and Asn^{28**} are illustrated as sticks, and receptor residues Leu^{32}, Trp^{39}, and Arg^{121} are also shown as sticks below the surface. (B) is rotated clockwise by approximately 90° relative to (A).

hydrophobic interactions are conserved when GLP-1 and exendin-4 bind to the ECD. The hydrophobic interactions are probably the major driving force for ligand binding to the ECD. The hydrophilic ligand-receptor interactions are not conserved to the same extent. Exendin-4 has a nice arrangement of oppositely charged residues (Glu^{16**}, Glu^{17**}, Arg^{20**}, Glu^{24**}, and Lys^{27**}) separated by one turn on the α-helix, which stabilizes the α-helical structure of exendin-4 both in solution and in the receptor-bound state (Runge *et al.*, 2008). Arg^{20**} and Lys^{27**} play a dual role by providing both structural stability to the ligand itself and direct interactions with the ECD (Fig. 9.8A). For peptide ligands of the ECD, a positive correlation has been established between their α-helical propensity in solution and their binding affinity for the ECD (Runge *et al.*, 2007).

The hydrophilic face of GLP-1 is less developed than that of exendin-4 (Figs. 9.5A and 9.8A) and indeed GLP-1 has both a lower α-helical propensity in solution (even in TFE) (Fig. 9.9) and a lower affinity for the ECD compared to exendin-4 (Andersen *et al.*, 2002; Chang *et al.*, 2001; Neidigh *et al.*, 2001; Runge *et al.*, 2007). Hence, the entropy change is more favorable upon binding of exendin-4 to the ECD compared to binding of GLP-1. In addition, the hydrophilic face of exendin-4 forms superior direct interactions with the ECD compared to GLP-1. This is supported by site-directed mutagenesis studies of the full-length GLP-1R, as substitution of Glu^{127} with Ala reduced the affinity of exendin-4 by sevenfold without reduction of GLP-1 affinity (Underwood *et al.*, 2010). Taken together, the differential affinity of the ECD for binding of GLP-1 and exendin-4 is explained as a combined effect of the α-helical propensity of the ligands in solution and direct receptor interactions.

The C-terminal extension of exendin-4 folds back on the α-helix forming the so-called "Trp-cage," in solution containing TFE (Fig. 9.9;

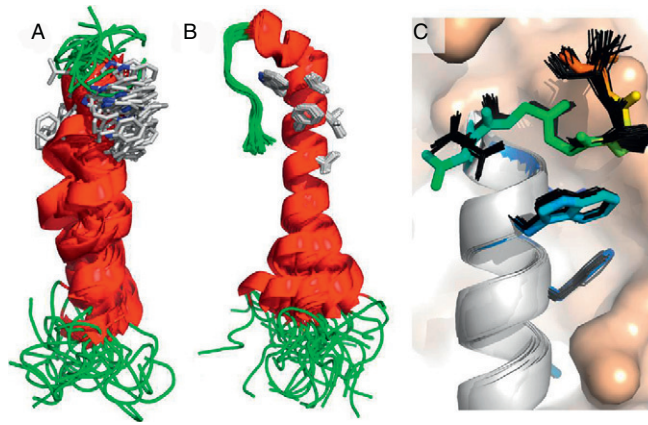

Figure 9.9 Solution structures of GLP-1 and exendin-4 in TFE/water: (A) Solution NMR structure ensemble of GLP-1 in 35% TFE (PDB ID: 1D0R; Chang *et al.*, 2001) and (B) solution NMR structure ensemble of exendin-4 in 30% TFE (PDB ID: 1JRJ, (Neidigh *et al.*, 2001)). The α-helices and loops are shown in red and green, respectively. Residues shown as sticks: Phe^{28*}, Ile^{29*}, Trp^{31},* and Leu^{32*} of GLP-1 and Val^{19**}, Phe^{22**}, Ile^{23**}, Trp^{25**}, and Leu^{26**} of exendin-4. Leu^{21**}-Pro^{38**} of exendin-4 form a tertiary structure known as the "Trp-cage", which shields the side chain of Trp^{25**} from solvent exposure (Neidigh *et al.*, 2001). (C) Superposition of exendin-4 in TFE solution (1JRJ) and receptor-bound exendin-4 (9–39) (Neidigh *et al.*, 2001; Runge *et al.*, 2008). Receptor-bound exendin-4 (9–39) is colored according to B-factors showing Val^{19**}, Phe^{22**}, Ile^{23**}, Trp^{25**}, Leu^{26**}, Asn^{28**}-Ser^{32**} as sticks. The structure ensemble of solution exendin-4 is shown in gray ribbon (α-helix) and black lines (residues Val^{19**}, Phe^{22**}, Ile^{23**}, Trp^{25**}, Leu^{26**}, Asn^{28**}-Ser^{32**}).

Neidigh *et al.*, 2001). In aqueous buffers, the Trp-cage is only partially populated and it is not necessary to maintain a superior helicity in aqueous buffer compared to GLP-1 nor is it necessary for high-affinity binding to the isolated ECD or full-length GLP-1R (Neidigh *et al.*, 2002; Runge *et al.*, 2007). A specific Trp-cage conformation was not observed in the crystal structure of ECD-bound exendin-4 (9–39) and the C-terminal tail ($Ser^{33\star\star}$-$Ser^{39\star\star}$) does not interact with the ECD (Runge *et al.*, 2008). However, the ECD-bound structure of the $Gly^{29\star\star}$-$Ser^{32\star\star}$ segment is strikingly similar to the solution NMR structure of exendin-4 (1JRJ) which suggests that also in the receptor-bound state, the C-terminal tail of exendin-4 ($Ser^{33\star\star}$-$Ser^{39\star\star}$) probably folds back on the α-helix, albeit in multiple conformations—hence the lack of clear electron density for this segment of exendin-4 (Fig. 9.9; Neidigh *et al.*, 2001; Runge *et al.*, 2007).

4. Ligand-dependent structural differences in the ECD

The tertiary structure of the ECD in the GLP-1- and exendin-4-bound forms is almost identical. However, one diverging residue in the two ligands ($Val^{33\star}$ of GLP-1 and $Lys^{27\star\star}$ of exendin-4 (9–39)) causes a shift in the side-chain conformations of four residues in or near the binding pocket of the ECD (Fig. 9.10). In exendin-4 (9–39), $Lys^{27\star\star}$ interacts with Glu^{127}, and positioning of the $Lys^{27\star\star}$ side chain appears to be guided by a hydrophobic interaction with Leu^{123} (Fig. 9.10A). $Val^{33\star}$ of GLP-1 cannot interact with Glu^{127} causing Glu^{127} to change rotamer conformation and point its side chain away from GLP-1.

This results in a shift in the side-chain conformations of Leu^{123}, Arg^{121}, and Pro^{119} compared to the exendin-4 (9–39)-bound structure leading to closure of an otherwise water accessible cavity (Fig. 9.10A). The GLP-1 specific conformations also affect the conserved core of the ECD by rotating the guanidine group of Arg^{102} and by decreasing the distance between Asp^{67} and Arg^{102} compared to the exendin-4 (9–39)-bound structure. In this way, Asp^{67} and Arg^{102} interact directly through a hydrogen bond in the GLP-1-bound structure of ECD, but indirectly via a water molecule in the exendin-4 (9–39)-bound structure (Fig. 9.10B). The functional consequences of the ligand-specific conformational differences are not known, but the ligand-specific effect of the Glu^{127}-Ala mutation in the full-length GLP-1R and the ligand-dependent ECD side-chain conformations point to subtle differences in the binding modes of GLP-1 and exendin-4.

C. The transmembrane and C-terminal domain

No full-length class B GPCR has been crystallized to date, leaving crystal structures of class A receptors the only source of information regarding the tertiary structure of the TM domain. Throughout the last decade, several homology models have attempted to predict the three-dimensional

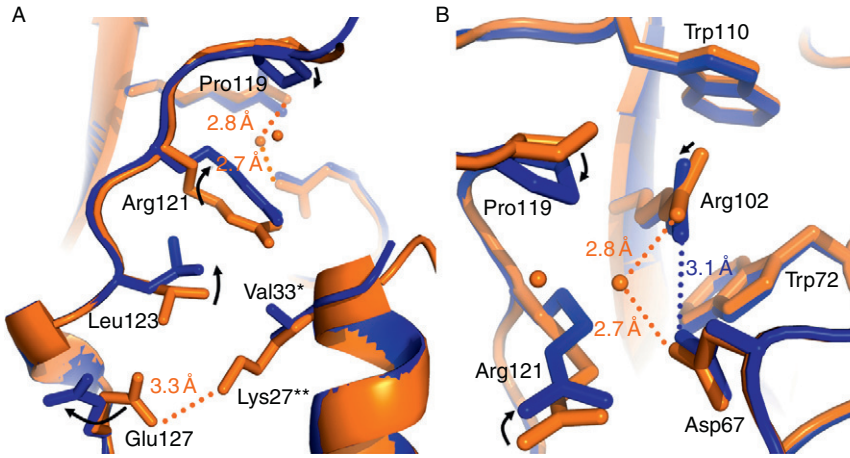

Figure 9.10 Comparison of the GLP-1- and exendin-4 (9–39)-bound structure of GLP-1R ECD: (A) One diverging residue, Val^{33*} of GLP-1 and Lys^{27**} of exendin-4 (9–39), causes a shift in the conformation as indicated with arrows, of four residues of the ECD namely Glu^{127}, Leu^{123}, Arg^{121}, and Pro^{119}. The GLP-1-bound structure is shown in blue and the exendin-4 (9–39)-bound structure is shown in orange. (B) The GLP-1 specific conformations affect the conserved core of the ECD by rotating the guanidine group of Arg^{102} and by decreasing the distance between Asp^{67} and Arg^{102} compared to the exendin-4 (9–39)-bound structure without affecting the relative position of Trp^{72} and Trp^{110}. Water molecules are present only in the exendin-4 (9–39)-bound structure and they are shown as orange spheres.

structure of the GLP-1R TM domain by using rhodopsin as a template structure (Frimurer and Bywater, 1999; Lin and Wang, 2009). However, the sequence identity between class A and class B GPCRs is approximately 10%, which is below the level at which sequence alignment-based structural models can be trusted. Chimeric receptor studies and site-directed mutagenesis have been employed to characterize determinants of ligand binding and activation in the TM part and C-terminal domain of incretin receptors.

A pair of polar residues in the second transmembrane helix (TM2) is a conserved feature among class B GPCRs, and they appear to be important for agonist binding and activation of several class B receptors (Perret *et al.*, 2002; Runge *et al.*, 2003b; Turner *et al.*, 1996). Neighboring Arg and Ser residues make up a polar face of TM2 of GLP-1R (Fig. 9.11), which may provide a surface for interaction with either GLP-1 or another TM helix during receptor activation. The latter is supported by site-directed mutagenesis studies of the PTH receptor, which suggest that Arg^{190} in TM2 could be functionally linked to Gln^{394} in TM7 (Fig. 9.11; Gardella *et al.*, 1996). Alternatively, side-chain interactions between the Ser and Arg residues in TM2 could be important for stabilizing TM2 in an active

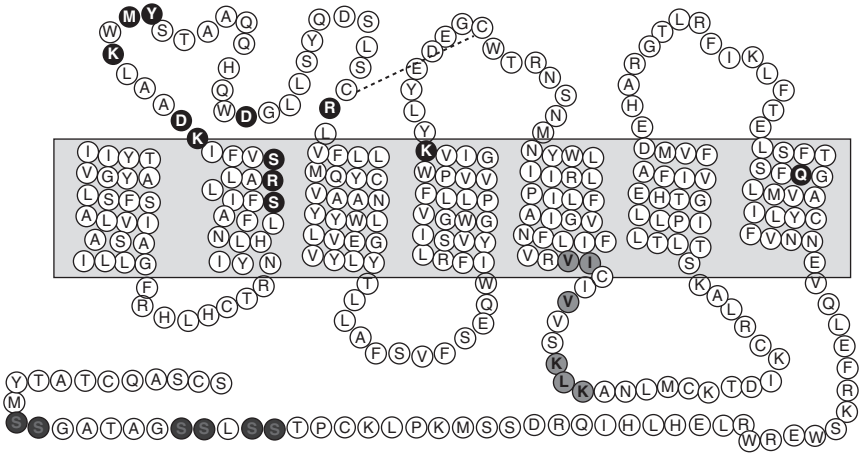

Figure 9.11 Snake diagram of the GLP-1R TM domain: Residues in the TM domain that are experimentally determined to be important for peptide ligand binding and/or activation of the receptor are shown as white letters in black circles. Residues thought to be important for G_s-protein coupling are shown as black letters in gray circles and serine doubles involved in homologous desensitization and internalization are shown as gray letters in black circles. The conserved disulfide bridge that connects ECL1 and ECL2 is indicated with a dashed line.

receptor conformation upon peptide agonist binding (Turner *et al.*, 1996). The importance of polar residues in GLP-1 binding to the TM domain is further supported by the observation that five additional polar residues (Lys^{197}, Asp^{198}, Lys^{202}, Asp^{215}, and Arg^{227}, Fig. 9.11) in the first extracellular loop (ECL1) and one polar residue in TM4 (Lys^{288}, Fig. 9.11) probably contribute to the binding determinants of GLP-1R (Al-Sabah and Donnelly, 2003a; Xiao *et al.*, 2000). Nonpolar residues may also be important for the binding of GLP-1 to ECL1, as substitution of Met^{204} and Tyr^{205} with Ala (Fig. 9.11) markedly reduces the affinity of GLP-1 (López de *et al.*, 2004). A disulfide bridge connecting ECL1 and ECL2 is a conserved feature among all GPCRs (Fig. 9.11). Mutational studies of the VIP receptor suggest that this linkage is essential for high-affinity ligand binding, possibly by stabilizing an active receptor conformation (Knudsen *et al.*, 1997). Taken together, residues in TM2 and ECL1 appear to be important for GLP-1 binding, but to less extent for binding of exendin-4 (López de and Donnelly, 2002; López de *et al.*, 2004). Subtle differences in the binding modes to the TM domain may explain why the two ligands bind to the full-length GLP-1R with similar affinity despite the differential affinity of the isolated ECD. The existence of differences in the binding modes of GLP-1 and exendin-4 to the GLP-1R TM domain is also supported by the observation that N-terminal truncation of exendin-4 by two amino acids

generates a high-affinity antagonist (Montrose-Rafizadeh *et al.*, 1997). This is not observed after N-terminal truncation of GLP-1 by two amino acids (Montrose-Rafizadeh *et al.*, 1997).

Specific determinants for the coupling of GLP-1R to adenylyl cyclase are thought to be located mainly in the intracellular end of TM5 and in N-terminal part of the third intracellular loop (ICL3). Mutation of hydrophobic residues in the intracellular end of TM5 (Val^{327}, Ile^{328}, Fig. 9.11) and in ICL3 (Val^{331}, Fig. 9.11) significantly lowered the production of cAMP without reducing receptor expression (Mathi *et al.*, 1997). In addition, deletion studies of GLP-1R suggest that a conserved Lys-Leu-Lys motif in the N-terminal part of ICL3 (Fig. 9.11) plays a critical role for G_s-protein coupling (Takhar *et al.*, 1996), which is further supported by studies of synthetic peptide sequences derived from ICL3 of the GLP-1R (Hällbrink *et al.*, 2001). Truncation studies indicate that the C-terminal part of GLP-1R is not involved in the signal transduction process, as deletion of the last 44 amino acids of GLP-1R does not compromise receptor activity (Vazquez *et al.*, 2005). Instead, phosphorylation of three serine doublets in the C-terminal part of GLP-1R (Ser^{441}/Ser^{442}, Ser^{444}/Ser^{445}, and Ser^{441}/Ser^{452}, Fig. 9.11) appears to be important for homologous desensitization and internalization of the receptor (Widmann *et al.*, 1997).

III. The GIP Receptor

The receptor of glucose-dependent insulinotropic polypeptide (GIPR)—also a member of class B GPCRs—displays approximately 40% sequence identity to GLP-1R. Hence, the two incretin receptors are expected to show the same overall topology. GIPR was initially cloned from a rat cerebral cortex cDNA library (Usdin *et al.*, 1993), followed by cloning of the hamster (Yasuda *et al.*, 1994) and human receptors (Yamada *et al.*, 1995). In contrast to the GLP-1R, several splice variants of the GIPR have been identified, resulting in functionally distinct receptor isoforms (Boylan *et al.*, 1999; Gremlich *et al.*, 1995; Harada *et al.*, 2008; Volz *et al.*, 1995). Premature stop codons have been reported in a few cases leading to truncated GIPRs (Boylan *et al.*, 1999; Harada *et al.*, 2008). The functional properties of the truncated receptors are not fully established, but they may be involved in regulating the expression of wild-type GIPRs at the cell surface (Harada *et al.*, 2008). The full-length GIPR binds GIP with high affinity (K_d = 0.3 nM) and high specificity (Amiranoff *et al.*, 1984). Studies of chimeric GIP/GLP-1 receptors first established the importance of the ECD in ligand binding (Gelling *et al.*, 1997a). Recent binding studies of the isolated ECD of the GIPR showed that GIP binds to the ECD with reduced affinity

(K_d = 1.1 μM) compared to the full-length receptor (Parthier *et al.*, 2007). Hence, as for GLP-1, other receptor domains appear to be important for GIP recognition.

A. The N-terminal extracellular domain (ECD)

The ECD of the GIP receptor also adopts the "secretin family recognition fold" (Fig. 9.12A) including an N-terminal α-helix (residues Ala^{32}-Ala^{52}) that packs against a central core consisting of two regions of antiparallel β-sheets (β-strands β_1–β_4) (Parthier *et al.*, 2007). Residues Ser^{64}-Phe^{65} (β_1) and Cys^{70}-Trp^{71} (β_2) constitute the first antiparallel β-sheet, and the second β-sheet is comprised of residues Ala^{78}-Ser^{83} (β_3) and Phe^{98}-Cys^{103} (β_4). In addition, the ECD contains two short α-helical segments in the C-terminal (His^{91}-Val^{94} and Thr^{116}-Cys^{118}) similar to GLP-1R ECD. Five loop regions (L1–L5) are present between the β-strands (Parthier *et al.*, 2007). The tertiary structure is stabilized by three conserved disulfide bonds also found in the GLP-1R ECD. Cys^{46} forms a disulfide bridge with Cys^{70} connecting the α-helix and β_2. The two regions of antiparallel β-sheets are

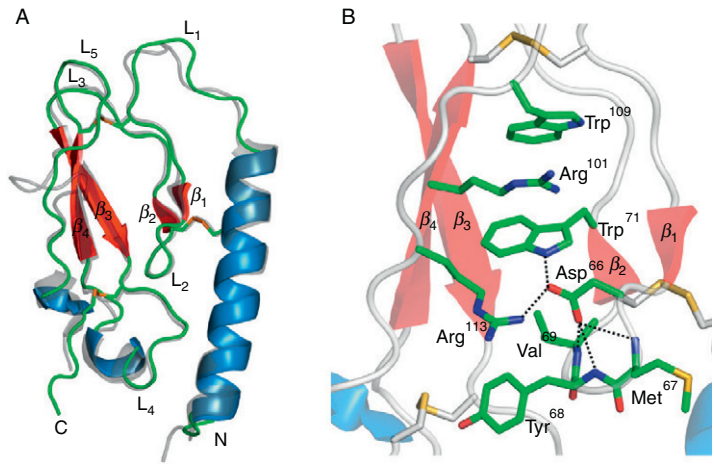

Figure 9.12 The extracellular domain of the GIP receptor: (A) GIPR ECD adopts the "secretin family recognition fold". The secondary structure elements (α-helices colored in blue, β-strands in red) are connected by five loop regions (L1–L5). The fold is stabilized by three disulfide bonds (orange sticks) present also in other ECDs of class B GPCRs. Superimposition of the GLP-1R ECD structure (gray ribbon) shows the high structural similarity between the ECDs of GIPR and GLP-1R. (B) Stick representation of GIPR residues forming the core of the ECD by intramolecular interactions (residues colored according to atom type: carbon—green, nitrogen—blue, oxygen—red, sulfur—yellow, hydrogen bonds displayed as dots).

joined through a disulfide bridge between Cys^{61} (β_1) and Cys^{103} (β_4), and the last disulfide bridge connects the end of β_3 (Cys^{84}) to the C-terminal part of the ECD (Cys^{118}) (Fig. 9.12A).

The tertiary structure of the GIPR ECD is also stabilized by intramolecular interactions between residues that are conserved in class B GPCRs. Similar to the GLP-1R ECD, Arg^{101} is sandwiched between the side chains of Trp^{71} and Trp^{109}, forming a central cluster, but the side chain of Arg^{101} does not interact with Asp^{66} as observed in the GLP-1R structure (Fig. 9.3B). As also observed in the GLP-1R, Asp^{66} inhabits a central role by stabilizing the arrangement of discontinuous ECD segments in spatial proximity (Fig. 9.12B). It interacts via hydrogen bonds with Met^{67}, Tyr^{68}, Val^{69}, Trp^{71}, and Arg^{113}. In this way, Asp^{66} helps to stabilize the loop region (L2) at the base of the ligand binding pocket. As observed in the GLP-1R, the main ligand binding site is comprised of residues of the α-helical segment (Leu^{35}, Val^{36}, and Trp^{39}), loop regions (Met^{67}, Tyr^{68} of L2 and Tyr^{87}, Leu^{88}, Pro^{89}, and Trp^{90} of L4) and the C-terminal part of the ECD (His^{115}) (Parthier *et al.*, 2007). In the following, GIP residues are designated with ′.

1. The binding of GIP to the ECD

NMR studies have shown that native GIP (1-42) adopts an α-helical conformation in water (Alana *et al.*, 2006). GIP (1-42) and the truncated but equipotent GIP (1-30) are α-helical between $Phe^{6'}$ and $Ala^{28'}$ in 50% TFE (Alana *et al.*, 2004, 2007). This correlates very well with the receptor-bound structure of GIP, as GIP is a continuous but slightly curved α-helix from $Phe^{6'}$ to $Ala^{28'}$ when bound to the ECD of GIPR (Parthier *et al.*, 2007). Residues 33–42 of GIP, not seen in the complex structure, have been linked to somatotropic properties of GIP and do not contribute to the insulinotropic action of the hormone (Morrow *et al.*, 1996).

GIP binds the ECD in agreement with the two-domain binding model (Fig. 9.1B), as C-terminal residues between $Asp^{15'}$ and $Lys^{30'}$ interact with the ECD (Fig. 9.13A; Parthier *et al.*, 2007). As for GLP-1 and exendin-4, the α-helical segment of GIP is amphiphilic, facilitating hydrophilic and hydrophobic interactions with the ECD. The hydrophobic face of GIP is defined by residues $Phe^{22'}$, $Val^{23'}$, $Leu^{26'}$, and $Leu^{27'}$ (Parthier *et al.*, 2007), which are exposed toward the complementary hydrophobic binding pocket of ECD (Fig. 9.13B). The importance of $Phe^{22'}$, $Val^{23'}$, $Leu^{26'}$, and $Leu^{27'}$ in GIP binding has been confirmed by Ala-substitution (Parthier *et al.*, 2007), emphasizing the significance of hydrophobic interactions for ligand binding to the GIPR ECD. The backbone carbonyls of $Leu^{26'}$ and $Leu^{27'}$ also form hydrogen bonds to Arg^{114} and the conserved Asp^{66}, respectively (Parthier *et al.*, 2007). Residues on the hydrophilic face of GIP ($Asp^{15'}$, $Gln^{19'}$, and $Gln^{20'}$) interact with the ECD through hydrogen bonds partially mediated by water molecules in the binding pocket (Fig. 9.13C). $Asp^{15'}$ forms hydrogen bonds to the backbone amide of Ala^{32}, and $Gln^{19'}$ forms hydrogen bonds

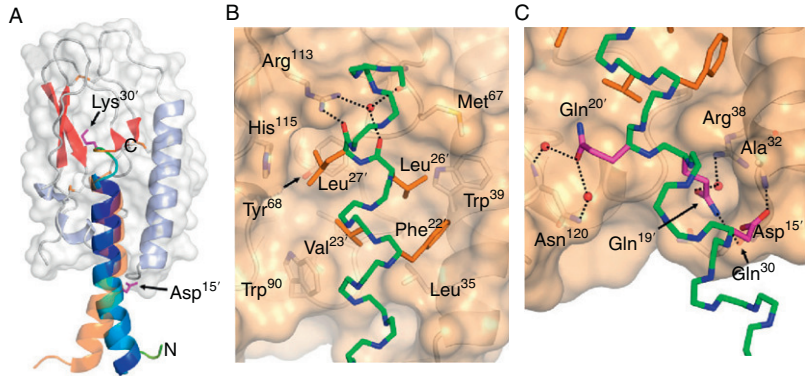

Figure 9.13 Ligand binding by the GIPR extracellular domain: (A) The ligand GIP (ribbon representation, color gradient according to B-factors; blue: low B-factors; red: high B-factors) is bound to the ECD (surface and ribbon representation) in α-helical conformation. Residues from the C-terminal part of the ligand spanning $Asp^{15'}$ to $Lys^{30'}$ (shown as magenta sticks) are involved in ECD binding. (B) GIP residues $Phe^{22'}$, $Val^{23'}$, $Leu^{26'}$, and $Leu^{27'}$ (side chains shown as orange sticks, the ligand main chain shown as green and blue sticks) are involved in hydrophobic interactions to a binding groove formed by the residues Leu^{35}, Trp^{39}, Met^{67}, Tyr^{68}, Trp^{90}, and His^{115} of GIPR ECD (shown as sticks under surface). The carbonyl oxygens of $Leu^{26'}$ and $Leu^{27'}$ form also hydrogen bonds to the ECD (showns as dotted lines), directly or mediated by water (red sphere). (C) Hydrophilic GIP residues $Asp^{15'}$, $Gln^{19'}$, and $Gln^{20'}$ (side chains shown as magenta sticks, ligand main chain shown as green and blue sticks) interact via hydrogen bonds (shown as dotted lines) with the ECD resdiues Gln^{30}, Ala^{32}, Arg^{38}, and Asn^{120} (shown as sticks under surface). Water molecules are shown as red spheres.

to Gln^{30} and a hydrogen bond to Gly^{29}, Thr^{31}, and Arg^{38} through a water molecule. The side chain of $Gln^{20'}$ is involved in hydrogen bonds to the side chain and backbone amide of Asn^{120} via two different water molecules.

B. The transmembrane and C-terminal domain

The TM domain of the GIPR has not been studied to the same extent as that of GLP-1R. However, chimeric GIP/GLP-1 receptor studies have demonstrated the importance of ECL1 in GIP binding, and TM1 appears to be involved in GIPR activation (Gelling *et al.*, 1997a). In contrast to GLP-1R, the wild-type GIPR displays agonist-independent activity. Site-directed mutagenesis studies suggest that residues in TM5 and TM6 could be involved in mediating constitutive activity, as mutation of Arg^{316} (TM5) and Glu^{354} (TM6) reduced basal activity (Fortin *et al.*, 2010). However, this could be a result of reduced receptor expression (Fortin *et al.*, 2010). On the contrary, mutation of Thr^{343} in the intracellular end of TM6 (Fig. 9.14) has

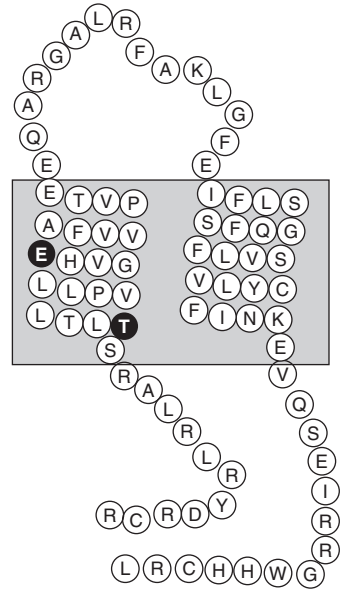

Figure 9.14 Snake diagram representing TM6, ECL3, and TM7 of GIPR. Residues Thr^{343} and Glu^{354} in TM6 may be involved in mediating constitutive activity of the receptor. The residues are shown as white letters in black circles.

been shown to increase agonist-independent activity without affecting the expression level of the receptor (Tseng and Lin, 1997). Similar studies of the human PTH receptor showed that mutation of Thr^{410} in TM6 increased basal activity without changing receptor expression (Schipani *et al.*, 1997).

Little is known about the mechanisms that control G-protein coupling or desensitization and internalization of GIPR. Complete removal of the C-terminal abolishes ligand binding (Tseng and Zhang, 1998), but truncation of the GIPR C-terminus by Arg^{418} does not affect the expression level or the functionality of the receptor compared to the wild type (Wheeler *et al.*, 1999). Hence, as for GLP-1R, most of the GIPR C-terminal is not required for signaling. Instead, serine doublets in the C-terminal part of the receptor appear to be involved in receptor internalization (Wheeler *et al.*, 1999).

IV. Common and Divergent Features of GLP-1R and GIPR Ligand Binding

The GLP-1R and GIPR demonstrate complete specificity for their endogenous ligands (Gelling *et al.*, 1997a; Graziano *et al.*, 1996; Runge *et al.*, 2003a). GLP-1R does not bind GIP and vice versa, although GIPR show

some exendin-4 binding at very high concentrations of 1–10 μM (Gremlich *et al.*, 1995). Until now, the basis for the high specificity of the two incretin receptors is not well understood.

From a sequence alignment of GLP-1 and GIP (Fig. 9.15), it is obvious to note, that the N-terminal part of the ligands show a higher similarity (~60% identity) than the C-terminal part (~25% identity). This suggests that the specificity may be largely determined by the C-terminal ligand residues.

It is well established that the C-terminal part of an incretin ligand is bound by the ECD of the respective incretin receptor. The ECD of the GLP-1R and GIPR share approximately 34% sequence identity, and the crystal structures of the ligand-bound ECDs are very similar (Fig. 9.12A). The fold of the ECD is the same and conserved residues (Asp^{67}, Trp^{72}, Arg^{102}, and Trp^{110} of GLP-1R) in the center of the ECD help to stabilize the tertiary structure and to form the ligand binding pocket (Figs. 9.3B and 9.12B), although the molecular details are slightly different for each ligand and receptor. For example, Asp^{67} and Arg^{102} interact directly through a hydrogen bond in the GLP-1-bound structure of the GLP-1R ECD, but indirectly via a water molecule in the exendin-4 (9–39)-bound structure of the GLP-1R ECD, causing Arg^{101} to point away from Asp^{66} (Fig. 9.16A). Therefore, Asp^{66} and Arg^{101} do not interact in the GIPR ECD despite the presence of a water molecule between the two residues as observed in the exendin-4 (9–39)-bound structure of the GLP-1R ECD. It is indeed possible that rather subtle differences as such in this region of the ECD reflect different ligand specificity.

The crystal structures as well as Ala-scanning experiments of GLP-1 and GIP (Adelhorst *et al.*, 1994; Parthier *et al.*, 2007; Runge *et al.*, 2008; Underwood *et al.*, 2010) suggest that hydrophobic ligand-receptor interactions play a central role in ligand binding to the ECD of incretin receptors. Hydrophobic residues in the C-terminal of the peptide interact with a complementary hydrophobic binding pocket on the surface of the ECD (Figs. 9.5B, 9.8B, and 9.13B). In each case, the hydrophobic binding pocket is comprised of conserved residues from the α-helix of the ECD and from loops 2 and 4 (Parthier *et al.*, 2007; Runge *et al.*, 2008; Underwood *et al.*, 2010). The apparent similarity of the ECD of incretin receptors suggests that minor structural differences are responsible for the high ligand specificity of

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          7   10    15    20    25    30    35
GLP-1     HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
GIP       YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
          1    5    10    15    20    25    30    35    40
```

Figure 9.15 Sequence alignment of GLP-1 and GIP. Identical residues are shaded in gray. Note the different residue numbering system of GLP-1 and GIP. The residues referred to in the text as 'N-terminal part' of the ligands spans the first 14 amino acids. The remaining residues are referred to as the 'C-terminal part'. GIP residues 33–41 do not contribute to the insulinotropic activity of the hormone.

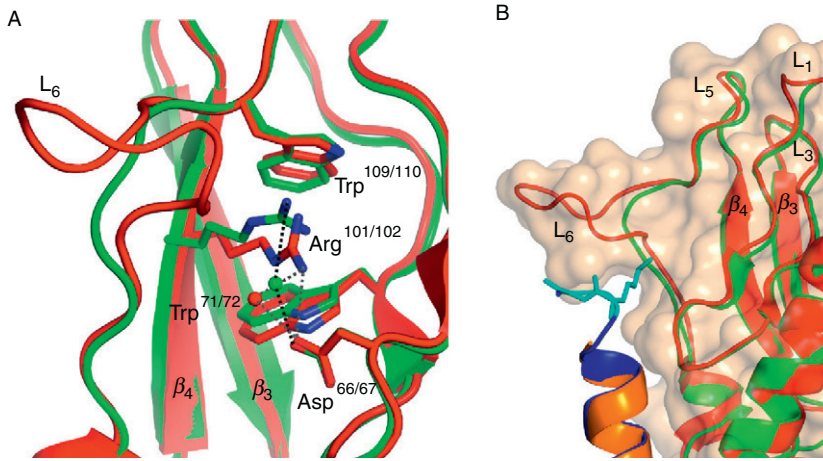

Figure 9.16 Structural differences between the GLP-1R ECD (red) and the GIPR ECD (green): (A) The arrangement of central residues of the ECDs (shown as sticks) differ only slightly, for example, the side chain of Arg^{102} of GLP-1R ECD and Arg^{101} of GIPR ECD, respectively. Water molecules are shown as spheres, hydrogen bonds by dotted lines. (B) An extra loop (L_6) is present in GLP-1R ECD (red ribbon and surface representation), which is not seen in GIPR (green ribbon representation). The C-terminus of bound GLP-1 (orange ribbon) locates near L_6. Binding of GIP (blue ribbon) to GLP-1R would lead to a clash of C-terminal GIP residues (cyan sticks) with L_6.

the receptors. A comparison of the crystal structures reveals, for example, that the α-helix of GIP is one helix turn longer in the C-terminus compared to GLP-1. Superposition of the GLP-1R and GIPR ECDs shows that GIP may not fit in the ECD binding pocket of GLP-1R, as residues in the C-terminal part of GIP may clash with loop 6 of GLP-1R, which is not present in GIPR (Fig. 9.16B). This is further supported by binding studies, as GIP does not bind to the soluble GLP-1R ECD (unpublished). Vice versa, GLP-1 binds to the soluble GIPR ECD with only 10-fold lower affinity compared to GIP ($K_d = 14 \pm 0.8$ μM for GLP-1 vs. 1.1 ± 0.1 μM for GIP) (Parthier *et al.*, 2007), indicating that loop 6 of GLP-1R may indeed be involved in ligand selectivity. However, GLP-1 does not bind to the full-length GIPR (Amiranoff *et al.*, 1984) suggesting that ligand selectivity cannot be entirely determined by the ECD and that additional determinants of ligand selectivity are present in the TM domain.

Several receptor-binding models have been proposed with respect to the full-length class B receptors (Al-Sabah and Donnelly, 2003b; Ceraudo *et al.*, 2008; Dong *et al.*, 2008; Lin and Wang, 2009; Miller *et al.*, 2007). The models are based on studies of chimeric peptides and receptors, photoaffinity cross-linking or molecular modeling, and they are all variations of the

two-domain binding model. The recent advance in structural characterization of the isolated ECDs of class B receptors has paved the way for significantly improved models of ligand binding. Several studies show that the N-terminal part of incretins is important for receptor activation (Gelling *et al.*, 1997b; Hinke *et al.*, 2001; Montrose-Rafizadeh *et al.*, 1997; Thorens *et al.*, 1993) and that increasing the degree of α-helicity in the ligand can increase potency (Manhart *et al.*, 2003; Miranda *et al.*, 2008; Murage *et al.*, 2008). A novel model for ligand binding to full-length class B GPCRs involves α-helix formation of the ligand (Parthier *et al.*, 2009). The model is based on the observation that the N-terminal part of GLP-1, GIP, and exendin-4 (9–39) adopts an α-helical conformation even though this segment does not interact with ECD. In solution, the N-terminal part of the ligands is highly flexible. The C-terminal segment of the ligand is stabilized in an α-helical conformation as a consequence of binding to the ECD. Hence, binding to the ECD could subsequently induce or stabilize an α-helical extension toward the N-terminal part of the ligand. This hypothesis is supported by the solution structure of GLP-1 in different concentrations of TFE. TFE is a helix-stabilizing organic solvent and induces a transition from a disordered conformation to an α-helical conformation in the C-terminal part of GLP-1, which is gradually extended toward the N-terminal of the peptide by increasing concentrations of TFE (Chang *et al.*, 2001). Similarly, the helical propensity of other class B ligands depends on the solvent composition (Alana *et al.*, 2006; Motta et al., 1998; Sasaki et al., 1975). Consequently, it has been suggested that the α-helical conformation of the ligand—stabilized by binding to the ECD—restricts the conformation of the N-terminal residues in the ligand, thereby enabling the ECD to present the ligand N-terminus to the TM domain of the receptor, which subsequently leads to activation of downstream signaling cascades. This novel model of peptide binding to class B GPCRs is compatible with the two-domain binding model, and necessitates a movement of the ECD relative to the TM domain of the receptor. However, structural characterization of a full-length class B GPCR will be necessary to determine the orientation of the ECD relative to the TM domain and eventually clarify the binding and activation mechanism of incretin receptors.

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CHAPTER TEN

Epac2-Dependent Rap1 Activation and the Control of Islet Insulin Secretion by Glucagon-Like Peptide-1

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Contents

Abstract

Glucagon-like peptide-1 (GLP-1) binds its Class II G protein-coupled receptor to stimulate cyclic adenosine monophosphate (cAMP) production and to potentiate the glucose metabolism-dependent secretion of insulin from pancreatic β cells located within the islets of Langerhans. Prior clinical studies demonstrate that this cAMP-mediated action of GLP-1 to potentiate glucose-stimulated insulin secretion (GSIS) is of major therapeutic importance when evaluating the abilities of GLP-1 receptor (GLP-1R) agonists to lower levels of blood glucose in type 2 diabetic subjects. Surprisingly, recent *in vitro* studies of human or rodent islets of Langerhans provide evidence for the existence of a noncanonical mechanism of β cell cAMP signal transduction, one that may explain how GLP-1R agonists potentiate GSIS. What these studies demonstrate is that a

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cAMP-regulated guanine nucleotide exchange factor designated as Epac2 couples β cell cAMP production to the protein kinase A-independent stimulation of insulin exocytosis. Provided here is an overview of the Epac2 signal transduction system in β cells, with special emphasis on Rap1, a Ras-related GTPase that is an established target of Epac2.

I. Introduction

Glucagon-like peptide-1 (GLP-1) is an intestinally derived incretin hormone that potentiates the glucose metabolism-dependent secretion of insulin from β cells located within the islets of Langerhans. This action of GLP-1 to potentiate glucose-stimulated insulin secretion (GSIS) is achieved by the binding of GLP-1 to the β cell GLP-1 receptor (GLP-1R), a Class II GTP-binding protein-coupled receptor (GPCR) that is positive coupled to 3′-5′-cyclic adenosine monophosphate (cAMP) production (Thorens, 1992). Since GLP-1R agonists (e.g., exenatide, liraglutide) stimulate pancreatic insulin secretion and lower levels of blood glucose in patients diagnosed with type 2 diabetes mellitus (Campbell and Miller, 2009; Israili, 2009), there is considerable interest in identifying the molecular mechanisms of β cell stimulus–secretion coupling that are regulated by GLP-1 in a cAMP-dependent manner. Summarized here are recent findings that provide evidence for a functional coupling of the GLP-1R to a noncanonical mechanism of cAMP signal transduction, one that is mediated by the cAMP-regulated guanine nucleotide exchange factor designated as Epac2. In this regard, we focus on the likely involvement of Rap1, a Ras-related GTPase that is reported to couple Epac2 activation to the potentiation of GSIS (Shibasaki *et al.*, 2007). For additional information concerning Epac2 and Rap1, the reader is referred to prior reviews of this subject matter (Holz *et al.*, 2006; Seino and Shibasaki, 2005).

II. PKA and Epac2 Regulate Insulin Secretion from β Cells

Although it is widely accepted that cAMP mediates the insulin secretagogue action of GLP-1 in β cells, there is debate concerning the identities of the cAMP signaling pathways that mediate this action of the hormone. The principal issue concerns whether the secretagogue action of GLP-1 is explained by its ability to activate protein kinase A (PKA) or whether GLP-1 also signals through Epac2. A major, if not exclusive, role for PKA as the dominant cAMP-binding protein controlling glucose-dependent insulin secretion has been championed by Hatakeyama *et al.* (2006) in

live-cell imaging studies of mouse β cell exocytosis. These investigators reported that PKA mediated the cAMP-dependent potentiation of large dense core secretory vesicle exocytosis, whereas in these same cells Epac2 was implicated in the cAMP-dependent exocytosis of small synaptic vesicle-like structures (Hatakeyama *et al.*, 2007). Since insulin is present within large vesicles, whereas the neurotransmitter GABA is found within small vesicles of β cells, such findings argue for an unexpected segregation of cAMP signaling such that PKA and Epac2 differentially regulate the exocytosis of two distinct subpopulations of secretory vesicles. Importantly, these findings concerning mouse β cells are in general agreement with one earlier study of human β cells in which it was reported that GLP-1 potentiated exocytosis that was both depolarization-induced and Ca^{2+}-dependent, as determined by the measurement of membrane capacitance, and that this effect of GLP-1 was blocked by a cAMP analog (Rp-cAMPS) that is a selective antagonist of PKA activation (Gromada *et al.*, 1998). Thus, for both human and mouse β cells, it seems clear that PKA plays an important role in the stimulation of exocytosis. Yet, since none of the above-mentioned studies examined insulin secretion *per se*, and instead relied on biophysical methods to detect exocytosis, no firm conclusions can be reached concerning whether or not PKA is the primary cAMP-binding protein by which GLP-1 potentiates GSIS.

Given that the collective wisdom supports a role for PKA in the regulation of islet insulin secretion, it is remarkable that in studies of mouse islets performed by Kashima and coworkers, a dramatically different story emerged concerning the existence of a noncanonical mechanism of cAMP signaling in β cells. It was reported that an inhibitor of PKA activity (H-89) reduced but did not fully abrogate the action of GLP-1 to potentiate GSIS (Kashima *et al.*, 2001). Moreover, in these same islets, the action of GLP-1 to potentiate GSIS was reduced but not abrogated following treatment of islets with Epac2 antisense (AS) deoxyoligonucleotides. Since combined treatment of islets with H-89 and Epac2 AS suppressed the secretagogue action of GLP-1 in an additive manner, it was concluded that in addition to PKA, it is the cAMP-binding protein Epac2 that couples cAMP production to the potentiation of GSIS (Kashima *et al.*, 2001). This conclusion was reinforced by a parallel study using PKA inhibitors in which a PKA-independent action of GLP-1 to stimulate mouse islet insulin secretion was confirmed (Nakazaki *et al.*, 2002).

One important caveat to the interpretation of findings obtained using PKA inhibitors is that this approach can only establish PKA-independence and cannot definitively establish a role for Epac2 in the potentiation of GSIS by cAMP-elevating agents. A more convincing strategy would be to demonstrate antagonism of GLP-1-stimulated insulin secretion through the use of cAMP analogs that competitively inhibit cAMP-dependent activation of Epac2. Unfortunately, this approach is not feasible at the present time due to

the lack of suitable Epac2 antagonists (Holz *et al.*, 2008). There are, however, selective activators of Epac2, and these compounds are known to stimulate insulin secretion (Kelley *et al.*, 2009). Chepurny and coworkers reported that human islet GSIS was potentiated by an acetoxymethyl ester (AM-ester) of a cAMP analog (8-pCPT-2′-*O*-Me-cAMP-AM) that is a selective activator of Epac (Chepurny *et al.*, 2009, 2010). Importantly, this action of 8-pCPT-2′-*O*-Me-cAMP-AM was not secondary to PKA activation (Chepurny *et al.*, 2010). Studies of this sort are significant because until recently, the assessment of Epac2 signal transduction was hampered by the poor membrane permeability of first-generation Epac-selective cAMP analogs (ESCAs) that lack the AM-ester moiety (Chepurny *et al.*, 2009). Expanding on this analysis, it has now been demonstrated that 8-pCPT-2′-*O*-Me-cAMP-AM also exerts profound stimulatory effects on human β cell Ca^{2+} signaling (Chepurny *et al.*, 2010). As discussed below, this action of the ESCA to raise levels of cytosolic Ca^{2+} involves not only the mobilization of an intracellular source of Ca^{2+}, but it also results from Ca^{2+} influx that is secondary to β cell depolarization (Chepurny *et al.*, 2010).

Surprisingly, it has not yet been reported what effect GLP-1 exerts on insulin secretion from the islets of Epac2 knockout (KO) mice. However, Shibasaki *et al.* (2007) did report that 8-Br-cAMP, a cAMP analog that activates both PKA and Epac2, had a greatly reduced ability to potentiate first-phase GSIS from the β cells of Epac2 KO mice. Assuming 8-Br-cAMP activates PKA in these Epac2 KO mice, it would seem that first-phase GSIS is under the control of Epac2, and that the activation of PKA by 8-Br-cAMP does not allow for the normal cAMP-dependent potentiation of first-phase GSIS in the Epac2 KO mice. This is a remarkable finding, since it is dramatically at odds with the prior study of Hatakeyama and coworkers that was performed using wild-type mouse β cells. In that study, no evidence for Epac2-dependent regulation of GSIS was measurable, and instead it was found that all stimulatory effects of cAMP on GSIS were mediated by PKA (Hatakeyama *et al.*, 2007; Kasai *et al.*, 2010). Although it is not clear why such divergent findings have been reported by different teams of investigators, it seems likely that the source of confusion arises as a consequence of the use of different live-cell imaging techniques when evaluating exocytosis at the single cell level. Clearly, such findings need to be validated using whole islets and conventional immunoassays of secreted insulin. In summary, findings obtained using PKA inhibitors, Epac activators, and Epac2 KO mice provide evidence for an Epac2-mediated action of cAMP to potentiate GSIS from both human and mouse islets. However, it has yet to be definitively established that such a mechanism of cAMP signal transduction mediates the insulin secretagogue action of GLP-1.

III. Epac2 Activates Rap1 GTPase

If Epac2 does in fact mediate cAMP-dependent potentiation of GSIS by GLP-1, how is this effect achieved? The most likely explanation is that Epac2 couples cAMP production to the activation of Rap1, a Ras-related GTPase that plays an important role in the control of Ca^{2+}-dependent exocytosis, not only in β cells but also in other cell types (Bos, 2006). In fact, the Epac family of cAMP-regulated guanyl nucleotide exchange factors (GEFs) are cAMP-binding proteins first identified on the basis of their ability to stimulate the exchange of GDP for GTP at the guanine nucleotide-binding site of Rap1 (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Structure–function studies demonstrated that the CDC25 homology domain (CDC25-HD) of Epac2 catalyzes guanine nucleotide exchange on Rap1, thereby activating it (Gloerich and Bos, 2010). This is a regulated process that requires the binding of cAMP to a high-affinity cAMP-binding domain (CNBD-B) located within the regulatory region of Epac2 (Fig. 10.1A).

In the absence of cAMP, the regulatory region of Epac2 is responsible for autoinhibition of the CDC25-HD catalytic function, and this autoinhibition is relieved as a consequence of the binding of cAMP to CNBD-B. Importantly, the lower-affinity cAMP-binding domain (CNBD-A) that is also located in the regulatory region of Epac2 (Fig. 10.1A) does not play a role in the cAMP-dependent disinhibition of Epac2 GEF activity. It is instead reported to play a role in plasma membrane targeting of Epac2 within β cells (Niimura *et al.*, 2009). Just as interesting, within Epac2 there may exist binding sites for the sulfonylurea class of blood glucose-lowering agents, such that the binding of sulfonylureas to Epac2 may disinhibit this exchange factor's GEF activity. In fact, sulfonylureas such as tolbutamide are reported to activate Rap1, and in Epac2 KO mice there is a diminished ability of sulfonylureas to stimulate pancreatic insulin secretion (Zhang *et al.*, 2009). Although the ability of sulfonylureas to activate Rap1 might result from their direct binding to Epac2, it is also likely that indirect activation of Epac2 occurs as a consequence of the established ability of sulfonylureas to inhibit cyclic nucleotide phosphodiesterases (PDEs) and to elevate levels of cAMP in islets (Goldfine *et al.*, 1971). Thus, it will be of particular interest to validate that sulfonylureas do in fact directly activate Epac2, since this mechanism of Epac2 activation has recently been drawn into question (Leech *et al.*, 2010).

Although largely unexplored, Epac2-independent mechanisms controlling Rap1 activity may also exist in β cells since islets express Rap1 GTPase-activating protein (Rap1GAP), a protein that inactivates Rap1 by stimulating its GTPase activity. Rap1GAP is phosphorylated and inactivated

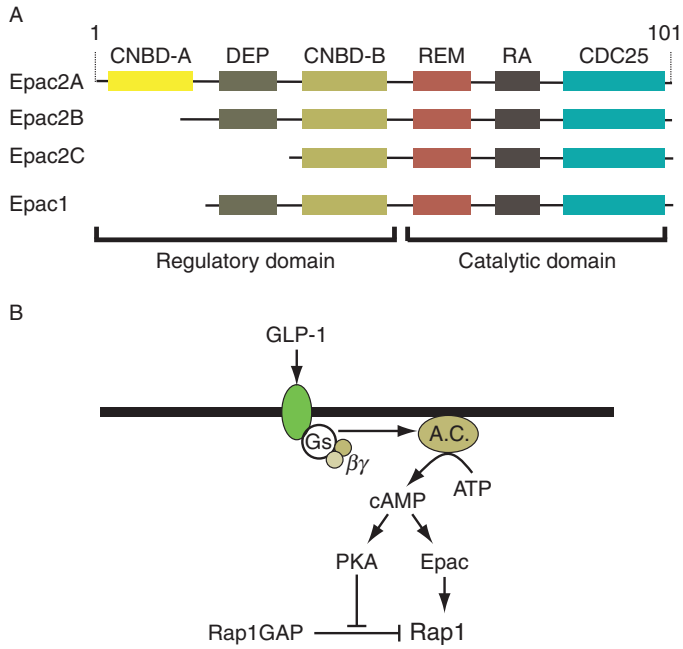

Figure 10.1 (A) Domain structure of Epac proteins. The two isoforms of Epac are Epac1 and Epac2, each of which is encoded by its own gene. Epac2 is the major isoform of Epac expressed in islets, and it is encoded by the *RAPGEF4* gene located at chromosome 2q31–q32. There are three splice variants of Epac2, with Epac2A being the variant expressed in islets. Epac2A has two cAMP-binding domains, a low-affinity site (CNBD-A), important for cellular localization, and a high-affinity site (CNBD-B), important for cAMP-dependent activation of GEF activity. A disheveled, Egl-10, pleckstrin (DEP) domain is responsible for association of Epac2 with intracellular membranes, a Ras exchange motif (REM) domain stabilizes the tertiary structure of the catalytic region, and a Ras association (RA) domain allows the interaction of Epac2 with activated Ras. The CDC25 homology domain (CDC25) catalyzes guanine nucleotide exchange on Rap1, thereby activating it. Epac2B is specifically expressed in the adrenal cortex and lacks the low-affinity cAMP-binding site (CNBD-A). Epac2C is found in the liver and lacks both CNBD-A and DEP domains. All three isoforms have GEF activity to activate Rap1. (B) Role of cAMP in Rap1 activation. Activation of the GLP-1 receptor stimulates G_s, adenylyl cyclase (AC), and cAMP production. The activation of Epac2 is likely to be the major pathway for Rap1 activation in β cells, although PKA can phosphorylate and inactivate Rap1GAP to prolong the activated state of Rap1.

by PKA (McAvoy *et al.*, 2009), and thus elevated PKA activity could prolong the active GTP-bound state of Rap1 (Fig. 10.1B). It remains to be determined whether such an effect of PKA to promote Rap1 activation explains the synergistic interaction of PKA- and Epac-selective cAMP analogs to stimulate insulin secretion, as reported in studies of rat INS-1 insulin-secreting cells (Chepurny *et al.*, 2009).

Epac2-independent activation of Rap1 may also be mediated by the Ras guanyl-releasing proteins (RasGRPs; also known as CalDAG-GEFs) expressed in islets (Ozaki *et al.*, 2005). RasGRPs link intracellular Ca^{2+} and diacylglycerol (DAG) signaling to Rap1 activation, and in this manner, it is predicted that Rap1-dependent insulin secretion should be synergistically stimulated by agents that activate both RasGRPs and Epac2. However, what role, if any, RasGRPs play in GLP-1R signal transduction remains to be determined. Finally, posttranslational modifications of Rap1 may also contribute to the overall process by which Rap1 activity regulates insulin secretion (Kowluru, 2008).

IV. Rap1 Effectors and Their Potential Roles in the Control of GSIS

A. Activation of phospholipase C-epsilon

One of the best characterized downstream effectors of Rap GTPase is phospholipase C-epsilon (PLCε). PLCε was first identified as a fourth class of PLC that is directly regulated by Ras (Kelley *et al.*, 2001). Subsequent studies showed that PLCε is also regulated by Rap (Schmidt *et al.*, 2001; Song *et al.*, 2002). Two isoforms of PLCε are generated from the *PLCE1* gene as a consequence of the alternative splicing of N-terminal exons resulting in the expression of long (2302 a.a.) and short (1994 a.a.) forms of the enzyme, each with similar domain structures (Fig. 10.2A). Both isoforms of PLCε are expressed in the pancreas (Sorli *et al.*, 2005), but their relative levels of expression within islets are not currently known. Although it was originally reported that Rap2B is the specific activator of PLCε (Schmidt *et al.*, 2001), more recent studies demonstrated that Rap1 is also capable of activating this phospholipase (Song *et al.*, 2002). Less well understood are the reported roles of Rho GTPase and heterotrimeric G protein subunits (Gα12/13 and Gβγ) in the regulation of PLCε (Wing *et al.*, 2001, 2003).

As indicated in the domain structure of PLCε (Fig. 10.2A), activated Rap binds to the phospholipase's RA2 domain to activate the enzyme. Intrinsic GEF activity at the N-terminal CDC25-HD of PLCε is then responsible for additional Rap activation (Fig. 10.2B). This constitutes a mechanism of positive feedback that is important for sustained Rap activation (Jin *et al.*, 2001). Once activated, PLCε hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the Ca^{2+}-mobilizing second messenger inositol 1,4,5-trisphosphate (IP_3) and the lipid metabolite DAG (Fig. 10.2B). Ca^{2+} and DAG then act via RasGRP3 to promote additional Rap activation with concomitant stimulation of PLCε (Fig. 10.2B). Ca^{2+} and DAG also participate in the activation of protein kinase C (PKC), and

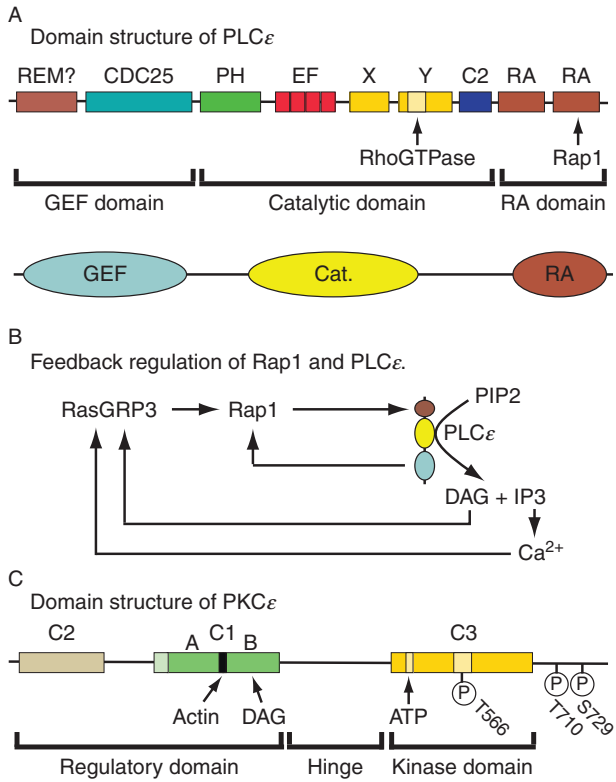

Figure 10.2 Domain structures of PLCε and PKCε. (A) The N-terminal region of PLCε has GEF activity and contains a CDC25 homology domain and possibly a REM domain. The catalytic region contains a pleckstrin homology (PH) domain, EF hand domains, the X and Y boxes, and a core C2 catalytic domain. The C-terminal Ras association (RA) domain contains two Ras association motifs that interact with Ras and Rap. Figure adapted from Bunney and Katan (2006). (B) Illustrated are feedback loops that might be important for sustained activation of Rap1 and PLCε. Rap1 activates PLCε and the GEF domain of PLCε activates Rap1. Note that PLCε-catalyzed hydrolysis of PIP_2 generates DAG and Ca^{2+}, both of which activate RasGRP3, thereby catalyzing additional activation of Rap1. (C) Located within the regulatory domain of PKCε is a C1 domain that contains an actin-binding motif and a DAG-binding site. The regulatory domain also contains a C2 domain that binds phospholipids. The kinase domain of PKCε contains a C3 domain, and within it there is an ATP-binding site. Note that phosphorylation of T566 in the activation loop of the C3 domain is essential for PKCε activity. A pseudosubstrate motif at which autophosphorylation occurs is located at the C-terminus of PKCε. Figure adapted from Akita (2002).

for cell types expressing the PLCε isozyme, it appears that DAG may be a particularly effective stimulator of PKC-epsilon (PKCε) activity (Akita, 2002) (Fig. 10.2C).

Given that both Ca^{2+} and PKC are key stimulators of islet insulin secretion, it is clear that the activation of PLCε by Rap1 could explain, at least in part, the action of cAMP-elevating agents to potentiate GSIS. However, it should be pointed out that earlier studies of islet phosphoinositide metabolism failed to demonstrate a stimulatory effect of GLP-1 on ^{3}H-inositol phosphate production (Fridolf and Ahren, 1991), as would be expected if GLP-1 activates any of the multiple PLC isozymes expressed in islets. This negative finding might be explained by the low sensitivity of biochemical assays of inositol phosphate production since PLCε is expressed in relatively low abundance in islets. In fact, in cardiac myocytes that express PLCε at low abundance, the activation of Epac1 by an ESCA results in the PLCε-mediated stimulation of sarcoplasmic reticulum Ca^{2+} release (Oestreich *et al.*, 2009), and this effect is not accompanied by significant inositol phosphate production. However, using more sensitive methods of live-cell imaging in combination with a fluorescent phosphoinositide biosensor (PHD-PLCδ-EGFP) expressed in rat INS-1 insulin-secreting cells, it was recently demonstrated that phosphoinositide hydrolysis at the plasma membrane can be stimulated by an ESCA (Leech *et al.*, 2010). Such findings are reminiscent of prior studies in which glucose metabolism-stimulated cAMP production in β cells was found to be measurable using imaging techniques (Dyachok *et al.*, 2008; Landa *et al.*, 2005), whereas conventional biochemical assays of bulk cAMP content failed to support a major role for glucose metabolism in the stimulation of β cell cAMP production (Schuit and Pipeleers, 1985).

B. Stimulation of PIP_2 hydrolysis

Since activation of PLCε by Rap1 results in PIP_2 hydrolysis, the resultant depletion of PIP_2 in the β cell plasma membrane might have important functional consequences, even without further downstream signaling. We have proposed a model (Fig. 10.3) in which the hydrolysis of PIP_2 by PLCε favors the ATP-dependent closure of ATP-sensitive potassium (K-ATP) channels in β cells (Kang *et al.*, 2006). This model is based on the established fact that the activity of this channel type, as well as its ATP sensitivity, is under the control of PIP_2 (Baukrowitz *et al.*, 1998; Shyng and Nichols, 1998). The model also takes into account the demonstration that Epac2 binds to nucleotide-binding fold-1 (NBF-1) of the sulfonylurea receptor-1 (SUR1) subunit of K-ATP channels (Kang *et al.*, 2006). Thus, the model predicts that there can exist a macromolecular signaling complex comprising K-ATP channels, Epac2, Rap1, and PLCε. The existence of this signaling complex is of primary significance because the model predicts that cAMP-dependent activation of Eapc2 associated with SUR1 will selectively deplete PIP_2 in the immediate vicinity of K-ATP channels, without exerting a global effect on PIP_2 content throughout the β cell plasma membrane (Fig. 10.3).

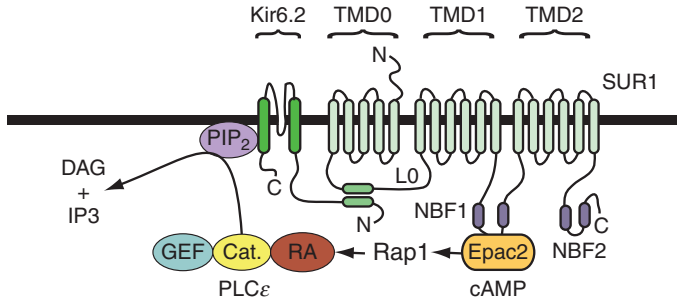

Figure 10.3 A model for Epac/Rap1 regulation of K-ATP channels. The K-ATP channel in β cells is a hetero-octamer formed by four SUR1 subunits and four Kir6.2 subunits. SUR1 has 17 transmembrane domains that are grouped into three units (TMD0, TMD1, and TMD2). The intracellular L0 loop between TMD0 and TMD1 interacts with the N-terminal of Kir6.2 (Bryan *et al.*, 2007). Note that Epac2 binds to nucleotide-binding fold-1 (NBF1) of SUR1 and that this interaction may allow cAMP to activate Rap1 and PLCε located within the immediate vicinity of K-ATP channels. Therefore, cAMP is predicted to regulate K-ATP channel activity by stimulating hydrolysis of PIP_2 associated with Kir6.2. Although not shown, it is also possible that the binding of Epac2 to NBF-1 of SUR1 allows Epac2 to allosterically regulate K-ATP channel activity.

One additional prediction of this model is that activation of PLCε by GLP-1 will enhance glucose-induced closure of K-ATP channels, and that this action of GLP-1 will explain how the hormone potentiates GSIS. To understand this model, it is must be realized that K-ATP channels close in response to a glucose metabolism-dependent increase of cytosolic [ATP]/[ADP] ratio. Furthermore, the association of PIP_2 with K-ATP channels increases the likelihood that these channels will open, while it also decreases the channel's apparent affinity for ATP (Baukrowitz *et al.*, 1998; Shyng and Nichols, 1998). Thus, the model predicts that under conditions in which PIP_2 hydrolysis is stimulated by GLP-1, a majority of K-ATP channels will be sensitized to the inhibitory action of ATP, and for this reason more K-ATP channels will close in response to β cell glucose metabolism (Kang *et al.*, 2008). In view of the fact that K-ATP channel closure promotes β cell depolarization and Ca^{2+} influx that initiates exocytosis, this model provides one simple explanation for how glucose metabolism and GLP-1 signal transduction interact to stimulate islet insulin secretion. In fact, the model also provides an explanation for how GLP-1 acts as a β cell glucose sensitizer (Holz *et al.*, 1993).

Interestingly, the above-summarized model may also provide an explanation for how GLP-1R agonists functionally interact with sulfonylureas to stimulate insulin secretion. This concept derives from the established observation that combined administration of a sulfonylurea with the GLP-1R

agonist exenatide can induce excessive pancreatic insulin secretion and hypoglycemia in patients diagnosed with type 2 diabetes mellitus (Kendall *et al.*, 2005). We proposed that such clinical findings are explained, at least in part, by the synergistic interaction of exenatide and sulfonylureas to inhibit K-ATP channels (Leech *et al.*, 2010). This concept is advanced because PIP_2 hydrolysis is expected to enhance the inhibitory action of sulfonylureas at K-ATP channels (Koster *et al.*, 1999), whereas exenatide is expected to activate PLCε in β cells. Thus, there may exist a mechanism of GLP-1R signal transduction that promotes PIP_2 hydrolysis, and that modulates K-ATP channel responsiveness to sulfonylureas while also controlling the channel's sensitivity to ATP derived from glucose metabolism.

C. Activation of protein kinase C-epsilon

PKCε is a member of the "novel" family of PKC isoforms, and it serves as an immediate effector of PLCε in various cell types, although this has yet to be demonstrated for β cells. A role for PKCε in the control of insulin biosynthesis is reported (Warwar *et al.*, 2008), but there are conflicting data concerning what role PKCε plays in regulated insulin secretion. Published findings demonstrate that PKCε translocates to insulin-containing secretory granules in response to elevated levels of glucose, whereas over expression of a dominant-negative PKCε abolishes GSIS (Mendez *et al.*, 2003; Zaitsev *et al.*, 1995). Interestingly, PKCε is also reported to mediate the inositol hexakisphosphate-induced exocytosis of insulin in β cells (Hoy *et al.*, 2003). What is surprising is that the findings summarized above are contradicted by reports that genetic deletion or inhibition of PKCε fails to influence GSIS (Cantley *et al.*, 2009; Schmitz-Peiffer *et al.*, 2007). For example, in PKCε KO mice, or when PKCε activity was inhibited, insulin secretion in response to glucose alone was not altered (Cantley *et al.*, 2009; Schmitz-Peiffer *et al.*, 2007).

It remains to be determined whether stimulatory effects of GLP-1 on insulin secretion involve its putative Epac2-mediated ability to activate PKCε. This might be the case in view of the fact that PKCε is involved in the regulation of Cdc42 (Akita, 2008), a Rho-family GTPase that stimulates cytoskeletal reorganization during exocytosis. Cdc42 is activated in β cells in response to glucose metabolism, and it activates a Rac1 GTPase that promotes actin remodeling (Wang *et al.*, 2007). Remodeling under the control of Cdc42 and Rac1 might favor exocytosis because the actin-containing cytoskeleton is a barrier that restricts access of secretory granules to the plasma membrane, whereas disruption of this barrier enhances insulin secretion (Orci *et al.*, 1972). Importantly, it is now known that Rac1 GEFs can be activated by Rap1 (Arthur *et al.*, 2004), and that Rap1 regulates insulin secretory granule dynamics (Shibasaki *et al.*, 2007). Thus, Rap1 acting through Rac1–GEFs and Rac1 itself might stimulate cytoskeletal

reorganization and the trafficking of secretory granules to the plasma membrane. Furthermore, such a signaling mechanism might be under the control of GLP-1 acting through Epac2. This hypothetical Rap1 and Rac1–GEF-mediated effect of GLP-1 would complement its putative ability to activate PKCε and to directly regulate Cdc42 function. Finally, it is interesting to note that in neurons, the activation of PKCε enables this kinase to bind to actin and to regulate synaptic vesicle exocytosis (Prekeris *et al.*, 1996). This finding leads to the prediction that in β cells, the GLP-1R-mediated activation of PKCε should facilitate Cdc42 dependent trafficking of insulin granules to their docking sites at the plasma membrane where insulin exocytosis occurs.

D. Elevation of cytosolic [Ca^{2+}]

Considerable evidence exists that stimulatory effects of GLP-1 on islet insulin secretion are attributable, at least in part, to its ability to increase levels of cytosolic [Ca^{2+}] in β cells. This action of GLP-1 to increase $[Ca^{2+}]_i$ requires exposure of β cells to elevated concentrations of glucose. One mechanism that underlies this action of GLP-1 involves its ability to enhance the action of glucose metabolism to trigger Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs). Such an ability of GLP-1 to sensitize β cells to the stimulatory action of glucose is consistent with its established action to promote glucose metabolism-dependent closure of K-ATP channels, and to also promote β cell depolarization (Holz *et al.*, 1993). These effects of GLP-1 to inhibit K-ATP channel activity, induce β depolarization, and promote Ca^{2+} influx through VDCCs are likely to be Epac2-mediated, since all such actions of GLP-1 are mimicked by ESCAs (Chepurny *et al.*, 2010; Kang *et al.*, 2006, 2008).

There is also good evidence that GLP-1 acts through cAMP to mobilize an intracellular source of Ca^{2+} in β cells (Fig. 10.4). This intracellularly released Ca^{2+} is likely to be an effective stimulus for insulin secretion, as is already established to be the case for Ca^{2+} that enters by way of VDCCs (Fig. 10.5). Although there was some earlier debate concerning whether or not the Ca^{2+} mobilizing action of GLP-1 is mediated by PKA or Epac2, it is now established that both cAMP-binding proteins participate in the regulation of $[Ca^{2+}]_i$ by the hormone (Kang *et al.*, 2005). Thus, prior to the discovery of Epac2, a role for PKA as the target of GLP-1 was demonstrated in studies of rat and human β cells (Holz *et al.*, 1999), and this finding was subsequently validated in studies of Ca^{2+} oscillations and intracellular Ca^{2+}-induced Ca^{2+} release (CICR) in mouse β cells (Dyachok and Gylfe, 2004; Kang *et al.*, 2005). The consensus that developed was that PKA promotes the release of Ca^{2+} from intracellular Ca^{2+} stores by sensitizing intracellular Ca^{2+} release channels to the stimulatory effects of Ca^{2+}-mobilizing second messengers (e.g., IP_3) or even Ca^{2+} itself (Fig. 10.4).

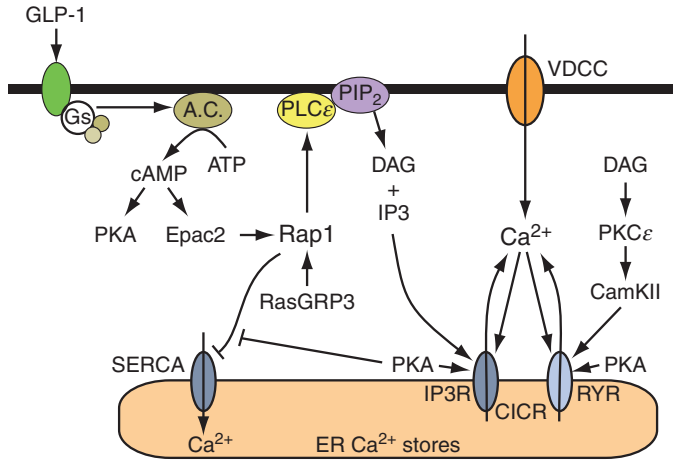

Figure 10.4 Regulation of CICR by cAMP. Ca^{2+} influx through VDCCs generates an increase of $[Ca^{2+}]_i$, and this acts as a stimulus for CICR from the ER. The Ca^{2+} release channel that mediates CICR is the ryanodine receptor (RYR). However, Ca^{2+} released from the ER acts as a coagonist with IP_3 to stimulate additional Ca^{2+} release from IP_3 receptor-regulated Ca^{2+} stores (IP3R). Ca^{2+} release via the RYR and IP3R is facilitated by PKA, possibly as a consequence of the phosphorylation of RYR and IP3R. It is also likely that PKA increases the ER Ca^{2+} load by promoting ER Ca^{2+} uptake. Epac2 acts through a pathway involving Rap1, PLCε, PKCε, and CamKII to exert a stimulatory effect at RYR. This action of Epac2 favors additional CICR. Note that Rap1 activation may be stimulated by RasGRP3 since the GEF activity of RasGRP3 is itself stimulated by Ca^{2+} and DAG derived from PLCε-catalyzed hydrolysis of PIP_2. Activated Rap1 may inhibit SERCA, so that leakage of Ca^{2+} from the ER may raise levels of $[Ca^{2+}]_i$ and favor CICR. The association of Rap1 with SERCA is inhibited by PKA.

Following the discovery of Epac2 in 1998, it was reported that the action of GLP-1 to raise $[Ca^{2+}]_i$ in mouse islets was not entirely explained by its ability to activate PKA (Bode *et al.*, 1999). Soon after, it was demonstrated that the action of cAMP-elevating agent forskolin to raise $[Ca^{2+}]_i$ was inhibited by transfection of INS-1 insulin-secreting cells with a dominant-negative Epac2 (DN-Epac2) that fails to bind cAMP (Kang *et al.*, 2001). More extensive studies revealed that the action of a GLP-1R agonist to raise $[Ca^{2+}]_i$ was similarly blocked by DN-Epac2 (Kang *et al.*, 2005). Final confirmation that Epac2 does in fact participate in the regulation of $[Ca^{2+}]_i$ was provided by studies in which it was demonstrated that ESCAs reproduced the Ca^{2+}-elevating action of GLP-1 in mouse and human β cells (Chepurny *et al.*, 2010; Kang *et al.*, 2003; Leech *et al.*, 2010).

The potential role of PLCε in the regulation of β cell Ca^{2+} handling by GLP-1 is an area of ongoing investigation, and preliminary studies favor such a mechanism. Thus, the ability of Epac activators to promote PIP_2 hydrolysis and to enhance K-ATP channel closure is fully consistent with

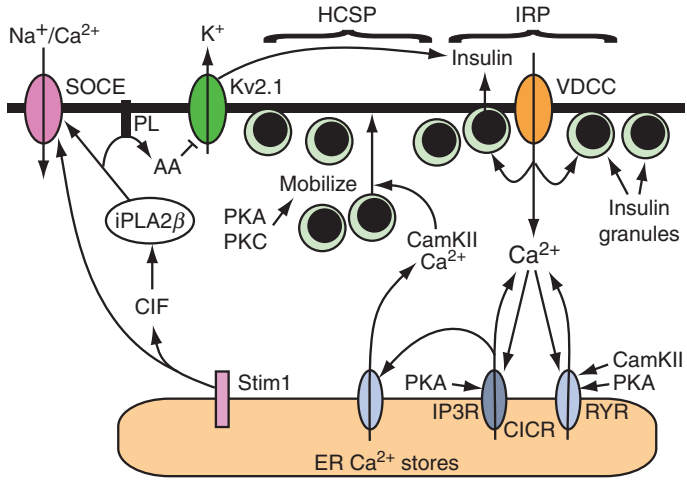

Figure 10.5 CICR regulates insulin secretion. Ca^{2+} influx through VDCCs is the main stimulus for insulin secretion under conditions in which β cells are exposed to elevated levels of glucose. Secretory granules located in an immediate releasable pool (IRP) adjacent to these VDCCs undergo exocytosis in response to the high [Ca^{2+}] that exists at the inner mouth of each VDCC. When β cells are exposed to both glucose and GLP-1, exocytosis is amplified due to the fact that CICR is initiated. Amplification of exocytosis occurs because PKA and PKC increase the number of secretory granules within a highly Ca^{2+}-sensitive pool (HCSP). These granules are then able to undergo exocytosis in response to CICR that is facilitated by both PKA and Epac2. Ca^{2+} released from the ER also plays a major role in stimulating the mobilization of insulin granules from the reserve pool, an effect mediated by CamKII. The role of Stim1 in β cells is not fully understood but upon store depletion it translocates to the plasma membrane where it may activate store-operated Ca^{2+} entry (SOCE) and also Na^{+} entry through nonselective cation channels. An alternative model suggests that Stim1 activation triggers the formation of a Ca^{2+} influx factor (CIF) that activates iPLA2β, and that iPLA2β regulates SOCE activity. iPLA2β hydrolyzes phospholipids (PL) in the plasma membrane to liberate arachidonic acid (AA) and this lipid metabolite can inhibit Kv2.1 delayed rectifier channels to potentiate membrane depolarization, Ca^{2+} influx, and insulin secretion.

the established action of GLP-1 to depolarize β cells and to stimulate Ca^{2+} influx. In view of the fact that PIP_2 hydrolysis produces IP_3, it may be speculated that PLCε plays a significant role as a determinant of Ca^{2+} release from an intracellular compartment where IP_3 receptors are located (Fig. 10.4). This concept is consistent with the demonstrated ability of GLP-1R agonists and ESCAs to mobilize Ca^{2+} in β cells (Chepurny *et al.*, 2010; Kang *et al.*, 2001, 2003).

The source of Ca^{2+} mobilized by Epac activators is not exclusively IP_3 receptor regulated, but also includes those Ca^{2+} stores that under the control of ryanodine receptors (RYRs). This is particularly true for

human and rat β cells that express the type-2 isoform of RYR (Dror *et al.*, 2008), whereas it may be less significant for mouse β cells, since the expression of RYR in this cell type is low (Beauvois *et al.*, 2004). Exactly how Epac2 activation leads to RYR-dependent release of intracellular Ca^{2+} remains to be elucidated, but studies of cardiac myocytes seem to indicate a role for PLCε, PKCε, and calcium–calmodulin kinase II (CamKII) (Oestreich *et al.*, 2009). Available evidence indicates that Ca^{2+} is mobilized from RYR-regulated Ca^{2+} stores via a process of CICR, and that this Ca^{2+} acts as a direct stimulus for insulin secretion from β cells (Kang *et al.*, 2003).

Since PKA and Epac2 activators mobilize Ca^{2+} from endoplasmic reticulum (ER) Ca^{2+} stores, it is significant that ER Ca^{2+} release plays a major role in regulating insulin granule trafficking (Hao *et al.*, 2005). This effect is achieved through the activation of CamKII (Gromada *et al.*, 1999). Thus, we propose a unifying model to explain how cAMP regulates insulin secretion (Fig. 10.5). In this model, cAMP acting through PKA and Epac2 enhances glucose-dependent influx of Ca^{2+} through VDCCs while also promoting CICR. Simultaneously, CamKII promotes insulin granule translocation to the plasma membrane. In the absence of cAMP, exocytosis is limited to the small numbers of docked secretory granules that are located in close to proximity to VDCCs. However, in the combined presence of cAMP and glucose, exocytosis can occur at a distance from VDCCs, and for this reason a larger number of secretory granules are released. This model is based on studies that demonstrated an ability of CICR to amplify exocytosis in an insulin-secreting β cell line (Kang *et al.*, 2003). Although the model emphasizes the role of CICR originating from the ER, the real situation is likely to be considerably more complicated because the ER is not the only Ca^{2+} store in β cells (Duman *et al.*, 2006), and the release of Ca^{2+} from these stores may be governed by additional Ca^{2+} mobilizing second messengers such as cyclic-ADP-ribose and NAADP (Kim *et al.*, 2008).

Finally, it is interesting to note that depletion of ER Ca^{2+} stores induces the reversible translocation of the ER Ca^{2+} sensor Stim1 with concomitant activation of store-operated Ca^{2+} entry (SOCE) in β cells (Tamarina *et al.*, 2008). Activation of Stim1 by store depletion is proposed to activate SOCE either directly through a conformational coupling model, or indirectly by inducing the generation of a soluble Ca^{2+} influx factor (CIF) that activates Ca^{2+}-independent phospholipase A2β (iPLA2β) (Bolotina, 2008). This raises the possibility that iPLA2β mediates an indirect effect of ER Ca^{2+} store depletion on insulin secretion (Fig. 10.5). It is known that β cells express iPLA2β and that it plays a role in GSIS (Turk and Ramanadham, 2004). It is also known that iPLA2β plays a role in generating arachidonic acid in β cells and that this lipid metabolite regulates Kv2.1 delayed rectifier potassium channels to facilitate Ca^{2+} entry and to stimulate insulin secretion (Jacobson *et al.*, 2007).

E. Activation of protein kinases

The ability of cAMP-elevating agents such as GLP-1 to enhance islet insulin secretion is not simply a consequence of their acute stimulatory influences on β cell GSIS. Instead, *in vivo* studies of rodents demonstrate that chronic administration of a GLP-1R agonist (exendin-4) stimulates the proliferation of β cells while also protecting against β cell death (apoptosis) (Drucker, 2003). This remarkable growth factor-like effect of exendin-4 leads to an increase of "β cell mass" with associated islet hypertrophy, and these effects are measurable as a long-term increase of pancreatic insulin secretory capacity (Holz and Chepurny, 2005). Although it has yet to be established that such growth factor-like effects of exendin-4 occur in the human pancreas, evidence exists that this may in fact be the case (Farilla *et al.*, 2003; Lupi *et al.*, 2008). Mechanistically, a role for Rap in this process of islet hypertrophy is indicated on the basis of new findings demonstrating that in rodent islets and INS-1 insulin-secreting cells, Rap1A promotes the activation of a serine/threonine protein kinase designated as ribosomal protein S6 kinase (S6K1) (Kelly et al., 2010). This kinase is a substrate of the mitochondrial target of rapamycin (mTOR), and it stimulates both protein synthesis and cellular proliferation in numerous cell types. Intriguingly, Rap1 may also play a significant role in the stimulation of the mitogen-activated protein kinases (MAPKs) that are known to control β cell growth. For example, in human islets, GLP-1 is reported to act through Rap to activate the serine/threonine protein kinase B-Raf that couples cAMP production to the activation of MAPKs of the extracellular signal-regulated kinase (ERK) family (Trümper *et al.*, 2005). Finally, it is important to note that one recent study provides evidence for Epac-mediated "noncanonical" activation of PKB in β cells (Widenmaier *et al.*, 2009). PKB is a serine/threonine protein kinase that has an established role as a stimulator of β cell growth (Assmann *et al.*, 2009), so it could be that cAMP-elevating agents such as GLP-1 act through Epac2 and Rap1 to promote PKB activation with attendant expansion of β cell mass.

V. Interactions of Epac2 with Secretory Granule-Associated Proteins

Epac2 binds directly to secretory granule-associated proteins and also to SNARE apparatus proteins that are involved in the regulation of exocytosis. These direct protein–protein interactions may explain, at least in part, how Epac2 regulates insulin secretion (Fig. 10.6). The proteins that Epac2 interacts with include Rim2 (Ozaki *et al.*, 2000), Piccolo (Fujimoto *et al.*, 2002), Rap1 (Shibasaki *et al.*, 2007), and SNAP-25 (Vikman *et al.*, 2009).

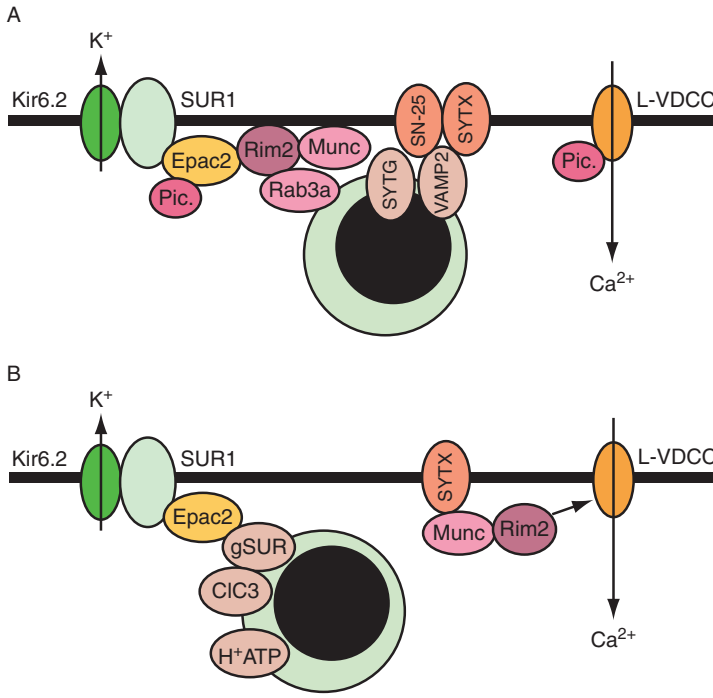

Figure 10.6 (A) Protein interactions that regulate exocytosis. Epac2 stimulates exocytosis by directly interacting with the Ca^{2+} sensor Piccolo (Pic.), Rim2 (Rab GTPase-interacting molecule), and SNAP-25 (SN-25; interaction not shown). Epac2 also indirectly interacts with secretory granule-associated proteins and SNARE apparatus proteins that control exocytosis. These include Rab3A, synaptotagmin (SYTG), VAMP2, and syntaxin (SYTX). Piccolo also interacts with L-type voltage-dependent Ca^{2+} channels (L-VDCC), but the significance of this interaction is not known. (B) Priming of secretory granules by Epac2. In addition to binding plasma membrane SUR1, Epac2 is proposed to bind granular SUR (gSUR) and regulate ClC3 chloride channels to promote granular acidification through the v-type H^+-ATPase (H^+ATP). Also shown is the interaction of Munc13-1 with SYTX and Rim2. The C-terminal tail of Rim2 binds weakly to L-VDCC but the significance of this interaction is not known.

Interestingly, the binding of Epac2 to the SUR1 subunit of K-ATP channels may influence exocytosis not simply by altering K-ATP channel activity, but by promoting the cAMP-dependent acidification of secretory granules, a priming step that renders the granules competent to undergo exocytosis (Eliasson *et al.*, 2003). A role for Epac2 in the priming of secretory granules is also suggested based on the observation that Epac2 exists within a macromolecular complex comprising Rim2, Munc13-1, and the SNARE protein syntaxin. Available evidence indicates that this complex is under the dual regulation of PKA and Epac2, and that priming of secretory granules results

from the action of Munc13-1 to unfold syntaxin (Leung *et al.*, 2007). Although electrophysiological findings first indicated that Epac2 increases the number of primed granules available for exocytosis (Eliasson *et al.*, 2003; Renstrom *et al.*, 1997), it should be noted that in studies of neurons, it was reported that Epac2 acts presynaptically to increase the probability that individual synaptic vesicles will undergo exocytosis (Gekel and Neher, 2008). Intriguingly, this action of Epac2 might reflect its ability to increase the number of vesicles located in close proximity to VDCCs. Thus, the action of Epac2 to influence exocytosis appears to be complex and may even be contingent on simultaneous activation of PKA, as suggested by the finding that Epac agonist-stimulated insulin secretion is abrogated after treatment of human islets with PKA inhibitors (Chepurny *et al.*, 2010).

The above-summarized protein interactions are also likely to explain the ability of Epac2 to influence insulin secretory granule dynamics. Using methods of live-cell imaging, Shibasaki *et al.* (2007) provided evidence for an Epac2- and Rap1-mediated action of cAMP to potentiate first-phase GSIS. Such studies revealed that cAMP potentiated GSIS by increasing the number of secretory granules newly recruited to the plasma membrane in response to glucose metabolism, and that these granules underwent exocytosis without pausing in a docked mode. Such granules were designated as "restless newcomers" since they not only appeared at the plasma membrane suddenly, but also underwent exocytosis without a pause. This cAMP-dependent stimulation of restless newcomer exocytosis was Epac2-mediated, since the action of cAMP was dramatically reduced in β cells of Epac2 KO mice. Moreover, the potentiation of GSIS by cAMP was abrogated following treatment of β cells with siRNA for Rap1, or after overexpression of Rap1GAP (Shibasaki *et al.*, 2007). Importantly, the existence of Epac2-regulated restless newcomer exocytosis is unanticipated in view of prior electrophysiological studies in which the action of Epac2 to enhance insulin secretion was demonstrated to result from its ability to facilitate the exocytosis of granules that were already docked and primed at the plasma membrane (Eliasson *et al.*, 2003; Renstrom *et al.*, 1997). Thus, considerable debate exists as to the nature of the secretory granule pool that undergoes exocytosis in an Epac2-regulated manner.

VI. Conclusions

From the data reviewed above, it is apparent that both Epac2 and Rap1 play a highly significant role in the cAMP-dependent stimulation of insulin secretion from pancreatic β cells. Complex feedback loops, possibly involving Ca^{2+}, DAG, and RasGRPs, may dictate the spatiotemporal activation of Rap1 that is required for an appropriate insulin-secretory

response. Furthermore, the possibility that Rap1 regulates the activity of PLCε provides an unexpected explanation for how cAMP influences K-ATP channel activity, cytosolic Ca^{2+} signaling, and PKC activation in β cells. It may be anticipated that a molecular genetics approach involving the use of Epac2 and PLCε KO mice will more fully reveal what roles these signaling proteins play as determinants of incretin hormone action.

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CHAPTER ELEVEN

Central GLP-1 Actions on Energy Metabolism

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Contents

Abstract

Glucagon-like peptide 1 (GLP-1) is secreted mainly by the intestine in a nutrient-dependent manner and stimulates glucose-induced insulin secretion, inhibits gastric emptying, food intake, and glucagon secretion. All these beneficial effects make GLP-1 as a promising, and currently in the market, drug candidate for the treatment of type 2 diabetes. More recently, it has been also demonstrated that within the central nervous system, GLP-1 also exerts important metabolic actions inhibiting food intake, increasing insulin secretion, and modulating behavioral responses. In this review, we will focus on the metabolic actions and mechanisms of the central GLP-1 system: modulation of energy intake, glucose metabolism, and fatty acid metabolism.

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I. Introduction

Glucagon-like peptide 1 (GLP-1) is a posttranslational product of proglucagon, that is, endogenously released mainly from two organs: (a) "L" cells within the gastrointestinal tract, mainly in the ileum, after the ingestion of nutrients and (b) neurons of the nucleus tractus solitarius (NTS) in the caudal brainstem, reviewed in Drucker (2006, 2007) and Goke *et al.* (1991). A small percentage of GLP-1 is also secreted from the pancreas following the ingestion of food. The amino acid sequence differs depending on the origin of the peptide. GLP-1(7-36) is derived from the intestine and represents the majority of circulating GLP-1 in plasma, whereas GLP-1 (1-36) is derived from the pancreas (Orskov *et al.*, 1994). The metabolism and degradation of endogenous GLP-1 by the enzyme dipeptidyl-peptidase-4 (DPP-IV) is very rapid. It is estimated that $<25\%$ of GLP-1 secreted from the gastrointestinal tract enters the portal vein in an active form before reaching the liver. Further degradation occurs rapidly within the liver (40–50% of the remaining GLP-1 not initially degraded by DPP-IV) and the peptide is also rapidly cleared from circulation via the kidney. Thus, at most, between 10% and 15% of secreted GLP-1 reaches circulation in the active form, and the total half-life of GLP-1 is 1–2 min, as reviewed in Drucker (2006, 2007).

The GLP-1 receptor (GLP1-R) is a G-protein-coupled receptor that is widely expressed in pancreatic islets, kidney, lung, heart, stomach, intestine, pituitary, or skin. In the central nervous system (CNS), numerous neuronal populations express GLP-1R, including relevant nuclei that are crucial for the regulation of energy balances such as the paraventricular nucleus (PVH), the dorsomedial nucleus (DMH), and the arcuate nucleus (ARC) of the hypothalamus, as well as caudal brainstem structures including the parabrachial nucleus, area postrema, and the NTS (Shimizu *et al.*, 1987; Uttenthal *et al.*, 1992).

The major stimulus for GLP-1 secretion is the ingestion of nutrients, including glucose, fatty acids, and dietary fiber (Kreymann *et al.*, 1987; Wang *et al.*, 1995). When nutrients are ingested, the release of GLP-1 into the circulation occurs in a biphasic manner, consisting of a rapid (within 10–15 min) early phase followed by a more prolonged (30–60 min) second phase (Roberge and Brubaker, 1993). Two different mechanisms are responsible of this biphasic pattern: the vagus nerve, the neurotransmitter gastrin-releasing peptide, and the hormone glucose-dependent insulinotropic peptide contribute to the rapid release of GLP-1 from distal L cells in response to nutritional stimuli (Kreymann *et al.*, 1987; Wang *et al.*, 1995). In contrast, a direct stimulation of the L cells by digested nutrients is responsible for the second phase of peptide release (Roberge and Brubaker, 1993). In both humans and rodents, GLP-1 augments glucose-induced insulin release,

an effect that occurs postprandially when glucose levels are elevated (Mojsov *et al.*, 1987). Moreover, GLP-1 inhibits glucagon release, slows gastric emptying and has islet-independent effects to reduce hepatic glucose production, reviewed in Drucker (2006, 2007).

Most of the evidence demonstrating a role for GLP-1 on glucose metabolism has focused on actions within the pancreatic islet. However, the importance of the central GLP-1 system regulating different physiological settings has been also clarified, and when endogenous GLP-1 rises, it will exert its actions through afferent sensory neurons relaying in the brainstem or the hypothalamus (Imeryuz *et al.*, 1997; Wettergren *et al.*, 1998). For instance, the central GLP-1 system has been linked to the control of food intake (Turton *et al.*, 1996), glucose metabolism (Knauf *et al.*, 2005), lipid metabolism (Nogueiras *et al.*, 2009), and other endocrine and behavioral responses to stress (Ulrich-Lai and Herman, 2009), visceral illness (van Dijk *et al.*, 1997), heart rate, or arterial blood flow (Cabou *et al.*, 2008). In this review, we will focus on the specific aspects of the central actions of GLP-1 on energy metabolism, including feeding behavior, glucose, and fatty acid metabolism.

II. CNS Glucagon-Like Peptide 1 and Energy Intake

As stated above, neurons that express GLP-1 are located in the hypothalamus and NTS, CNS regions that are thought to be important for regulating appetite and satiety (Merchenthaler *et al.*, 1999). Hypothalamic GLP-1 is a physiological satiety factor (Turton *et al.*, 1996), and its central administration dose dependently reduces feeding in rats (Meeran *et al.*, 1999; Tang-Christensen *et al.*, 1996), an effect that is reversed by coadministration of the GLP-1 receptor antagonist exendin (9-39) (Tang-Christensen *et al.*, 1996). Consistently, central administration of exendin (9-39) itself increases food intake (Turton *et al.*, 1996). It has been proposed that the inhibitory effects of GLP-1 on food intake can be mediated indirectly by its ability to slow gastric emptying, thereby promoting gastric distension and a sensation of satiety. However, it is clear that GLP-1 receptors in the hypothalamus mediate the reduction of food intake by acting through the normal pathways that control energy balance (Kinzig *et al.*, 2002; Larsen *et al.*, 1997; McMahon and Wellman, 1998). In this regard, central administration of GLP-1 stimulated neuronal activity, measured by c-fos immunoreactivity, in the PVH and supraoptic nucleus (SON) and neurons of the medial parvicellular subregion of the PVH (Larsen *et al.*, 1997). A slight induction of c-fos expression was seen in the ARC and the NTS, including the area postrema (Larsen *et al.*, 1997).

Evidence demonstrating that the hypothalamic GLP-1R mediates the actions of GLP-1 on feeding behavior is that the abolition of the ARC and parts of the sensory circumventricular organs with monosodium glutamate blunts the anorectic effect of GLP-1 (Tang-Christensen *et al.*, 1998). Moreover, the potent orexigenic action of neuropeptide Y (NPY) was reduced by coadministration of GLP-1, suggesting that GLP-1R mediating feeding inhibition is localized upstream to NPY neurons (Tang-Christensen *et al.*, 1998). NPY is one of the main hypothalamic targets for leptin actions. Leptin is secreted by adipose tissue in direct proportion to its mass, and plays a crucial role in the maintenance of energy balance (Badman and Flier, 2007). The results obtained for NPY are in agreement with later evidence showing that leptin pretreatment strongly enhanced anorexia and weight loss induced by GLP-1 or exendin 4, a potent GLP-1R agonist (Williams *et al.*, 2006). Conversely, during fasting, a state of hypoleptinemia attenuated the anorexic response to GLP-1 and exendin 4 treatment via a leptin-dependent mechanism (Williams *et al.*, 2006). Further support of the interaction between leptin and GLP-1 is that leptin administration increased hypothalamic GLP-1 content, indicating that the anorectic effect of leptin is partially exerted by GLP-1 (Goldstone *et al.*, 2000). Further support of a hypothalamic action of the GLP-1R is that hypothalamic NPY and AgRP levels were increased by fasting, whereas central GLP-1 administration blunted those fasting-induced increases (Seo *et al.*, 2008). Moreover, proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) mRNA expression was decreased by fasting, while GLP-1 treatment blunted those fasting-induced decreases (Seo *et al.*, 2008). Finally, fasting increased hypothalamic AMPKα2 levels and central GLP-1 injection abolished that increase (Seo *et al.*, 2008), suggesting that GLP-1 decreases food intake through its effects on neuropeptides and AMPK.

Another important signal acting through NPY is ghrelin, an hormone mainly synthesized in the stomach that stimulates food intake and adiposity (Nakazato *et al.*, 2001; Tschop *et al.*, 2000). GLP-1 and ghrelin exert several opposite actions, since ghrelin increases food intake, gastric acid secretion and motility, and fat deposition, whereas GLP-1 reduces food intake, gastric acid secretion, and gastric emptying and has lipolytic effects. Circulating GLP-1 and ghrelin levels also shows an inverse relationship after a glucose tolerance test in humans (Djurhuus *et al.*, 2002), and GLP-1 reduces circulating ghrelin levels *in vitro* (Lippl *et al.*, 2004). Consistently, the central or peripheral administration of exendin 4 reduces in a dose-dependent manner ghrelin levels in fasted (hyperghrelinemic state) rats (Perez-Tilve *et al.*, 2007), suggesting that the decrease in ghrelin levels induced by exendin 4 might explain the reduced food intake induced by GLP-1R activation. This interaction between ghrelin and GLP-1 has been also demonstrated in electrophysiological studies showing that ghrelin-stimulated ARC neurons respond to GLP-1 (Riediger *et al.*, 2010). Nevertheless,

several reports suggest the interaction between ghrelin and GLP-1 systems; there is no data assessing if central ghrelin administration would increase food intake in GLP-1R knockout mice, or if central GLP-1 injection would decrease feeding behavior in mice lacking the ghrelin receptor. Further studies using genetically modified mice will be necessary to elucidate the potential physiological interaction between both systems at central level.

The complexity of the central molecular pathways modulating GLP-1 actions is supported by a recent study indicating that GLP-1 and exendin 4, a potent GLP-1R agonist, decrease food intake through different central pathways (Barrera *et al.*, 2009). The efficacy was also different since exendin 4 was 100-fold more potent than GLP-1 at reducing food intake, and this effect was insensitive to GLP-1R antagonism, whereas GLP-1R antagonists completely blocked the anorectic action of intraperitoneal exendin 4 (Barrera *et al.*, 2009). This work suggests that exendin 4 requires GLP-1R in the brain to exert its functions, but the molecular mechanisms in the periphery and the brain are different, and overall, indicate that the pharmacology of GLP-1R ligands differs from GLP-1.

Whereas the actions of GLP-1 at hypothalamic levels are clear, GLP-1 receptors in the amygdala mediate the reduction of food intake by the activation of aversive signaling pathways that produce visceral illness (Kinzig *et al.*, 2002; Thiele *et al.*, 1997). This was demonstrated because central GLP-1 administration produces similar effects to those caused by lithium chloride, a commonly used procedure for inducing visceral illness in experimental animals (Lachey *et al.*, 2005; Rinaman, 1999; Thiele *et al.*, 1997). Moreover, GLP-1 and lithium chloride activate a similar pattern of Fos expression in the CNS, including neurons in the hindbrain, hypothalamus, and the central nucleus of the amygdala, and the NTS (Rinaman, 1999). However, the mechanisms by which GLP-1 reduces food intake through homeostatic signals and visceral illness can be dissociated (Kinzig *et al.*, 2002). Lateral and fourth ventricular GLP-1 administration resulted in reduction of food intake at similar doses, whereas only lateral ventricular GLP-1 resulted in conditioned taste aversion, indicating that both hypothalamic and caudal brainstem GLP-1R participate in the reduction of food intake, and the central nucleus of the amygdala is mediating the GLP-1-induced visceral illness (Kinzig *et al.*, 2002).

Independently of its mechanism of action, the stimulation of the central GLP-1 system is able to decrease food intake in obese animals. This was observed in obese Zucker rats treated with different doses of GLP-1 that showed a dose-dependent reduction of food intake (Hwa *et al.*, 1998). These obese Zucker rats respond not only to central GLP-1, but also to its peripheral administration, and the chronic administration of exendin 4 practically blocks weight gain on obese rats (Rodriquez de Fonseca *et al.*, 2000). Similar results were obtained in diet-induced obese mice treated

during several days with central GLP-1 (Nogueiras *et al.*, 2009), suggesting that in obese states the use of GLP-1 or GLP-1R agonists is an efficient pathway to induce a negative energy balance. This is important since obese rodents and humans often present a resistance to several potent anorexigenic signals, like leptin. Therefore, the GLP-1 system might provide an alternative useful method to fight obesity.

Peripherally administered GLP-1 has also an anorexigenic effect on healthy (Gutzwiller *et al.*, 1999a), obese (Naslund *et al.*, 1999), and diabetic humans (Gutzwiller *et al.*, 1999b; Toft-Nielsen *et al.*, 1999). Because the half-life of active GLP-1 is very short, the reduction of food intake is probably to the result of GLP-1's inhibitory effects on the gastrointestinal transit and reduced gastric emptying (Delgado-Aros *et al.*, 2002). However, peripherally administered GLP-1 can cross the blood–brain barrier (Kastin *et al.*, 2002); thus, its role on food intake is likely mediated through the CNS.

Another product generated by the posttranslational processing of proglucagon is GLP-2, which is also secreted from the intestinal L cells mainly located in the ileum and the colon. GLP-2 has beneficial effects on intestinal growth (Drucker, 1999; Jeppesen, 2003), and its actions are mediated by a specific GLP-2R (Munroe *et al.*, 1999). In addition to mediating increased small bowel absorption through induction of epithelial proliferation, GLP-2 has been described to decrease gastric emptying (Wojdemann *et al.*, 1998), increase intestinal transit time (Wojdemann *et al.*, 1999), and inhibit sham feeding-induced gastric acid secretion (Wojdemann *et al.*, 1999). Furthermore, administration of a potent protease-resistant analogue of GLP-2 has been demonstrated to augment the adaptive response to massive intestinal resection, specifically stimulating the digestion and absorption, in rodents (Scott *et al.*, 1998). However, there is limited evidence that GLP-2 has trophic effects on the pancreas, and even though GLP-2 is structurally very similar to GLP-1, its role on food intake is not clear. Some studies have found that central administration of GLP-2 in rats could decrease food intake (Tang-Christensen *et al.*, 2000), whereas a study in mice treated with GLP-2 for 9 days found no effect on food intake or weight gain (Tsai *et al.*, 1997). In humans, no effect has been observed on food intake, weight gain either, or gastric emptying (Schmidt *et al.*, 2003; Sorensen *et al.*, 2003).

III. CNS Glucagon-Like Peptide 1 and Glucose Metabolism

At peripheral level, the insulinotropic activity of GLP-1, which is strictly glucose dependent, is exerted through the actions of GLP-1R on pancreatic beta cells (Holst, 2007). Activation of the GLP-1R stimulated cyclic AMP formation and activation of downstream pathways coupled to

protein kinase A and cAMP-regulated guanine nucleotide exchange factors (Drucker, 2006, 2007; Holst and Orskov, 2004). GLP-1R agonists phosphorylate cyclic AMP response element binding protein (CREB) and regulate CREB activity. GLP-1R activation is also related to increased intracellular calcium, inhibition of voltage-dependent K+ channels and the activation of Erk1/2, protein kinase C, and phosphatidylinositol 3-kinase (PI3K) (Drucker, 2006, 2007).

Although most of the antidiabetic properties of GLP-1 have been attributed to its actions on the pancreatic islet, the central GLP-1 system has been also linked to the control of glucose homeostasis. The administration of a GLP-1R antagonist into the brain of mice increases total body glucose disposal, with increased formation of glycogen in skeletal muscle and reduced hepatic glycogen levels. The blockade of the central GLP-1R, during hyperglycemia only, increased muscle glycogen deposition independent of insulin signaling. Consistently, the activation of GLP-1R in the brain induced insulin resistance and increased insulin secretion, promoting the storage of glycogen in the liver (Knauf *et al.*, 2005). These effects were reduced by selective muscle denervation, suggesting that GLP-1 activity in the brain initiates peripheral neural signals that regulate systemic glucose metabolism (Knauf *et al.*, 2005). These findings suggest that during the hyperglycemia that follows a meal, the central GLP-1 system inhibits muscle glucose utilization and increases insulin secretion to favor hepatic glycogen storage, preparing the body for the next fasting state (D'Alessio *et al.*, 2005). To test this hypothesis, the authors performed a follow-up study blocking the central GLP-1R with exendin (9-39) during 1 month in rats fed on high fat diet. The blockade of the central GLP-1R reversed both the insulin resistance and hyperinsulinemia induced by the diet (Knauf *et al.*, 2008a). Interestingly, the chronic treatment with exendin (9-39) increased both food intake and energy expenditure, so there were no changes in body weight (Knauf *et al.*, 2008a). The mechanisms related to increased energy intake were not assessed, but the higher energy expenditure was explained by the increased thermogenesis, with no changes in physical activity. The beneficial effect of central GLP-1R blockade on insulin sensitivity was related to higher glucose utilization in muscle, adipose tissue, and heart (Knauf *et al.*, 2008a).

Although these studies clearly demonstrated that the central GLP-1 system plays a relevant role in the regulation of glucose homeostasis, the specific neuronal populations mediating those actions remained completely unknown. In this sense, a recent study has focused on the role of GLP-1R in the hypothalamic ARC, as the ARC plays a key role in the maintenance of glucose levels in response to anorectic peptides or nutrients by regulating glucose production (Lam *et al.*, 2005; Plum *et al.*, 2006) and GLP-1R are found in this area. When GLP-1 was specifically administered in the ARC after insulin infusion, the production of glucose was reduced (Sandoval *et al.*, 2008), suggesting that GLP-1 acts similarly to insulin and nutrients to regulated

glucose production (Obici *et al.*, 2002, 2003). GLP-1R is located in POMC neurons, which are related with the regulation of body weight and glucose metabolism, so it seems plausible to hypothesize that GLP-1 acts through these neurons to regulate glucose metabolism but no food intake (Sandoval *et al.*, 2008). The same work also investigated the potential role of ATP-sensitive K^+ channels (K_{ATP}), which are essential for glucose homeostasis (Pocai *et al.*, 2005), mediating the actions of central GLP-1. The authors demonstrate that the coinfusion of a K_{ATP} blocker together with GLP-1 blunted the effect of GLP-1 on glucose production and glucose uptake, indicating that K_{ATP} channels in the ARC regulate the action of GLP-1 (Sandoval *et al.*, 2008). Nevertheless, the mechanism of action at hypothalamic levels has been elucidated, it is still unknown how this system is activated. Circulating GLP-1 can cross the blood–brain barrier, so it might be possible that peripheral GLP-1 coming from the intestine activates the central GLP-1 system, or a mechanism of feedback to the hindbrain, or factors released after meal ingestion might also induce the activation (Sandoval, 2008). Another study aiming to investigate the signals transmitted by enteric glucose sensors showed that mice lacking the GLP-1R or mice injected with a GLP-1R antagonist showed whole-body lower glucose utilization and muscle glycogen synthesis after an oral glucose load (Knauf *et al.*, 2008b). Furthermore, this pathway does not work in diabetic mice, indicating that the central GLP-1 system is essential for the modulation of glucose metabolism.

In humans, the GLP-1R was colocalized in the brain with glucose transporter GLUT2 and glucokinase particularly in hypothalamic areas involved in feeding behavior (Alvarez *et al.*, 2005). Specific binding of 125I-GLP-1(7-36) amide to the GLP-1R was detected in several brain areas and was inhibited by unlabeled GLP-1(7-36) amide, exendin 4, and exendin (9-39) (Alvarez *et al.*, 2005). GLP-1, GLUT2, and glucokinase are important regulators of glucose-stimulated insulin secretion. Using PET technology, which allows the molecular imaging of biological actions *in vivo*, the authors found that the intravenous administration of GLP-1(7-36) amide increased the levels of 2-[F-18] deoxy-D-glucose (FDG) in selective areas of the hypothalamus and the brainstem. FDG is retained in the cell proportionally to the rate of glycolysis; therefore, this work suggests that GLP-1(7-36) amide increases glycolysis (Alvarez *et al.*, 2005).

IV. CNS Glucagon-Like Peptide 1 and Lipid Metabolism

The stimulation of the central GLP-1 system is able to decrease not only food intake, but also respiratory quotient relative to the control animals during the first 2 h of the nocturnal cycle (Hwa *et al.*, 1998). Interestingly,

low doses of GLP-1 (1 μg) in lean Zucker rats, which had minimal effects on food intake, caused an increase in oxygen consumption during the first 2 h of the nocturnal cycle (Hwa *et al.*, 1998). These data suggest that central GLP-1 may be an important factor controlling negative energy balance by mechanisms other than decreasing food intake. Supporting this hypothesis, it has been also recently demonstrated that CNS GLP-1 system directly controls adipocyte lipid metabolism (Nogueiras *et al.*, 2009). More specifically, the chronic central infusion of GLP-1 decreases the expression of several key enzymes involved in the synthesis of fatty acids, such as ACC, FAS, or SCD-1 in WAT (Nogueiras *et al.*, 2009). Importantly, these effects are independent of food intake. The sympathetic nervous system (SNS) seems to mediate those actions, as demonstrated by the fact that the central administration of GLP-1 stimulated the sympathetic nerve activity in WAT and the lack of response in mice lacking the beta-adrenoreceptors (Nogueiras *et al.*, 2009). Interestingly, the central effects of GLP-1 on adipocyte metabolism were completely blunted in diet-induced obese mice. Although obese mice showed a decrease in food intake and body weight, the amount of fat mass was unchanged after central GLP-1 administration. Consistent with body composition, the expression of lipogenic enzymes was not affected by central GLP-1, suggesting an obesity-induced adipocyte resistance to CNS GLP-1. Further findings supporting a role for the SNS as a mediator of GLP-1 are that GLP-1 receptor agonists rapidly increase heart rate and blood pressure (Cabou *et al.*, 2008). The effect of intravenously administered GLP-1 on arterial blood pressure and heart rate was eliminated by the intracerebroventricular or intravenous administration of the antagonist exendin (9-39) (Barragan *et al.*, 1999), consistent with a role for central GLP-1 receptor neurons in control of the cardiovascular response.

V. Future Directions

As summarized in Fig. 11.1, it seems clear that the actions of the central GLP-1 system produce strong effects on energy metabolism at several levels. Indeed, the most studied aspect is its potent anorectic action. Recent evidence have also shown a potent effect on glucose and lipid metabolism supporting the hypothesis that drugs targeting specific GLP-1 neuronal circuits within the brain might be a valid approach to treat obesity and/or diabetes. However, important issues must be taken in account, as GLP-1 has a potent anorectic action but it reaches some brain areas causing visceral illness. Since neuronal pathways mediating the anorectic action of GLP-1 *per se* and the brain nuclei activated by GLP-1 inducing visceral illness are known (Kinzig *et al.*, 2002), it seems clear that an approach

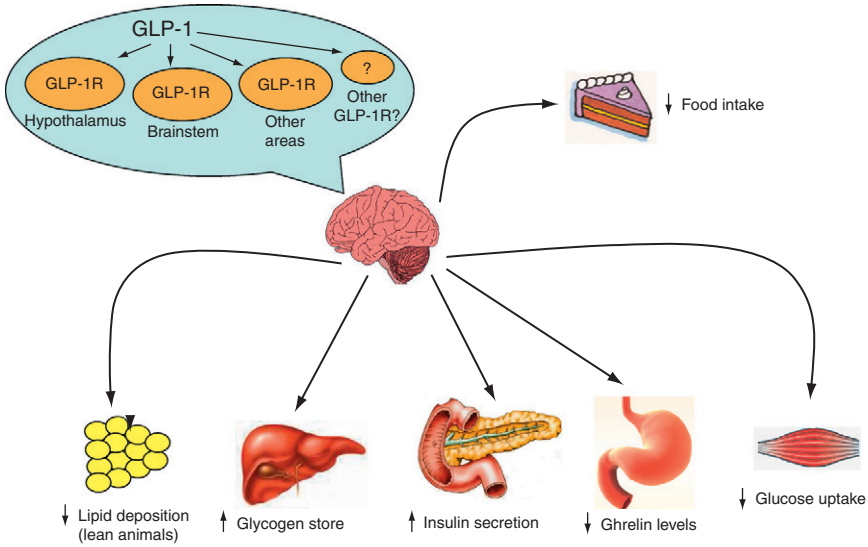

Figure 11.1 Schematic representation of the metabolic actions of the central GLP-1 system. GLP-1 is synthesized in neurons of the nucleus of the solitary tract (NTS) within the hindbrain, and reaches neuronal populations expressing the GLP-1R, which are widely distributed in the brain. Alternatively, it is plausible to hypothesize that there is another receptor mediating the actions of GLP-1 or GLP-1 agonists. Although the central GLP-1 system has been implicated in a variety of physiological functions, the most relevant in terms of regulation of energy homeostasis are as follows: decreased food intake, decreased lipid deposition, increased glycogen storage in the liver, increased insulin release by the pancreas, decreased ghrelin levels, and decreased glucose uptake in muscle.

reaching specifically the hypothalamus and/or the caudal brainstem but not the amygdala would be the most efficient without having undesirable side effects. Further information about the precise endogenous role of the GLP-1R in specific brain nuclei must be provided by the generation and characterization of mice lacking or overexpressing GLP-1R in specific brain areas. In this sense, global GLP-1R knockout mice are characterized by mild fasting hyperglycemia and a modest glucose intolerance in association with defective glucose-stimulated insulin secretion (Scrocchi *et al.*, 1996). Moreover, these mice have alterations in the development of islets and exhibit defective regeneration of beta-cell mass (Wang and Brubaker, 2002). Since the central GLP-1 system modulates different aspects of energy metabolism, it would be of great importance to know the alterations of mice lacking GLP-1R in specific brain areas.

Another interesting aspect is the fact that GLP-1 and GLP-1R agonists, like exendin 4, seem to act through different mechanisms within the brain (Barrera *et al.*, 2009). Although the receptor mediating the anorectic actions

of both compounds is the GLP-1R, the potency of exendin 4 diminishing food intake is much higher than GLP-1. These findings bring up some important questions such as, "does central exendin 4 administration also exert a more potent action than GLP-1 on glucose and lipid metabolism?" or "are other long-acting GLP-1R agonists that could improve the central actions of exendin 4?" In this regard, it would be interesting to assess the central actions of liraglutide, a GLP-1R agonist approved in 2010 by the Food and Drug Administration (FDA) to treat type 2 diabetes, which seems to reduce body weight and food intake in obese rats (Raun *et al.*, 2007) and obese patients (Astrup *et al.*, 2009). Very recently, it has been also demonstrated that the long-term administration of novel molecules with agonism for the glucagon and GLP-1 receptors decreased food intake, body weight, and enhanced insulin sensitivity in obese mice without causing any adverse effects (Day *et al.*, 2009). These coagonist compounds also normalized glucose and lipid metabolism and reduced liver steatosis in the diet-induced obese mice (Day *et al.*, 2009). These exciting findings trigger the question if a combination of more than two endogenous metabolically relevant peptides into a single molecule might provide a more potent action profile on obese animal models. Very likely, those new molecules will appear in the future and their potential clinical application will deserve further investigation.

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CHAPTER TWELVE

Glucagon-Like Peptide-1: Gastrointestinal Regulatory Role in Metabolism and Motility

Per M. Hellström

Contents

Abstract

Gastrointestinal (GI) motility, primarily gastric emptying, balances the hormonal output that takes place after food intake in order to maintain stable blood sugar. The incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), work together to reduce postprandial hyperglycemia by glucose-dependent insulin secretion and inhibition of glucagon release, as well as inhibition of GI motility and gastric emptying. GLP-1 is considered the more effective of the two incretins due to its additional inhibitory effects on GI motility. It is observed that patients on treatment with GLP-1 analogues or exenatide achieve a considerable weight loss during treatment. This is of benefit to improve insulin resistance in type 2 diabetes. Furthermore, weight loss *per se* is of considerable benefit in an even longer health perspective. The weight loss is considered to be due to the inhibition of GI motility. This effect has been studied in animal experimentation, and from there taken to involve studies on GI motility in healthy volunteers and patients with irritable bowel syndrome (IBS). Evolving to a phase II study in IBS, the GLP-1 analogue (ROSE-010) was recently shown to be effective for treatment of acute pain attacks in IBS. Taken together, data speak in favor of GI motility as a central component not only in metabolic disorders but also in IBS, be it due to a direct relaxing effect on GI smooth muscle or a slow emptying of gastric

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contents resulting in a less outspoken nutritional demand on hormonal regulatory functions in the GI tract.

I. Introduction

The reptile counterpart to glucagon-like peptide-1 (GLP-1) peptides exendin-3 and -4 were originally found in the salivary gland of the venomous lizards *Heloderma horridum* and *Heloderma suspectum* (Gila monster) species. In early work by Eng and Raufman, these peptides were isolated from the lizard *exo*crine gland and shown to have *end*o*cri*n*e* actions, thus named exendins (Eng *et al.*, 1990, 1992). The lizard venom contains a number of highly bioactive peptides where exendin-3 and -4 have found considerable interest for their usefulness as medicines.

Exendin-4 was originally found to stimulate amylase secretion from pancreatic acinar cells, and later proven to be a potent agonist for the mammalian GLP-1 receptor, consistent with 53% amino acid identity between exendin-4 and GLP-1. Furthermore, exendin-4 displays similar functional properties as native GLP-1, by regulating gastric emptying, insulin secretion, food intake, and glucagon secretion, thereby stabilizing blood glucose in normal as well as diabetic animals (Raufman, 1996). The peptide is also resistant to degradation by dipeptidyl peptidase-4 (DPP-IV), and consequently, a prolonged half-life (Abu-Hamdah *et al.*, 2009).

The feeding habits of reptiles is one of their fundamentally distinctive features, the Gila monster eats infrequently, only 5–10 times a year in the wild (Philadelphia Zoo staff, 2007) and then up to a third of its body weight with a correspondingly slow food passage rate, which is balanced by a low energy metabolism. The ingestion of intact prey by a lizard requires considerable processing by the stomach before passage into the small intestine, much of which demanding a considerable gastric energy workload to maintain low pH. There is also a direct relationship between body temperature and rates of acid and enzyme secretions, which can increase the duration of digestion (Christel *et al.*, 2007; Wright *et al.*, 1957). As exendin-4 in the salivary gland of the reptile is a high-affinity agonist for the mammalian GLP-1 receptor, it can be hypothesized that direct effects on the stomach and gastrointestinal (GI) tract may be achieved outside of pancreatic functions, especially during the digestive process when pH of the stomach is relatively high during the buffering capacity of ingesta. Since an analogue of exendin-4, the incretin mimetic exenatide, delays gastric emptying with ensuing weight loss and queasiness (Blase *et al.*, 2005; Edwards *et al.*, 2001), there are reasons to believe that other incretins as well would be able to influence GI functions. Our first observation in that direction was that the plasma glucose response to meal was seen *much* earlier than the expected

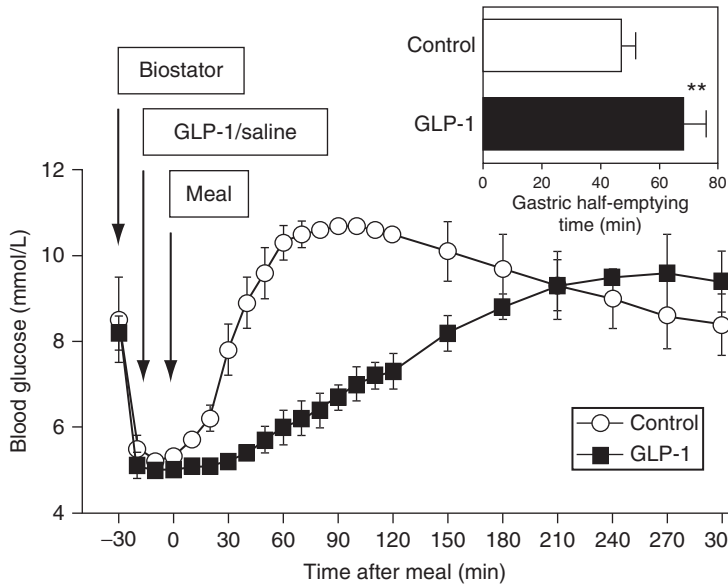

Figure 12.1 The inhibitory effect of an infusion of GLP-1 (0.7 pmol/kg/min) on blood glucose rise after a meal. Early inhibition of postprandial hyperglycemia is considered to be due to simultaneous inhibition of gastric emptying (see inset). All values are mean ± SE for $n = 8$, $^{**}p < 0.01$.

insulin release (Fig. 12.1) (Gutniak *et al.*, 2001). This was considered to be due to an initial powerful inhibition of gastric emptying, to be followed by an insulin release caused by GLP-1 as part of an activation of the entero-insular axis. Thus, the anorexic effect, and possibly also queasiness of GLP-1 is most likely due to the profound inhibitory impact of the peptide directly on gastric emptying. Subsequent studies using a radionuclide for scintigraphic gastric emptying studies have shown a consistent effect of GLP-1 at near-physiological plasma levels and without any sign of desensitization to repeat administration (Näslund *et al.*, 1999a,b; Fig. 12.2).

II. GLP-1 in Metabolism

GLP-1 is formed in intestinal L-cells by the proglucagon gene after cleavage from the proglucagon molecule by posttranslational processing. GLP-1 is constituted by the C-terminally amidated 7–36 amino acid peptide (Holst and Gromada, 2004). Sizeable amounts of GLP-1 are secreted into the blood stream from L-cells in the ileum and colon in response to intake of carbohydrates and fat, which increases the plasma levels fivefold (Näslund *et al.*, 1998a,b; Wang *et al.*, 1995). Then, circulating GLP-1

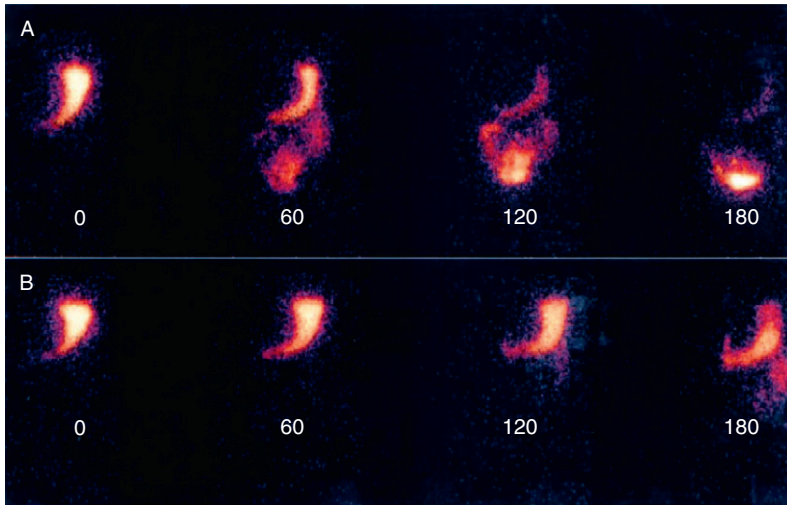

Figure 12.2 GLP-1 at 0.7 pmol/kg per min during 180 min slows total gastric emptying with retention of radionuclide-tagged omelet in the stomach (by courtesy of Dr. P. Grybäck).

enhances proinsulin release which is further cleaved to C-peptide and metabolically active insulin, a process that normally takes place within 40–60 min of food intake. Along with GLP-1, another incretin, glucose-dependent insulinotropic peptide (GIP), is simultaneously released from more proximal sites of the small intestine to promote insulin release. The action of GIP, however, is different from that of GLP-1. Apart from the common endocrine counter-regulatory actions of GLP-1 and GIP on postprandial hyperglycemia, GLP-1 has additional potentially beneficial effects related to a slowed gastric emptying rate and reduced food intake (Edholm *et al.*, 2010; Mudaliar and Henry, 2010). The glycated hemoglobin (HbA1c), which is indicative of the long-term management of diabetes over the last 3 months, also varies with the rate of gastric emptying (Cucchiara *et al.*, 1998). GLP-1 is one of several factors that regulate the rate of gastric emptying, and the rate and volume of gastric emptying accounts for as much as 34% of the variance in peak postprandial glucose concentrations (Horowitz *et al.*, 1993). The effect of GLP-1 on gastric emptying allows the rate of glucose appearance to better match the rate of glucose disappearance from the systemic circulation, thereby reducing postprandial glucose excursions. This effect of GLP-1 on gastric emptying is believed to be centrally mediated by vagal efferent nerves (Imeryüz *et al.*, 1997).

The most likely cause of poor glycemic control is a temporal mismatch between the onset of insulin action and the delivery of nutrients into the small intestine, both of which are dependent on the rate of gastric emptying.

Therefore, it can be advocated that unexplained poor glycemic control should be investigated for abnormality in gastric motility, specifically gastroparesis, which can appear at any time during the disease course of diabetes mellitus.

Among other beneficial effects possibly associated with the inhibitory actions of GLP-1 on gastric emptying are the positive effects seen on blood lipids and arterial blood pressure, which may be important benefits among diabetes type 2 patients with potential cardiovascular risk factors (obesity, high blood pressure, and smoking) (Mudaliar and Henry, 2010). In line with this, clinical experience has shown that treatment with insulin pump and fast-acting insulin over 24 h is another approach to achieve metabolic control as monitored by HbA1c, where the HbA1c levels are being shifted in a normal direction (Edelman *et al.*, 2010). Another safe approach to this condition might be use of GLP-1 analogues, which would enhance insulin secretion and suppress glucagon through glucose-dependent effects; as glucose concentrations fall to the normal range, the insulinotropic and glucagonostatic effects subside. In agreement with this, exenatide administration does not impair normal counter-regulatory hormone responses to hypoglycemia (Degn *et al.*, 2004).

III. GLP-1 in Satiety

Early studies of GLP-1 effects in diabetes type 2 showed an early blunting of the expected blood sugar rise after a meal, long before insulin was expected to be released, most likely as a result of an inhibited gastric emptying rate (Gutniak *et al.*, 1996). Later, this finding in man was confirmed with a profound inhibitory action of GLP-1 on gastric emptying, appearing at near-physiological plasma levels of the peptide (Näslund *et al.*, 1999b). Further animal experimentation in depth revealed this to be a nerve-mediated effect possibly through enteric or central nervous mechanisms (Tolessa *et al.*, 1998a,b), specifically involving vagal afferents (Bucinskaite *et al.*, 2009) and nitrergic mechanisms (Tolessa *et al.*, 1998b, 2001).

Further investigations showed that the inhibitory action of GLP-1 on gastric emptying rate was followed by a satiety effect that did not occur in conjunction with food intake but instead some 3–4 h after the meal was completed (Fig. 12.3; Näslund *et al.*, 1998a). From this we concluded that GLP-1 was not able to inhibit the ongoing food intake as this is a short-lasting process usually terminated within 15 min. Instead, as the gastric emptying process was slowed by GLP-1, the satiety period after the meal was prolonged over many hours hence increasing the satiety period in anticipation of the next meal (Näslund *et al.*, 1998b). Studied with meals taken at regular intervals, this effect led to increased satiety and diminished intake at the subsequent meal (Näslund *et al.*, 1999a). This satiety-inducing effect of GLP-1 was further analyzed and in a two-meal experimental series,

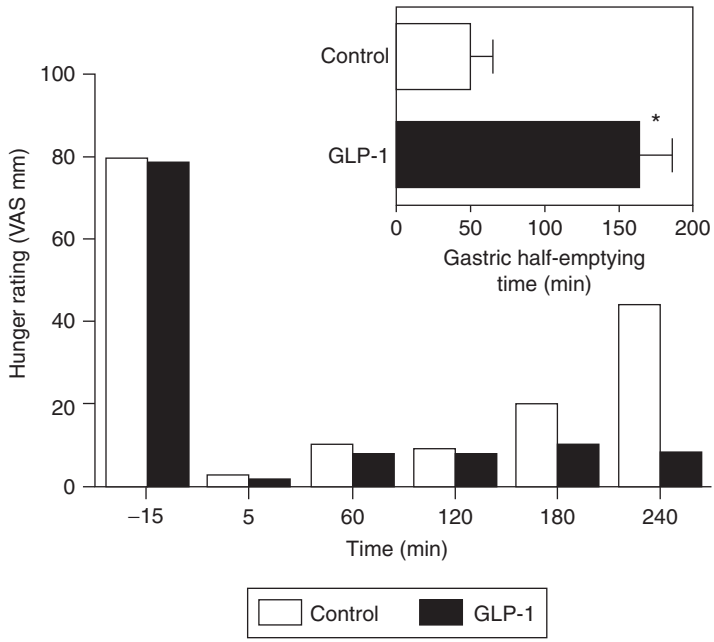

Figure 12.3 GLP-1 at 0.7 pmol/kg/min during 180 min reduces hunger ratings during a prolonged period after termination of GLP-1 infusion in obese men. At the same time, gastric half-emptying time is prolonged with GLP-1 as shown with the acetaminophen absorption technique (see inset). All values are mean, or mean ± SE for $n = 6$, $^{\star}p < 0.05$.

and over a 5-day long period, where reduced food intake was found together with decreased body weight in obese humans (BMI > 30 kg/m^2) by about 0.5 kg per week (Näslund *et al.*, 2004). These findings were later supported by studies of the exendin-4 analogue exenatide showing metabolic and weight control over prolonged periods of time (Blonde *et al.*, 2006), similarly, studies with the log-acting GLP-1 analogue liraglutide, improved glycemic control, and body weight (Feinglos *et al.*, 2005). Our animal experiments investigating possible mechanisms involved in the GLP-1 response with recordings of nerve activity from the left vagus nerve showed that GLP-1 increased the firing rates in afferent vagal fibers. This speaks in favor of a direct link from the L-cells of the GI tract to the brain relaying signals of satiety and post-meal influences alike directly to the brain (Bucinskaite *et al.*, 2009).

IV. GLP-1 in GI Motility

The clear actions of GLP-1 on gastric emptying raised the question whether GLP-1 may have a general inhibitory action on smooth muscle activity throughout the GI tract. Early experiments on small bowel

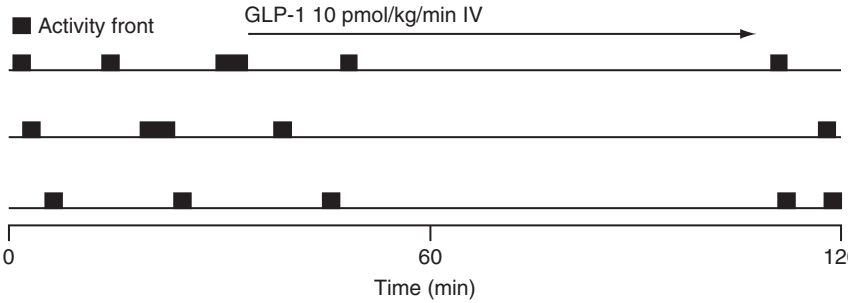

Figure 12.4 Schematic presentation of the result of GLP-1 infusion on the migrating myoelectric complex in the rat (adapted after Tolessa *et al.*, 1998b).

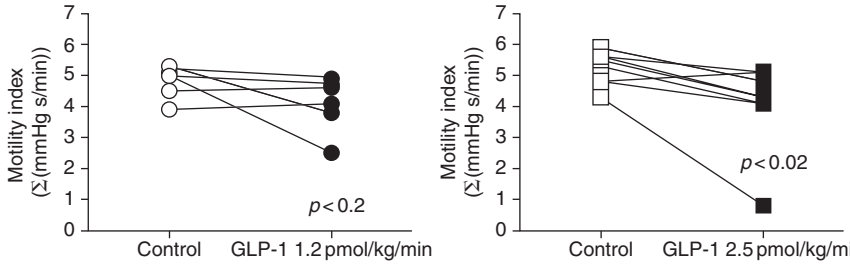

Figure 12.5 Inhibitory action of GLP-1 on small bowel motility computed as motility index over a 4-h infusion period with two doses of GLP-1 and compared with saline.

myoelectric activity in the rat showed a clear direct dose-related and partly nitric oxide-dependent inhibitory effect of GLP-1 on the migrating myoelectric complex (MMC) (Tolessa *et al.*, 1998b, 2001; Fig. 12.4). Further studies with small bowel manometry using near-physiological doses in healthy volunteers and approaching pharmacological levels in patients with irritable bowel syndrome (IBS) resulted in limited effects on the MMC pattern in humans, most likely due to its binary sporadic occurrence at irregular intervals. However, when the total contractile activity of the small intestine was computed as a motility index, infusions of GLP-1 resulted in reduced motility during an infusion period of 4 h (Hellström *et al.*, 2008; Fig. 12.5).

The inhibitory effects of GLP-1 on small bowel motility in healthy volunteers and IBS patients promoted a clinical phase II study of the actions of GLP-1 in IBS patients. A major hurdle to overcome in this project was the DPP-IV activity in plasma, which quickly inactivates GLP-1 with a half-life of about 1 min. Substituting the second amino acid residue alanine (amino acid no. 8) in the structure of GLP-1 (7–36 amide) for valine, a compound with the following structure was obtained: H_2N-His-Val-Glu-

Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Gly-COOH. This substitution created a peptide that is resistant to degradation by DPP-IV, has a prolonged half-life to 60 min, but retains biological activity on the GLP-1 receptor. This GLP-1 analogue was named ROSE-010. A randomized, double-blind clinical study was carried out with this compound in 166 IBS patients defined according to the Rome II criteria (Hellström *et al.*, 2009). The common feature of the IBS patients' disorder was a prominent pain component of IBS exceeding 4 on an 11-graded scale (VAS 0-10) as an inclusion criterion. ROSE-010 was given by subcutaneous injection at a dose of either 100 or 300 μg and compared to placebo. The primary efficacy endpoint of the study was the fraction of patients with a greater than 50% maximum total pain relief response from 10 to 60 min after a subcutaneous injection of ROSE-010. After decoding the data, twice as many patients responded with pain relief after either dose of ROSE-010 compared with placebo (24%, $p = 0.011$; 23%, $p = 0.005$; and 12% after 300 μg, 100 μg, and placebo, respectively; Fig. 12.6). Similar results were obtained for the secondary endpoints including the maximum summed pain intensity difference, time to meaningful pain relief, and patient ratings of satisfaction with treatment. The time to meaningful and total pain relief was also shorter with ROSE-010 at both doses compared with placebo. More patients ($p < 0.05$)

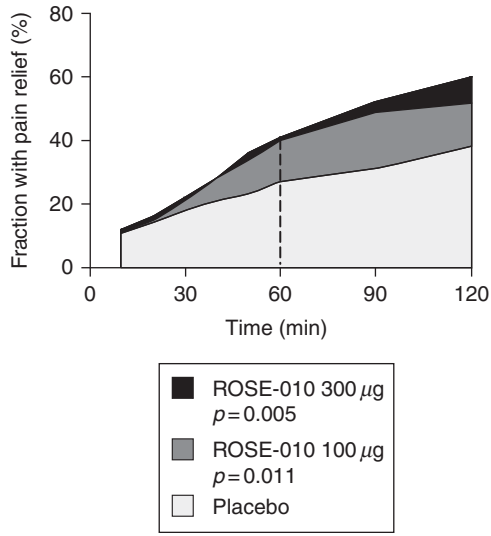

Figure 12.6 Fraction of patients responding with total pain relief defined as the primary endpoint at 60 min (broken vertical line) after injections with the GLP-1 analogue ROSE-010 at doses 100 and 300 μg and placebo. (Adapted after Hellström *et al.*, 2009).

were also satisfied with ROSE-010 and considered ROSE-010 better than previous IBS treatments. There were no severe adverse events, but nausea was the most common side effect.

IBS is a functional disorder with a paucity of positive morphological findings, but a yet undefined abnormality, possibly as a motility aberration. No firm biomarkers exist that can aid the clinician's diagnostic skills in IBS. Instead, we rely on the Rome criteria, which are regularly updated, lastly in 2006. Bearing the fact in mind that motility disorders, and possibly IBS, are dysfunctional in their character and do not include major tissue injury and destruction, it seems reasonable to assume that the mechanism action of a physiological gut peptide hormone, with high receptor specificity and few adverse effects, might be a reasonable the choice for searching new ways of medical treatment. In order to accomplish such research activity and implement findings in the clinic, the researcher has to be observant on physiological principles in nature and animal kingdom, which can be applied within the medical framework as a diagnostic or pharmaceutical tool.

V. GLP-1 in Perspective

This first clinical study with a GLP-1 analogue in IBS needs to be followed by future studies in an outpatient IBS population with self-administration of the drug. Most likely, a study cohort including ambulatory patients "at home" and "at work" will increase the variability of the response to the treatment. Thus, such a study has to be carefully planned and carried out in a carefully defined IBS population.

The new "incretin mimetics" constitute a new class of pharmacological agents of which GLP-1 is the prototype. These agents exhibit marked glucoregulatory effects similar to those of GLP-1, albeit their actions may not solely be mediated through glucose-dependent enhancement of insulin secretion via the pancreatic GLP-1 receptor, but also through other effects such as gastric emptying and GI motility. As new "incretin mimetics" are brought into practice, it seems crucial to evaluate their effects on gastric emptying and GI motility as these actions are the first regulatory steps in metabolism after a meal.

ACKNOWLEDGMENTS

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CHAPTER THIRTEEN

The Role of GLP-1 in Neuronal Activity and Neurodegeneration

Christian Hölscher

Contents

Abstract

Type 2 diabetes has been identified as a risk factor for Alzheimer's disease (AD). The underlying mechanism behind this unexpected link is most likely linked to the observed desensitization of insulin receptors in the brain. Insulin acts as a growth factor in the brain and supports neuronal repair, dendritic sprouting, and differentiation. Several drugs have been developed to treat type 2 diabetes which resensitize insulin receptors and may be of use to prevent neurodegenerative developments in AD. The incretin glucagon-like peptide-1 (GLP-1) is a hormone that facilitates insulin release under high blood sugar conditions. Interestingly, GLP-1 also has very similar growth factor like properties as insulin, and has been shown to protect neurons from toxic effects. In preclinical studies, GLP-1 and longer lasting analogues reduce apoptosis, protect neurons from oxidative stress, induce neurite outgrowth, protect synaptic plasticity and memory formation from the detrimental effects of β-amyloid, and reduce plaque formation and the inflammation response in the brains of mouse models of AD. An advantage of

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GLP-1 is that it does not affect blood sugar levels in nondiabetic people. Furthermore, recent research has shown that some GLP-1 analogues can cross the blood–brain barrier, including two that are on the market as a treatment for type 2 diabetes. Therefore, GLP-1 analogues show great promise as a novel treatment for AD or other neurodegenerative conditions.

Abbreviations

ADP	adenosine diphosphate
cAMP–GEFs	cAMP–guanine-nucleotide-exchange factors
CREB	cAMP responsive element-binding protein
ERK	extracellular signal-regulated kinase
MEK	MAPK kinase
Akt/PKB	protein kinase B complex
CPD3B	cyclic phosphodiesterase 3 β
Grb2/SOS	growth factor receptor-binding protein 2/son of sevenless protein
IRS	insulin receptor substrates that get phosphorylated after activation
MAPK	mitogen-activated protein kinase
PDK	phosphatidylinosite-dependent kinase
Pi3K	phosphatidylinositol 3 kinase
Raf	regulation of alpha-fetoprotein
Ras	rat sarcoma virus peptide
Shc	Src homology collagen peptide

I. A Causal Link Between Diabetes and Alzheimer's Disease

Type 2 diabetes mellitus (T2DM) is a condition where insulin is no longer able to control the levels of blood sugar. Insulin levels may be even increased as an attempt by the physiological system to overcome the reduced effectiveness of insulin. This is often due to insulin receptor desensitization. Receptor desensitization is a reaction to continuously increased levels of insulin, which are in turn induced by continuously increase blood sugar levels. A western style diet in combination with lack of exercise is the main factor for the increase of T2DM. While in previous times people only developed T2DM at older age ("old age diabetes"), now even teenagers

develop T2DM due to the change in lifestyle. Consequently, a lot of research is conducted in the discovery of the underlying mechanisms and in new treatments for T2DM (Dailey, 2008; Frias and Edelman, 2007; Pi-Sunyer, 2008; Scheen, 2008). As insulin is increasingly losing its effectiveness in treatment, one novel strategy is to make use of other signaling pathways that do not desensitize and can keep control of blood sugar levels. One new strategy is the use of the so-called incretins, a group of hormones that has positive effects on blood sugar control. Incretins will increase insulin release during episodes of high blood sugar levels, the so-called incretin effect (Baggio and Drucker, 2007; Drucker and Nauck, 2006; Green, 2007).

Recently, type 2 diabetes has been identified as a risk factor for Alzheimer's disease (AD). Epidemiological studies of patient data sets have found a clear correlation between T2DM and the risk of developing AD or other neurodegenerative disorders (Haan, 2006; Luchsinger *et al.*, 2004; Ristow, 2004; Strachan, 2005). For example, an analysis of patient databases showed a clear correlation between AD and type 2 diabetes. In this study, 85% of AD patients also had diabetes or increased fasting glucose levels, compared to 42% in age-matched controls (Janson *et al.*, 2004). Reduced insulin sensitivity, the stage before developing T2DM, is also observed in the majority of elderly people and contributes to the development of AD (Carro and Torres-Aleman, 2004; Hoyer, 2004). This connection between T2DM and AD motivated novel research initiatives to identify what the basis for this may be. Insulin is well known for its role in reducing blood sugar levels. However, current research shows that insulin has many other functions in the body. Its general physiological profile of activities is more in line with that of a growth factor. Every cell has insulin receptors, and blocking these has detrimental effects on cell growth and survival. Therefore, it is not surprising to find that neuronal insulin receptors have been shown to induce dendritic sprouting, neuronal stem cell activation, and general cell growth, repair, and neuroprotective effects (Cohen *et al.*, 2007; Holscher, 2005; Hoyer, 2004; Li and Hölscher, 2007; Stockhorst *et al.*, 2004; van Dam and Aleman, 2004). Furthermore, insulin and the related insulin-growth factor (IGF-I) are potent neuroprotective factors, and also regulate levels of phosphorylated tau, the major component of neurofibrillary tangles found in AD (Carro and Torres, 2004; Li *et al.*, 2007). Not surprisingly, insulin also improves brain function such as attention, memory, and cognition in humans (Okereke *et al.*, 2008; Reger *et al.*, 2008a; Watson and Craft, 2004; Zhao *et al.*, 2004). Nasal application of insulin, an application route where it enters the brain more directly, had clear effects on attention and memory formation (Reger *et al.*, 2008a,b).

In animal models, a decrease in insulin receptor signaling produces cognitive impairments and a reduction in hippocampal synaptic neurotransmission and synaptic plasticity, which is linked to memory formation (Biessels *et al.*, 2006; Trudeau *et al.*, 2004). Conversely, insulin injected into the brain can improve performance in memory tasks in animals similar to the enhancement

in performance of attention tasks in humans when applied via the nasal route (Stockhorst *et al.*, 2004). This effect might be linked to the fact that long-term potentiation (LTP) of neuronal synaptic transmission is impaired if insulin signaling is impaired, as shown in animal models of diabetes. Treatments of the diabetic animals with insulin rescued the impairment in neurotransmission (Biessels *et al.*, 2004; Gault *et al.*, 2010; Gispen and Biessels, 2000).

In T2DM, the insulin receptors have become desensitized, and the insulin signal to increase cell metabolism and cell growth and repair appears to be functionally impaired. Recently, it was shown that insulin receptors in the brain are desensitized in AD patients, and this condition has been named "type 3 diabetes" by some authors (Lester-Coll *et al.*, 2006; Steen *et al.*, 2005). The impairment of insulin signaling in the brain could well play a role in the development of neurodegenerative disorders, as it leaves neurons more exposed to degenerative influences. Attempts to improve cognition in patients with AD by applying insulin have shown promise but have not been successfully developed into a treatment yet (Craft, 2005, 2007; Hallschmid and Schultes, 2009).

II. An Insulin-Supporting Messenger: Glucagon-Like Peptide-1

Since insulin receptors are often desensitized in T2DM, and injection of insulin itself loses its effectiveness over time, researchers are investigating different strategies by which to improve blood glucose level maintenance. Fortunately, several parallel signaling systems exist that also modulate blood glucose levels, for example, the incretin hormone signaling pathways—in particular, glucagon-like peptide-1 (GLP-1) (Frias and Edelman, 2007).

GLP-1 is an endogenous 30-amino acid peptide hormone, which is released by intestinal L-cells after a meal and has several physiological roles in the body to control cell metabolism. GLP-1 binds to the GLP-1 receptor that is coupled to a second messenger pathway via G proteins (Green *et al.*, 2004b; see Fig. 13.1). GLP-1 receptor stimulation enhances β-cell proliferation, glucose-dependent insulin secretion and lowers blood glucose in patients with T2DM (Green *et al.*, 2006; Lovshin and Drucker, 2009).

A. The development of longer lasting GLP-1 analogues

GLP-1 only has a half-life of several minutes in the blood stream. In order to treat T2DM in a more effective way, long-lasting analogues have been developed that are not broken down by the endogenous protease DPP-IV (Gallwitz, 2006; Green *et al.*, 2003; Irwin *et al.*, 2005). Several such analogues have been developed by modulating the GLP-1 amino acid sequence to

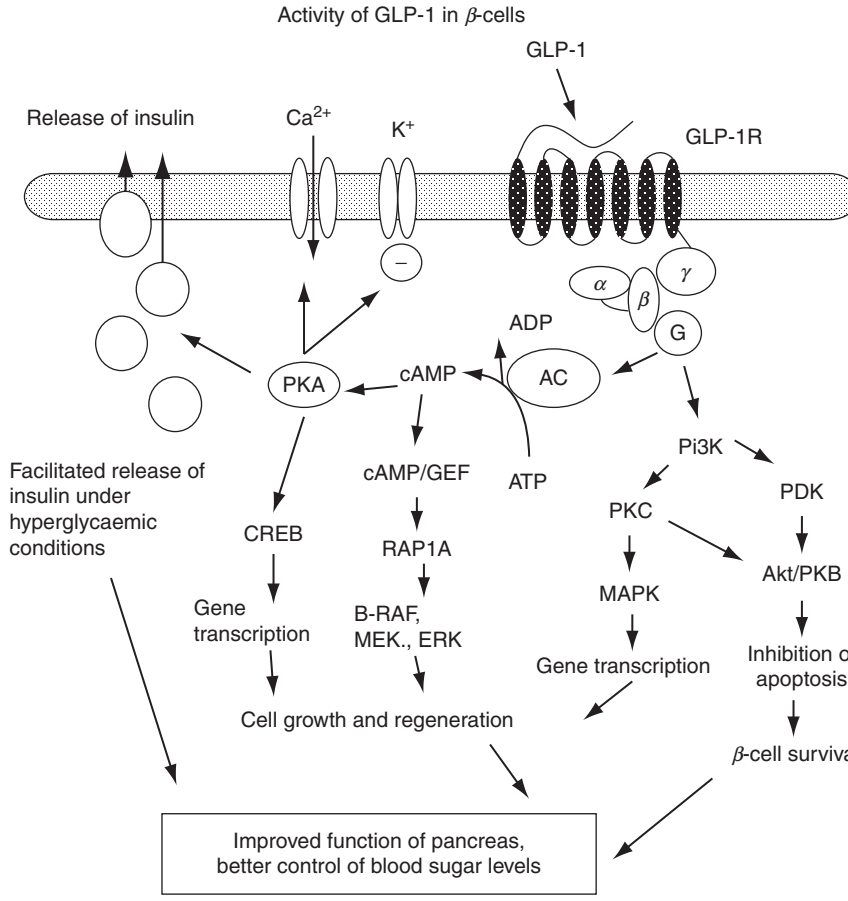

Figure 13.1 An overview of the roles and functions of the GLP-1 receptor in the pancreatic β-cell. Activation of the GLP-1 receptor under hyperglycemic conditions will activate a G protein, which in turn activates the adenylyl cyclase (AC) system (Green *et al.*, 2004b). The Ga subunit of the GLP-1 receptor stimulates AC, which leads to an increase in intracellular cAMP and activation of protein kinase A (PKA). PKA activity can increase vesicle release in β-cells to enhance glucose-stimulated insulin secretion. GLP-1 can also block K^+ channels, this leads to a slow depolarization of the cell membranes following closure of K^+ channels and reduced repolarization of cell membranes, which enhances the release of insulin vesicles (Doyle and Egan, 2003). Furthermore, this can increase the opening of voltage-dependent L-type Ca^{2+} channels and increase cytosolic Ca^{2+}, which not only induces depolarization and vesicle release but also in turn acts as a second messenger that can activate Ca^{2+} sensitive kinases (Jacobo *et al.*, 2009). Activation of GLP-1 receptors also leads to an increase in cytosolic Ca^{2+} levels as a result of activation of voltage-dependent L-channels following phosphorylation of PKA and/or mobilization of intracellular Ca^{2+} stores. An increase in phosphatidylinositol 3-kinase (PI3K) levels via the G protein activation also can activate intracellular Ca^{2+} stores (Fagni *et al.*, 1991). The increase in PI3K levels also activates mitogen-activated protein kinases (MAPK). This pathway activates gene expression,

reduce the affinity to DPP-IV while maintaining the affinity for the GLP-1R (Green *et al.*, 2004a; Lovshin and Drucker, 2009). Drug companies are investing greatly into the development of novel GLP-1 analogues that can be used as treatments for T2DM. A GLP-1 analogue that has been extracted from the saliva of the Gila monster lizard has entered the market as a treatment for T2DM in 2005 (exendin-4, *Exenatide*, *Byeatta*®) (Murphy and Bloom, 2007). This drug has a half-life of about 90 min in the blood stream and is injected subcutaneously twice-daily after a meal (DeFronzo *et al.*, 2005; Larsen and Holst, 2005). A second GLP-1 analogue called liraglutide has entered the market which is a modified GLP-1 peptide with a fatty acid associated to it to attach this molecule to blood proteins, which ensures a slow release into the blood stream, and to increase the half-life to about 10 h (Marre *et al.*, 2009; Russell-Jones, 2009; Vilsboll, 2007). This drug has been released to the market as a treatment for T2DM in 2009 (Holscher, 2010; Zinman *et al.*, 2009).

B. GLP-1 also plays important roles in the brain

GLP-1 receptors are found on neurons in the brains of rodents and humans (Goke *et al.*, 1995; Perry and Greig, 2005). They are predominately expressed on large neurons, on cell bodies and also on dendrites, indicating that they are located presynaptically (Hamilton and Holscher, 2009). Similar to insulin, GLP-1 is predominately known for its action on blood sugar levels. However, just as insulin, GLP-1 is principally a growth factor that has the typical properties that growth factors have (Holscher and Li, 2008). GLP-1 increases cell growth, proliferation, and repair and inhibits apoptosis (Perfetti *et al.*, 2000). In the brain, GLP-1 has been documented to induce neurite outgrowth and to protect against excitotoxic cell death and oxidative injury in cultured neuronal cells (Perry *et al.*, 2002, 2003). In one study, neurons were

which controls expression of peptides that are required for cell growth, repair, and differentiation of β-cells and also in neuronal cells. Inhibition of PI3K (with LY294002) or MAPK (with PD98059) reduced the GLP-1-stimulated neurite outgrowth (for a review, see Perry *et al.*, 2007). GLP-1-mediated activation of PI3K and downstream transcription factors regulate expression of the genes that encode insulin, β-cell growth and differentiation phenotype (Buteau *et al.*, 1999; Lee *et al.*, 2002; Stoffers *et al.*, 2000). cAMP activates multiple intracellular messenger systems via PDA or independent of activation of PKA (shown here). A PKA-independent pathway had been found in β-cells, which involves cAMP–GEFs. GEFs are activated by binding to cAMP and activation of Rap1A (Kawasaki *et al.*, 1998). Rap1A activates PKC and B-Raf, leading to activation of MAPK (Leech *et al.*, 2000). An IP3- and PKC-dependent pathway also has been described that activates the Akt/PKB-dependent pathway which inhibits apoptosis (see Baggio and Drucker, 2007 for a review). The processes described here activate gene expression and protein synthesis and form the growth factor like properties of insulin and GLP-1. These trophic properties potentially protect and repair cells from degenerative developments that are developing over time.

found to be protected against cell death induced by β-amyloid 1–42, the peptide that aggregates in the brains of Alzheimer patients, and against oxidative stress and membrane lipid peroxidation caused by iron (Perry and Greig, 2005). In addition, GLP-1 showed neuroprotective properties in pyridoxine-induced peripheral neuropathy (Perry *et al.*, 2007). Furthermore, mice that overexpress GLP-1 receptors in the hippocampus (a brain area that is involved in memory formation) showed increased neurite growth and improved spatial learning abilities (During *et al.*, 2003). Enhanced progenitor cell proliferation in the brain was also found in this study. GLP-1 analogues also enhance stem cell proliferation in the pancreas (Green *et al.*, 2006). Theoretically, it might be possible to regenerate neuronal tissue and to regain some of the lost cognitive functions in patients with AD similar to the regeneration of β-cells in the pancreas (Sugaya *et al.*, 2007).

C. GLP-1 and stable analogues cross the blood–brain barrier

An important aspect of the effects of substances in the brain is the permeability of the compounds across the blood–brain barrier (BBB). The BBB prevents chemicals in the blood to reach the brain in an uncontrolled manner, and is a highly controlled filter system between the blood and the brain parenchyma. Its role is to protect the brain from chemicals that are potentially dangerous to neurons. Numerous drugs that showed great promise in treating conditions of the brain failed, because they did not cross the BBB (Begley, 2004). Several studies have shown that the native peptide GLP-1 as well as long-lasting GLP-1 analogues such as exendin-4 cross the BBB (Kastin and Akerstrom, 2003; Kastin *et al.*, 2002). The GLP-1 analogue Val(8)GLP-1 also crosses the BBB (Gengler *et al.*, 2010), as does the novel GLP-1 analogue liraglutide (McClean *et al.*, 2009).

As outlined below, these analogues not only cross the BBB after peripheral injection, but most importantly also show physiological effects in the brain by increasing neuronal progenitor proliferation, enhancing LTP in the hippocampus, improving learning, and reducing plaque formation and inflammation in the brain, and even increasing neuroneogenesis (McClean *et al.*, 2010a).

It appears that there is a specific transporter for GLP-1 across the BBB which also can be utilized by GLP-1 analogues, very similar to the insulin transporter (Banks, 2004) that also transports longer lasting insulin analogues such as Detemir across the BBB (Hallschmid and Schultes, 2009).

D. Synaptic transmission is modulated by β-amyloid and GLP-1

Another surprising observation is that insulin as well as GLP-1 not only have growth factor like properties in the brain, but also modulate synaptic activity. One study showed that the injection of GLP-1 into the basal

ganglia induced the release of the neurotransmitter glutamate, showing that synaptic transmitter vesicle release may be modulated by GLP-1 (Mora *et al.*, 1992). GLP-1 also increased the spontaneous firing rate of pyramidal neurons in the hippocampus (Oka *et al.*, 1999). Interestingly, β-amyloid fragments can directly affect synaptic transmission and the use-dependent upregulation of synaptic transmission (LTP). Since such a mechanism could be used for storing information in the brain (Hölscher, 2001), this amyloid-induced block of LTP may be in part responsible for impaired memory formation in patients with AD (Freir *et al.*, 2001; Hölscher *et al.*, 2007). In addition, a recent study has shown that soluble β-amyloid fragments directly bind to and decrease insulin receptor densities on neuronal dendrites (Xie *et al.*, 2002; Zhao *et al.*, 2008). This may be a mechanism by which insulin signaling in the brain becomes impaired in people with AD.

Further studies showed that direct injection of GLP-1 or long-lasting GLP-1 analogues into the brain markedly enhanced LTP in the hippocampus, a brain area that is involved in memory formation. Agonists such as (Val8)GLP-1 showed a clear upregulation of LTP, while the selective antagonist exendin (9–36) blocked LTP (Gault and Holscher, 2008). The novel GLP-1 analogue liraglutide that has been released to the market as a treatment for T2DM also upregulated LTP (McClean *et al.*, 2010b). Importantly, GLP-1 analogues were able to prevent the impairment of LTP that was induced by β-amyloid fragments (Gault and Holscher, 2008; Fig. 13.2). This effect is most remarkable, and underlines the fact that β-amyloid has numerous independent effects on cell physiology; some of which may occur very early on in AD, long before amyloid aggregates appear and neuronal death is observed (Gong *et al.*, 2003; Townsend *et al.*, 2006). Moreover, GLP-1, liraglutide, and exendin-4 have been shown to reduce endogenous levels of β-amyloid in a mouse model of AD, and to reduce levels of β-amyloid precursor protein (APP) in neurons (McClean *et al.*, 2010a; Perry *et al.*, 2003) (see Fig. 13.3). In contrast, the elimination of the GLP-1R in a KO model severely impaired learning abilities and also strongly reduced synaptic plasticity (Abbas *et al.*, 2009). Interestingly enough, spatial learning and synaptic plasticity is also impaired in mouse models of diabetes, and exendin-4 is able to reverse these impairments (Gault *et al.*, 2010). These results suggest that treatment with GLP-1 or long-lasting analogues beneficially affect a number of the therapeutic targets associated with AD, such as impaired memory, impaired neuronal synaptic transmission, increased neurodegenerative processes, and reduced neuronal regeneration.

E. Do incretins modulate synaptic transmission and vesicle release?

What could be the mechanism for the effects of GLP-1 on LTP? We know that GLP-1 receptors on β-cells in the pancreas modulate insulin release via a mechanism that involves closure of K^+ channels and depolarization of the

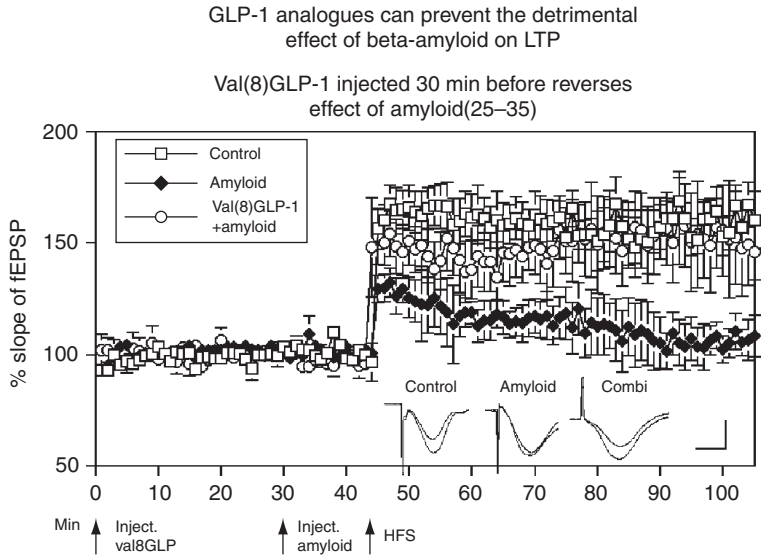

Figure 13.2 Injection of the protease resistant GLP-1 derivative (Val^8)GLP-1 30 min before β-amyloid injection reversed the impairment of LTP that was induced by β-amyloid fragments. No difference was found between the control group and the amyloid plus (Val^8)GLP-1 combination group. Averaged EPSPs are shown recorded 5 min pre-HFS and 1 h post-HFS. Calibration bars are 10 ms horizontal and 1 mV vertical. Averaged EPSPs are shown recorded 5 min pre-HFS and 1 h post-HFS (for details, see Gault and Holscher, 2008).

cell membrane which activates voltage-dependent calcium channels (VDCC). The subsequent influx of Ca^{2+} then activates Ca^{2+} sensitive enzymes such as PLA_2, PLC, or an adenylate cyclase that produces cAMP and thereby activates PKA, which in turn activates the mechanisms of vesicle exocytosis to release insulin into the extracellular space (Green *et al.*, 2004b; Leech and Habener, 1997; Suzuki *et al.*, 1997) (see Fig. 13.1). The same biochemical machinery that controls the release of neurotransmitters into the synaptic cleft via cAMP level regulation is found in neurons (Okamoto *et al.*, 1994; Wheeler *et al.*, 1994; Winder and Conn, 1993). Indeed, it has been shown that in neuronal cell cultures, GLP-1 modulates glutamate-induced Ca^{2+} influx. This effect was due to altered VDCC activity. Ca^{2+} influx was increased by reduced K^+ conductance, which slowed repolarization and enhanced the depolarization. GLP-1 furthermore induced cAMP formation, activated PKA, MAP kinases, and more (Doyle and Egan, 2003, 2007; Gilman *et al.*, 2003; Jacobo *et al.*, 2009; Perry and Greig, 2005) (see Fig. 13.4). However, a unique difference between neurons and β-cells has to be mentioned here. Another mechanism that regulates insulin release in β-cells is via the GLUT2 transporter. At high glucose levels, the GLUT2 transporter (and sensor) will increase intracellular

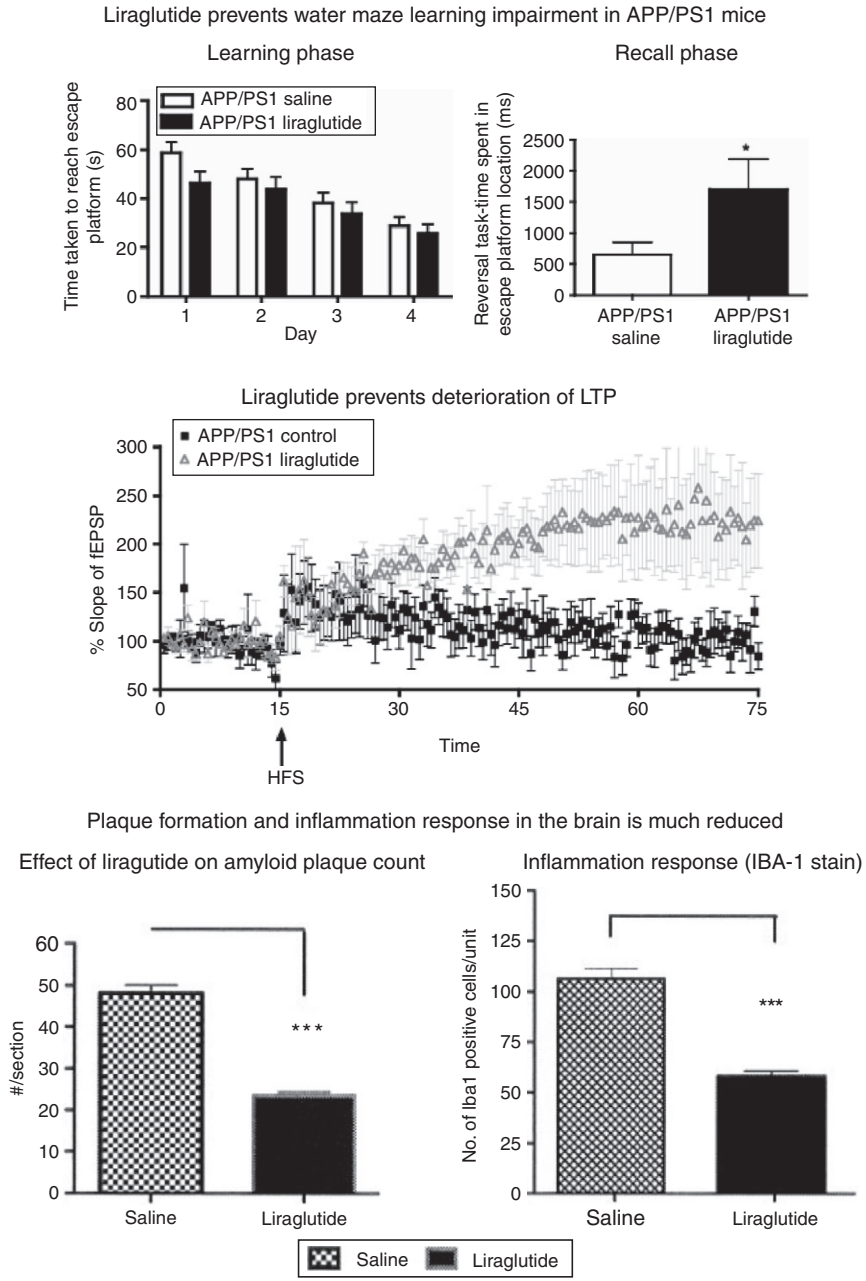

Figure 13.3 Injection of liraglutide 200 μg/kg b.w. i.p. once daily for 8 weeks greatly improved learning, LTP, reduced plaque formation, and inflammation response in the brain of 9-month-old APP/PS1 mice. App/PS1 mice are impaired as they get older, and the injection of liraglutide prevented the observed impairments in age-matched

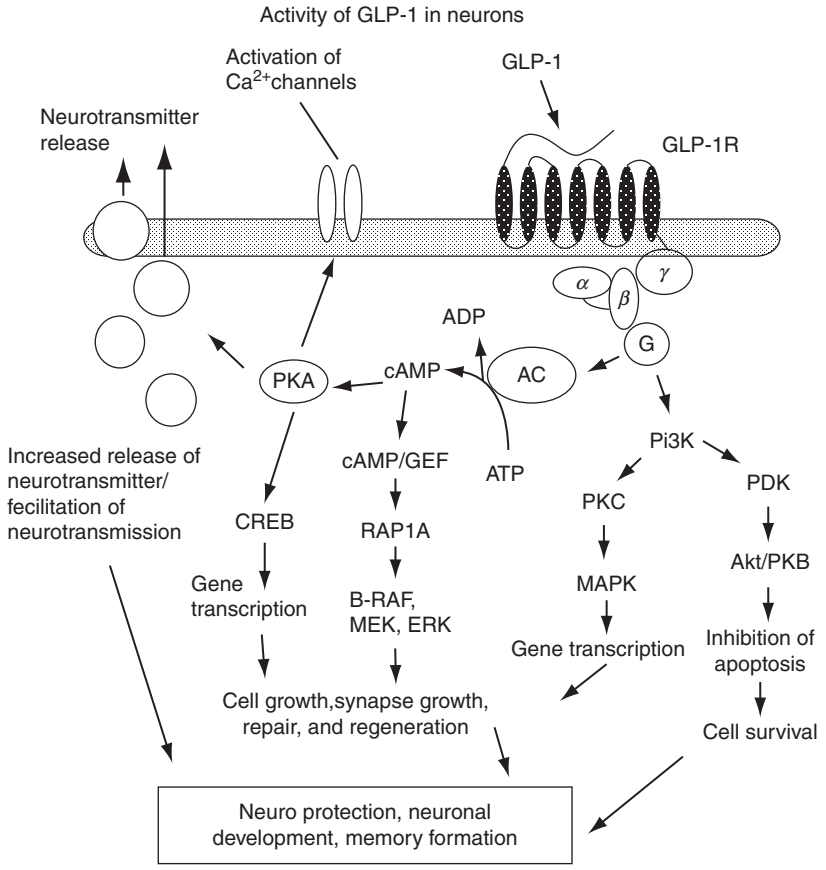

Figure 13.4 Overview of the main pathways induced by GLP-1 in neurons. As compared to Fig. 13.1, the overall mechanisms are very similar. The main physiological effects of GLP-1 on cell growth, proliferation, regeneration, and inhibition of apoptosis are identical. Differences can be seen in the control of vesicle release, which is glucose-dependent in β-cells but not in neurons. As shown in Fig. 13.5, activation of GLP-1R will not affect synaptic transmission directly since baseline transmission is not affected. A separate signal has to coincide with the GLP-1R activation, for example, the activation of neurons by high-frequency stimulation. Importantly, there is no effect on K_{ATP}+ channels that is linked to increased glucose uptake (e.g., via the GLUT4 transporter), and no direct effects of GLP-1 on K+ activity in neurons have been reported (Holscher and Li, 2008; see text for details).

controls. While the acquisition of the task was not impaired, APP/PS1 controls were not able to remember the location of the submerged platform on the next day. In contrast, liraglutide injected APP/PS1 animals were quite capable in recalling the location. Also, the inducibility of LTP in the hippocampus is reduced as the animals age, and this reduction is prevented by liraglutide. Furthermore, the numbers of plaques in the cortex and hippocampus and also the inflammation response in the brain as shown in the number of IBA-1 positive-activated microglia (McClean *et al.*, 2010a).

glucose levels, which will enhance ATP production in mitochondria via glycolysis. The increased levels of ATP affect ATP sensitive K+ channels in the cell membrane, which will reduce repolarization of the membrane, and therefore increase depolarization and enhance insulin vesicle release (Baggio and Drucker, 2007; Doyle and Egan, 2007; Perry *et al.*, 2003).

Interestingly enough, most neurons do not express the GLUT2 glucose transporter and only some neurons express the GLUT4 transporter (Arluison *et al.*, 2004). Consequently, glucose uptake in most neurons is not insulin dependent, and the glucose-induced modulation of K_{ATP}+ channels found in β-cells is not observed in neurons (Colom *et al.*, 1998). The exceptions of the rule are large neurons such as the pyramidal neurons in the hippocampus and in the cortex that have a very high metabolism and appear to need additional glucose uptake. They express the GLUT4 transporter and can increase their insulin-induced glucose uptake (Grillo *et al.*, 2009; Watson and Pessin, 2001). However, it is unlikely that neurons do express the same mechanism of high-glucose-induced enhancement of vesicle release, since this would interfere with neuronal communication.

F. GLP-1 analogues enhance memory formation

GLP-1 and longer acting analogues that can cross the BBB have beneficial effects on cognition. A behavioral study showed that the GLP-1 analogue exendin-4 can prevent the learning impairments induced by the injection of β-amyloid fragments (Oka *et al.*, 2000). Another study showed that GLP-1 when injected i.c.v. can enhance memory formation. The study also showed that the GLP-1 analogue Ser(2)exendin(1–9) can enhance learning of a spatial task when injected i.p., indicating that this analogue crosses the BBB (During *et al.*, 2003). The overexpression of the GLP-1 receptor also enhanced learning of a spatial task, while the deletion of GLP-1 receptor in KO mice impaired learning (During *et al.*, 2003). In a different study, GLP-1 receptor KO mice were impaired in learning spatial and recognition tasks, while LTP in the hippocampus was severely impaired (Abbas *et al.*, 2009). These results show that GLP-1 receptors do play an important role in cognitive processes in the brain, and that GLP-1 analogues can enhance learning even when injected i.p. (Fig. 13.5).

III. GLP-1 Analogues Have Neuroprotective Effects in Mouse Models of AD

Importantly, analogues of GLP-1 have neuroprotective effects in mouse models of AD. In one study, the GLP-1 analogue Val(8)GLP-1 had neuroprotective effects in a mouse model of AD. This mouse model

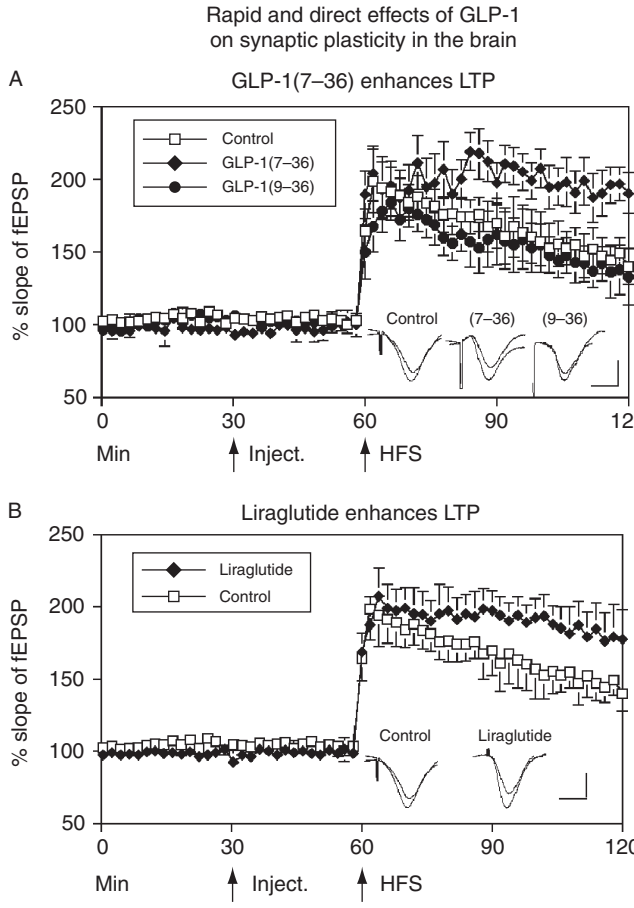

Figure 13.5 GLP-1 injected i.c.v. enhances weak LTP. Using a weak stimulation protocol, injection of (A) GLP-1-injected i.c.v. clearly enhanced LTP, while the inactive metabolite GLP-1(9–36) showed no effect. There was no difference between the GLP-1(9–36) group and the control group. (B) Injection of liraglutide i.c.v. (15 nmol/5 μl) also clearly enhanced LTP, demonstrating the effect of GLP-1R activation on neurotransmission. Averaged EPSPs are shown recorded 5 min pretetanus and 1 h posttetanus. Calibration bars are 10 ms horizontal, 1 mV vertical (for details, see Gault and Holscher, 2008; McClean *et al.*, 2010a). GLP-1 analogues can prevent the detrimental effect of β-amyloid on LTP.

overexpresses the human Swedish mutated form of APP and a human mutated form of presenelin-1. The mice quickly develop β-amyloid plaques in the cortex and hippocampus, starting at 2–3 months of age (Radde *et al.*, 2006). When injecting Val(8)GLP-1 chronically i.p. at a dose of 25 nmol/kg i.p. once daily for 3 weeks, synaptic plasticity in the hippocampus of these

mice was protected from the effects of plaque formation. LTP was completely preserved at 18 months of age compared to wild-type controls. In addition, the number of Congo red positive dense-core amyloid plaques in the brain was reduced. LTP was also protected in 18-month-old wild-type mice when compared to saline-injected wild types, indicating that Val(8)GLP-1 also protects from age-related synaptic degenerative processes (Gengler *et al.*, 2010) (see Fig. 13.6). The GLP-1 analogue exendin-4 which is currently on the market as a treatment of T2DM (Byetta®) had been tested in a triple transgenic mouse model of AD. This model also expresses the Swedish mutated form of human APP and a PS-1, and in addition expresses a mutated form of tau protein. The mice develop plaques at around 12 months. They also develop tangles similar to humans with AD. Exendin-4 was applied subcutaneously via osmotic pumps. To test the effects of a combination of diabetes and AD, a group of transgenic mice were made diabetic by injection of streptozotocin. This drug is taken up by β-cells and has cytotoxic effects. The main findings were that in the diabetic mouse model of AD, β-amyloid production had increased and plaque formation in the brain was enhanced. The treatment with exendin-4 treated the diabetes and reduced β-amyloid production and plaque formation (Li *et al.*, 2009).

In another study, the novel GLP-1 analogue liraglutide that is also on the market as a T2DM treatment (Victoza®) enhanced memory formation and synaptic plasticity in the brain after i.p. injection for 8 weeks (200 μg/kg b.w., once daily). The learning impairments observed in the APP/PS1deltaE9 mouse model of AD were reversed by liraglutide, and the impairment of hippocampal synaptic plasticity that develops over time in this mouse model was also prevented. More importantly, amyloid plaque formation was reduced to 50%, and the formation of Congo red dense plaques was reduced by half. In addition, the inflammation response (activated microglia) was also halved (see Fig. 13.3). Furthermore, neurogenesis was observed in the dentate gyrus of the brains (McClean *et al.*, 2010a). In this context, it is of interest to note that inhibitors of DPP-IV that prolong the half-life of endogenous GLP-1 in the body also have effects in AD mice. In the APP/PS1deltaE9 mouse model, the DPP-IV inhibitor sitagliptin administered orally for 12 weeks prevented impairment in a contextual fear condition task and produced reductions of nitrosative stress and inflammation hallmarks within the brain, as well as a reduction in plaque formation (D'Amico *et al.*, 2010).

These findings show that GLP-1 analogues do cross the BBB and have pronounced protective effects on the main hallmarks of AD as seen in mouse models. This suggests that treatment of patients that are developing AD with novel stable GLP-1 analogues would have the potential to prevent the early phase of neurodegeneration and potentially prevent the late phase

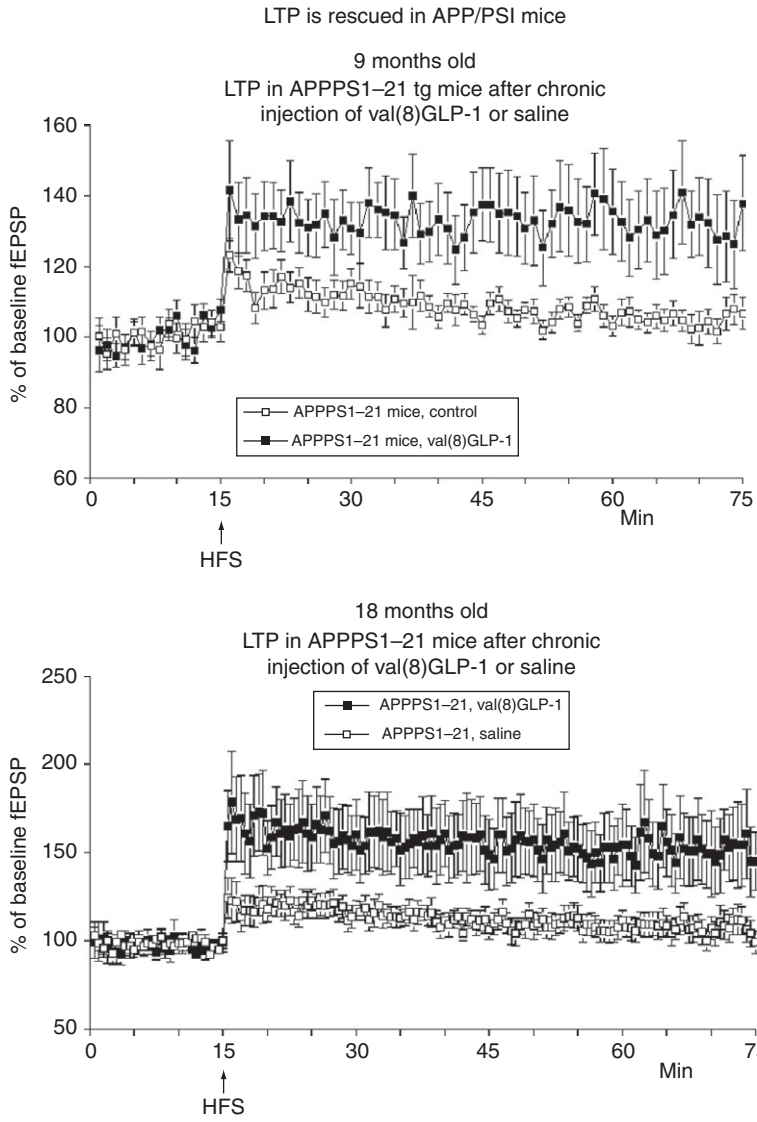

Figure 13.6 Val(8)GLP-1 protects LTP and learning abilities in a APP/PS1 mouse model of AD. Injection of 25 nmol/kg b.w. i.p. once daily for 3 weeks greatly improved LTP in the hippocampus of 9- and of 18-month-old mice (Gengler *et al.*, 2010).

of degeneration also. Importantly, clinical trials of the effects of exendin-4 in patients with AD are on the way now (Holscher, 2010) (see an update on www.clinicaltrials.gov).

IV. Many Other Growth Factors Show Neuroprotective Effects

The effects that GLP-1 analogues such as Val(8)GLP-1 or liraglutide have on memory formation and on the preservation of synapses from the detrimental effects of plaque formation are very similar to the neuroprotective effects of other growth factors. For example, brain-derived neurotrophic factor (BDNF) has been shown to protect synapses in mouse models of AD. Injecting BDNF i.c.v. improved cognition, prevented impairments of LTP, and led to an enhancement of hippocampal synaptic density (Blurton-Jones *et al.*, 2009). Increasing BDNF production in the brain by gene delivery vectors also had protective effects on synapses. Increase of BDNF levels, when administered after disease onset, reversed synapse loss, improved synaptic plasticity, and restored learning abilities of a mouse model of AD (Nagahara *et al.*, 2009; Poon *et al.*, 2009). The effects of BDNF are therefore very similar to those of Val(8)GLP-1 and liraglutide, with one vital difference: BDNF does not cross the BBB, and therefore a gene delivery system to the brain has to be developed, or it has to be injected directly into the brain (Schulte-Herbruggen *et al.*, 2007; Zuccato and Cattaneo, 2009). This clearly limits the application of BDNF as a treatment for AD. A different growth factor that has shown promise as a treatment for neurodegenerative disorders is nerve growth factor (NGF). Again, NGF was found to protect synapses, LTP, and learning abilities in AD mouse models or in nonprimate monkeys without affecting amyloid plaque load (Clarris *et al.*, 1994; Covaceuszach *et al.*, 2009; Kordower *et al.*, 1997). However, NGF does not cross the BBB either, and therefore gene delivery systems have been developed to be able to use NGF as a treatment of CNS disorders. Such attempts to increase the amount of NGF production in the CNS have not been successful so far (Bradbury, 2005; Covaceuszach *et al.*, 2009; Heese *et al.*, 2006; Schulte-Herbruggen *et al.*, 2007). Still, clinical trials are ongoing to test the effects of gene delivery via a viral vector when injected in the brains of patients, for example, by the company Ceregene (Mueller and Flotte, 2008). Other growth factors have similar protective effects on neurons in AD models, for example, insulin-like growth factor 1 (IGF-1) (Nagano *et al.*, 2005; Shi *et al.*, 2005), vascular endothelial growth factor (VEGF) (Azzouz *et al.*, 2004; Hwang *et al.*, 2009; Lee *et al.*, 2007), and glial cell line-derived growth factor (GDNF) (Aubert-Pouessel *et al.*, 2004). These growth factors have shown promising results in protecting neurons from the effects of β-amyloid, promoting cell repair, and protecting synaptic functions and cognitive performance. Again, the main stumbling block for these growth factors is the fact that they do not readily cross the BBB. As a consequence, special delivery systems are under development in order to deliver these growth factors,

which opens up a whole range of problems (Aubert-Pouessel *et al.*, 2004; Azzouz *et al.*, 2004; Gregory-Evans *et al.*, 2009; Hwang *et al.*, 2009; Sorenson *et al.*, 2008; Terzi and Zachariou, 2008).

The similarity of the activity profile of these different growth factors is surprising. It is possible that these growth factors activate similar or identical signaling mechanisms in neurons and synapses. Therefore, it is important to note that GLP-1 analogues such as Val(8)GLP-1 and liraglutide cross the BBB in a dose- and time-dependent fashion. At a dose that is effective in treating T2DM in a mouse model and also protects synaptic plasticity *in vivo*, Val(8) GLP-1 enters the brain and has physiological effects (Gengler *et al.*, 2010; Fig. 13.6). In addition, liraglutide had surprising effects in protecting an APP/PS1 mouse model of AD from the detrimental effects of amyloid plaque formation (Fig. 13.3). Since novel GLP-1 analogues are given to patients peripherally, and gene delivery systems are far away from having any practical application, the fact that analogues such as liraglutide and Val(8)GLP-1 cross the BBB makes such GLP-1 analogues a potential novel treatment for neurodegenerative diseases of the CNS. Further clinical trials in AD patients will have to be conducted to analyze the actual effects in humans.

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CHAPTER FOURTEEN

Wnt and Incretin Connections

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Contents

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Abstract

WNT signaling is emerging as a global regulator of metabolism, targeting multiple tissues. This is achieved either directly through Wnt receptors, or indirectly through the action of incretins, hormones that enhance glucose-stimulated insulin secretion and target extrapancreatic organs that cooperatively control whole body energy balance. WNT increases expression of incretins through evolutionarily conserved elements located within their proximal promoters. Wnt-responsive elements at the Incretin promoters may exhibit a high degree of selectivity for specific WNT effectors. Additionally, Incretins may modulate Wnt signaling and *vice versa*. Wnt-dependent modulation of incretin action in β-pancreatic cells is suspected because the expression levels of Incretin receptors correlate with those of the Wnt effector TCF7L2. Conversely, Wnt signaling is enhanced by Incretin binding to their receptors which induces cAMP accumulation followed by stabilization of the Wnt effector: β-catenin. High glucose and/or lipids control the number of Incretin receptors exhibited by β cells. Whether these nutrients and/or the Incretins control Wnt receptors (either their expression or signaling) remains to be further elucidated. Thus, Wnt controls the expression of incretins and modulate their signaling at pancreatic cells. Signaling by Wnt and incretins appears to be interconnected at multiple levels. The *in vivo* significance of incretin induction by Wnt is unknown as it is the nature and origin of Wnt signals in enteroendocrine cells but opens an intense research that promises many surprises; *in vitro* approaches may be used for mechanistic studies and animal models for physiological perspectives.

I. What Are Incretins, What They Do, Where, and How

A. The incretin effect

Intravenous glucose administration results in a much smaller pancreatic insulin secretion than oral administration (about 30%), suggesting a connection between the gastro intestinal (GI) tract and the pancreas. This observation led to the discovery of incretins, peptide hormones that sensitize pancreatic β cells and stimulate glucose-dependent production and secretion of insulin (Elrick *et al.*, 1964; McIntyre *et al.*, 1965). Incretins are released after nutrient ingestion by enteroendocrine cells scattered along the intestinal epithelium. Glucose-dependent insulinotropic peptide (GIP) is the major human incretin (75% of plasma levels) followed by glucagon-like peptide 1 (GLP-1) (Nauck *et al.*, 1993). In healthy individuals up to 60% of postprandial insulin release is due to effect of incretins (Nauck *et al.*, 1986b). GIP production and incretin effects predominate during the first hour postingestion and GLP-1 predominates afterward (Orskov *et al.*, 1994; Vilsboll *et al.*, 2003). The incretin connection between the GI tract and the pancreas led to the concept of an *enteroinsular axis* as an exocrine/endocrine unit. The enteroinsular axis—together with liver, adipocytes, muscle, and

bone—coordinates the digestion and absorption of nutrients, especially glucose and lipids, with their disposal. Nervous and endocrine connections with brain centers, especially in the hypothalamus, control whole body metabolism and energy balance. GIP and GLP-1 target the pancreas as well as these extrapancreatic tissues (Green and Flatt, 2007; Vella and Rizza, 2004) with overlapping or complementary actions cooperating to survey and control energy expenditure and metabolism. The actions of GIP and GLP-1 in these tissues are summarized in Table 14.1.

B. Incretin receptors and actions in the enteroinsular axis

The receptors for GIP (GIPR) and GLP-1 (GLP-1R) belong to the super family of seven transmembrane G(s) protein-coupled receptors. The coding sequences for rat, human, and hamster GIPR share 40–47% identity with those of GLP-1R (Usdin *et al.*, 1993; Yasuda *et al.*, 1994) and the crystal structure of the extracellular domains of the GIPR and GLP-1R bound to GIP or GLP-1 have been solved (Parthier *et al.*, 2007; Runge *et al.*, 2008; see also Lin and Wang, 2009). Upon incretin binding, signaling by incretin receptors increases intracellular cAMP to activate PKA, although other pathways such as PI3K are also activated in different cell types (Flamez *et al.*, 1999; Widenmaier *et al.*, 2009). Given their importance and their potential as therapeutic targets, the biology of these receptors is a matter of intense interest.

The actions of GIP and GLP-1 in the enteroinsular axis are complementary and not identical. In the pancreas, GIP and GLP-1 induce early and late β cell secretion of insulin, respectively. GIP also induces delayed α cell secretion of glucagon, while GLP-1 may inhibit glucagon secretion, although the presence of GLP-1R in α cells is controversial (de Heer *et al.*, 2008; Moens *et al.*, 1996). In the GI tract, GIP enhances GLP-1 production (Brubaker, 1991; Dumoulin *et al.*, 1995), while GLP-1 may inhibit secretion of GIP in rodents since mice deficient in GLP-1R show augmented secretion and insulinotropic function of GIP (Pederson *et al.*, 1998). However, while GLP-1R is expressed along the intestine (Pederson *et al.*, 1998), it has not been identified specifically in GIP producing K cells.

C. The GIP and GLP-1 genes

The human and rodent proglucagon (Gcg) genes have a conserved structure with six exons and five introns (Kieffer and Habener, 1999). Exons 1–2 comprise the 5′-untranslated region and signal peptide, whereas the last part of exon 2 to the beginning of exon 6 encode the prohormone proglucagon that will be processed differently in different cells to give rise to a variety of hormones among which are the three major peptide hormones: glucagon, GLP-1, and GLP-2 (Drucker, 2005; Sinclair and Drucker, 2005). The Gcg gene is expressed in intestinal L cells, α-pancreatic cells, and selected neurons in the brain (Kieffer and Habener, 1999).

Table 14.1 GIP, GLP-1, and Wnt target tissues

Impact	GIP	GLP-1	WNT/β-catenin
GI tract	– GLP-1 secretion in rodents (Herrmann-Rinke *et al.*, 1995)	– Gastric emptying – Gastric acid production – GIP secretion? (reviewed in Baggio and Drucker, 2007)	– Expression of GIP (Wnt3A) (Garcia-Martinez *et al.*, 2009) – Expression of GLP-1 (Ni *et al.*, 2003) – Maintenance of stem cell niche and others (see text)
Pancreas	– Insulin expression/secretion – Glucagon secretion – β cell mass (reviewed in McIntosh *et al.*, 2009)	– Insulin expression/secretion – β cell mass (reviewed in Nauck, 2009)	– Insulin expression/secretion? – β and α cell mass? – Incretin receptor expression? (Shu *et al.*, 2009; reviewed in Kubota *et al.*, 2009)
Liver	No receptors Indirect actions – Vasodilation of portal vein/ vasoconstriction of hepatic artery (Ding *et al.*, 2004) – Glycogenolysis (Hartmann *et al.*, 1986)	– Glycogenolysis (Ikezawa *et al.*, 2003) – Glycogen synthesis (Redondo *et al.*, 2003; reviewed in D'Alessio *et al.*, 2004)	– Metabolic zonation (Benhamouche *et al.*, 2006), regeneration, carcinogenesis (Gebhardt and Hovhannisyan, 2010) – Metabolical switch: inhibition of gluconeogenesis and induction of glycolysis (Chafey et al., 2009; Strathmann et al., 2007)
Adipose tissue	– Lipid deposition – Adipokine secretion (Yip *et al.*, 1998), reviewed (Weaver *et al.*, 2008)	– Lipogenesis and lipolysis (Sancho *et al.*, 2005) (Gao and Wang, 2007; Kim *et al.*, 2007)	– Adipogenesis (Wnt10b) (Longo *et al.*, 2002; Ross *et al.*, 2000; Wright *et al.*, 2007)
Bone	– Proliferation – Apoptosis osteoblast (Asmar and Holst; Bollag *et al.*, 2000)	– Osteoclasts (Yamada, 2009)	– Osteogenesis, reviewed (Kubota *et al.*, 2009)
CNS	Receptors in *cortex*, hippocampus, and olfactory bulb (Usdin *et al.*, 1993) – Proliferation in *Hippocampus* (Nyberg *et al.*, 2005) – Memory and motor function (Ding *et al.*, 2004)	Neurotrophic (Belsham *et al.*, 2009; Perry *et al.*, 2002) – Appetite and satiety at *Hypothalamus* (Christophe, 1998)	– Neurodegeneration (De Ferrari and Moon, 2006) – Wiring and axonal growth (Salinas and Zou, 2008) – Neuroprotection (Toledo *et al.*, 2008)

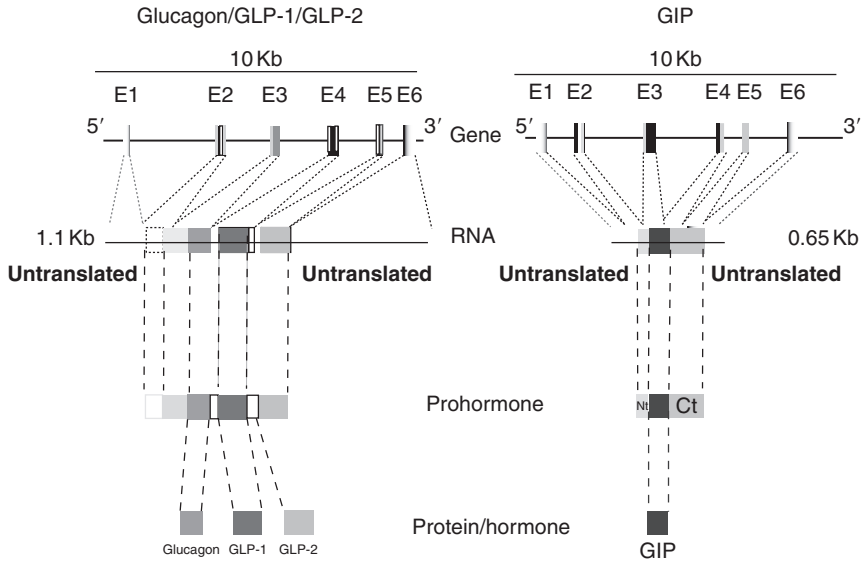

Figure 14.1 Comparison of Incretin encoding genes. Mouse genes encoding glucose-dependent insulinotropic peptide (mGip), and glucagon-like peptide 1 (GLP-1) transcribed from the Proglucagon gene (mGcg) with Ensemble database entry numbers: ENSMUSG00000014351 and ENSMUSG00000000394, respectively. Both mGcg and mGip expand a region of 10 kb and contain six exons numbered E1–E6. In both cases exons 1 and 2 codify for the 5′-untranslated region and leader peptide and E6 for the 3′-untranslated region. mGcg is transcribed into an RNA of 1.1 kb length and mGip into an RNA of 0.65 kb length. GLP-1 is encoded by E4 of the mGcg gene and GIP sequence is encoded by exons 3–4 in the mGip gene. In both cases the mRNA is translated into a preprohormone that is cleaved into several peptide products some of them with known hormonal activity.

The human and rodent GIP genes are also structurally conserved, again comprising six exons and five introns (Inagaki *et al.*, 1989). The precursor preproGIP is encoded by exons 2–5 and is processed to give the active peptide ($GIP_{1–42}$) which is encoded by exons 3–4 (Inagaki *et al.*, 1989). The GIP gene is expressed in intestinal K cells, the submandibular salivary gland, stomach, and selected neurons in the brain (Kieffer and Habener, 1999; reviewed in McIntosh *et al.*, 2009). Figure 14.1 presents a scheme of the mouse Gcg and Gip genes.

D. Incretin response in health and disease

Consistent with their central role in metabolism, incretin signaling is altered in animal models and human patients with metabolic disorders related to obesity and diabetes. GIPR-knockout mice are resistant to diet-induced

obesity and associated risk of diabetes (Hansotia *et al.*, 2007; Miyawaki *et al.*, 2002; Yamada *et al.*, 2007). Chronic desensitization of GIP receptor and hyperGIPemia progressively increases in the Zucker obese (Chan *et al.*, 1984), JCR:LA-cp (hyperinsulinemic and obese) (Pederson *et al.*, 1991), and streptozotocin-induced diabetic rats (Tseng *et al.*, 1996). Similarly, human diabetic and/or obese patients have increased plasma GIP levels after an oral glucose load (Brown and Otte, 1979) as well as increased fasting responses to GIP (Jones *et al.*, 1989a,b). The pancreatic sensitivity to GIP insulinotropic effects is reduced or lost in type 2 diabetes patients and their first-degree relatives (Knop *et al.*, 2007; Meier *et al.*, 2001; Nauck *et al.*, 1986a), while the response to GLP-1 is conserved, even though a small reduction in GLP-1 production has been described (Vilsboll *et al.*, 2001). Thus, a progressive enteroinsular communication failure starting with altered GIP synthesis and function may underlie the onset of type 2 diabetes. GIP and GLP-1 production and signaling must therefore be carefully controlled to avoid metabolic disorders such as diabetes.

Given the key roles for GIP and GLP-1 in regulating the response to glucose intake, understanding what controls the regulation of these genes is a key issue. Recently, it has become evident that expression of GIP and GLP-1 by enteroendocrine cells is governed by canonical Wnt/β-catenin signaling. Moreover, the levels of GIPR and GLP-1R in β-pancreatic cells reflect the levels of the Wnt effector Tcf7l2 (Shu *et al.*, 2009). The connection between the Wnt signaling pathway and GIP and GLP-1 expression is underscored by the fact that the strongest genetic link for diabetes relies on certain variants of the Wnt effector Tcf7l2. Taken together, these data strongly support a previously unsuspected role for altered Wnt/β-catenin signaling in metabolic disorders such as diabetes.

II. WNTs: What They Are and What They Do

A. WNT ligands

The Wnts comprise a large family of secreted cys-rich glycoprotein ligands that coordinate cell fate decision making in a broad range of developmental and homeostatic contexts (Mikels and Nusse, 2006). Many cells, particularly undifferentiated or inflammatory cells and adipocytes, secrete WNT proteins. WNTs act at short distances as autocrine or paracrine signals, and accumulating evidence suggests the existence of soluble Wnt molecules able to act over long distances in an endocrine fashion (Berndt *et al.*, 2003; Brack *et al.*, 2007; Liu *et al.*, 2007; Tian *et al.*, 2003). Wnt ligands may bind different types of membrane surface receptors: the G protein-coupled receptors of the Frizzled (Fzl) family, or the single-pass transmembrane receptors of the "low-density lipoprotein-related protein" (lrp) family.

Different Wnt molecules bind to one receptor type, the other or both, with Fzl-lrp coreceptors activating different signaling pathways (see below), and can be stimulatory, for example, Wnt3A, Wnt5A depending on the cell type, or inhibitory, such as Dkk1, sFrp.

B. WNT pathways

The intracellular pathways activated by Wnt ligands are classified as β-catenin-dependent (or canonical) and β-catenin-independent pathways, reviewed by Komiya and Habas (2008). The canonical, β-catenin-dependent pathway relies on WNT binding to Fzl-lrp coreceptors and is by far the best understood. The two main branches of the β-catenin-independent pathways are the *Wnt/Ca+2 pathway*, dependent on G-protein association to Fzl receptors, and the *planar cell polarity* (PCP) pathway which relies in lrp coreceptors other than lrp5/6 and which leads to transcription-independent actin cytoskeleton reorganization that determines cell polarity and migration. In the best understood WNT canonical pathway, WNT interaction with Fzl-lrp5/6 coreceptors at the cell surface recruits the scaffolding proteins disheveled and axin to dismantle the so-called destruction complex. This complex contains several kinases that act sequentially to prime proteins for proteasomal degradation. At the core of the "destruction complex," glycogen synthase kinase 3β (GSK3β) constitutively phosphorylates β-catenin and other factors, priming them for degradation. Dismantling the "destruction complex" results in accumulation of proteins like β-catenin.

C. WNT effector

Wnt-induced β-catenin stabilization leads to its entry into the nucleus where it binds members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. These include TCF7 (formerly known as TCF1), LEF1, TCF7L1 (formerly known as TCF3), and TCF7L2 (formerly known as TCF4) activating transcription of target genes mainly involved in proliferation. Thus, β-catenin acts as an intracellular cofactor that activates TCF/LEF factors in a WNT-dependent fashion. Understanding the outcome of Wnt signaling is complicated by the fact that a given cell type may contain multiple TCF/LEF members. Moreover, the diversity of effectors is multiplied by different promoter usage, and alternative splicing generates multiple LEF/TCF factor isoforms with differing transcriptional properties. The relative proportions of different isoforms will define the output of Wnt signaling. TCF7L2 followed by LEF1 are the most abundant through the adult mouse intestine, but Wnt3A signaling upregulates Lef1 (Driskell *et al.*, 2007) and TCF7L2 binds and transactivates the Lef1 promoter in colorectal carcinoma (Hovanes *et al.*, 2001). Two different

isoforms of LEF1 result from usage of two alternative promoters, and up to 13 isoforms of TCF7L2 might result from alternative splicing (reviewed in Waterman, 2004; see also Prokunina-Olsson *et al.*, 2009). Some LEF1 and TCF7L2 isoforms lack the β-catenin-interacting domain and behave as constitutive repressors, and different factors and variants will exhibit different interactions and properties. TCF7L2 and LEF1 bind the same DNA elements and a number of common cofactors including β-catenin and histone deacetylases. A major distinguishing feature of TCF7L2 is a C-terminal repressor domain that interacts with carboxy-terminal-binding proteins 1 and 2 (Ctbp1 and Ctbp2) which is absent from LEF1. Importantly, TCF7L2 appears to modulate GLP-1 production, GLP-1 pancreatic actions on β cells, and also controls adipogenesis.

D. Cross talk

In addition to β-catenin-dependent and -independent pathways, Wnt ligands have been reported to activate MAPK pathways: Ras-Raf-MEK-ERK in fibroblasts (Yun *et al.*, 2005) and the stress-activated p38 MAPK in mesenchymal cells (Caverzasio and Manen, 2007; Chang *et al.*, 2007). p38 in turn can modulate Wnt signaling through phosphorylation and inhibition of GSK3β (Bikkavilli *et al.*, 2008; Thornton *et al.*, 2008). TCF/LEF, β-catenin, GSK3β, and upstream components are targeted by other pathways serving as integrative nodes in a cell-specific signaling network. For example, GSK3β is targeted and inactivated by multiple signals including Wnt signaling, PKA (Liu and Habener, 2008; Suzuki *et al.*, 2008), PI3K/Akt (Naito *et al.*, 2005; Sharma *et al.*, 2002) as well as p38 MAPK as stated before. In addition, β-catenin itself might be targeted by PKA or GSK3β (Hino *et al.*, 2005) leading to opposing effects on its stability, as well as deacetylases of the sirtuin family (Firestein *et al.*, 2008), and acetylases like CBP/p300 (Hecht *et al.*, 2000; Levy *et al.*, 2004; Li *et al.*, 2007) that affect its localization. Moreover, β-catenin binds a variety of transcriptional effectors targeted by other pathways such as TGFβ, PI3K/Akt, JNK, PKA, or ERK, for example, the Smads (Letamendia *et al.*, 2001), FoxO1 (Essers *et al.*, 2005; Hoogeboom *et al.*, 2008; Wang *et al.*, 2007), or CBP/p300 (Li *et al.*, 2007; Wolf *et al.*, 2002). Thus, cross talk sustained by these integrative nodes provides an opportunity to tune and coordinate levels of transcriptional activation/silencing and its duration in a cell-specific manner. This concept is illustrated by the competition between FoxO and TCF/LEF factors for β-catenin binding that leads to decreased Wnt/β-catenin signaling which is important for glucose and lipid metabolism, β cell mass and function, and for incretin production. Insulin-induced cytosolic relocation of FoxOs may then restore Wnt/β-catenin signaling, reviewed by Jin and Liu (2008). Conversely, Sirtuin accumulation after caloric restriction relocates β-catenin to the cytosol but retains FoxO at the nucleus.

E. Physiological actions

Wnt signaling is a global regulator of embryonic development, being required for tissue renewal in postembryonic animals and when corrupted may promote unrestricted cell growth and cancer. For example, constitutive activation of the Wnt signaling pathway may lead to colorectal cancer or hepatocellular carcinoma in mice and humans (Kinzler and Vogelstein, 1996; Phelps *et al.*, 2009; Gao and Yao, 2009; Merle *et al.*, 2005; Laurent-Puig and Zucman-Rossi, 2006). WNT signaling is necessary for Paneth cell maturation (van Es *et al.*, 2005) and the development and homeostasis of tissues from the digestive tract are particularly dependent on Wnt/β-catenin (Clevers, 2006). Wnt signaling is vital for maintenance of the stem cell niche (Korinek *et al.*, 1997) and intestinal glands because inhibitors of Wnt signaling such as *DKK1* block proliferation in the small intestine and colon and promote degeneration of the *intestinal* glands (Kuhnert *et al.*, 2004). A proximal to distal gradient of Wnt-activated cells has been defined using Wnt-reporter mice (Davies *et al.*, 2008).

Remarkably, Wnt also induces incretin expression (GIP and GLP-1) in enteroendocrine cells (Garcia-Martinez *et al.*, 2009; Yi *et al.*, 2005), suggesting that Wnt signaling plays an important role in differentiated cell function as well as regulating stem cell activation. As mentioned above, the recent discovery of polymorphism variants in Tcf7l2 that represent the strongest genetic link to diabetes (Saxena *et al.*, 2006; Florez *et al.*, 2006; Grant *et al.*, 2006; reviewed in Lyssenko, 2008) has fueled interest in the role of Wnt signaling in the enteroinsular axis. Since then it has been shown that Wnt molecules govern both pancreatic β cell proliferation and insulin secretion (Rulifson *et al.*, 2007; Schinner *et al.*, 2008; Shu *et al.*, 2008) with the Wnt coreceptor lrp5 and β-catenin being necessary to mediate these responses (Fujino *et al.*, 2003; Murtaugh *et al.*, 2005). The incretin GLP-1 signals through Wnt effectors in the β cell (Liu and Habener, 2008). Thus, endocrine pancreas development and mature β cell functions including insulin secretion, survival, and proliferation are controlled by Wnt signals (Welters and Kulkarni, 2008).

The global control exerted by Wnt in the enteroinsular axis suggests that it might be a master controller for whole body metabolism. The extrapancreatic actions of Wnt signaling related to metabolism include targeting multiple tissues that are also targeted by incretins (see Table 14.1), raising the possibility of Wnt and incretin cosignaling in the same cells. Thus, liver and adipocytes play a central role in energy balance. In hepatocytes Wnt is essential for zonation (Benhamouche *et al.*, 2006) and for the metabolic switch leading to inhibition of gluconeogenesis and activation of glycolysis (Chafey *et al.*, 2009; Strathmann *et al.*, 2007). Wnt is also critical for the control of cholesterol metabolism through lrp receptors other than lrp5/6 (May *et al.*, 2007; Terrand *et al.*, 2009). In adipocytes, Wnt agonists and

antagonists drive adipocyte differentiation (Christodoulides *et al.*, 2006; Longo *et al.*, 2002; MacDougald and Mandrup, 2002; Ross *et al.*, 2000) and importantly, adipocytes themselves secrete Wnt ligands (Schinner *et al.*, 2007, 2009). Indeed, neighboring adipocytes might be the source of Wnt ligands for enteroendocrine or β-pancreatic cells, and the possibility that soluble Wnt ligands may act systemically in an endocrine fashion (Brack *et al.*, 2007) deserves further exploration. Consistent with this, an increasing number of publications in the past 5 years indicate a prominent role for Wnt signaling in endocrine and metabolic processes especially signaling modulation through lrp receptors (May *et al.*, 2007; Terrand *et al.*, 2009).

In addition, while Wnt signaling plays a prominent role in the control of energy balance at almost every level, either alone or cosignaling with incretins, altered Wnt signaling is altered in both metabolic diseases and cancer. Indeed the increased frequency of cancers in obese and/or diabetic patients suggests that deregulation of the molecular mechanisms underlying the onset of diabetes may be linked to certain types of cancer, such as colorectal (Gerber, 2009) or hepatocarcinoma (Gao and Yao, 2009) where deregulation of Wnt signaling is a prominent contributory factor.

III. WNT/β-catenin Increases the Synthesis of Incretins

The emerging evidence suggest that Wnts play a central role in coordinating body-wide metabolism of the GI tract, pancreas, liver, adipose tissue, muscle, bone, brain both directly through different Wnt receptors and indirectly via the induction of the incretin genes for GIP and GLP-1 that control insulin secretion and circulating levels of glucose and fat. Beyond their importance in the control of energy balance, GIP and GLP-1 may be considered differentiation markers for K and L intestinal cells, respectively. The finding that WNT ligands induce GIP and GLP-1 expression in enteroendocrine cells suggests that Wnt signaling plays a role in the maintenance of enteroendocrine differentiation.

A. WNT/β-catenin induces Gcg expression for GLP-1 production

The group of Jin reported in 2003 that lithium used the Wnt effector β-catenin to induce rat proglucagon (Gcg) gene transcription (Ni *et al.*, 2003). The authors identified an element in a region of the rat Gcg promoter known as the "G2 enhancer" (Fig. 14.2), which mediates lithium induction specifically in an enteroendocrine cell line named GLUTag. GLUTag cells derive from a glucagon-producing enteroendocrine cell tumor that arose in

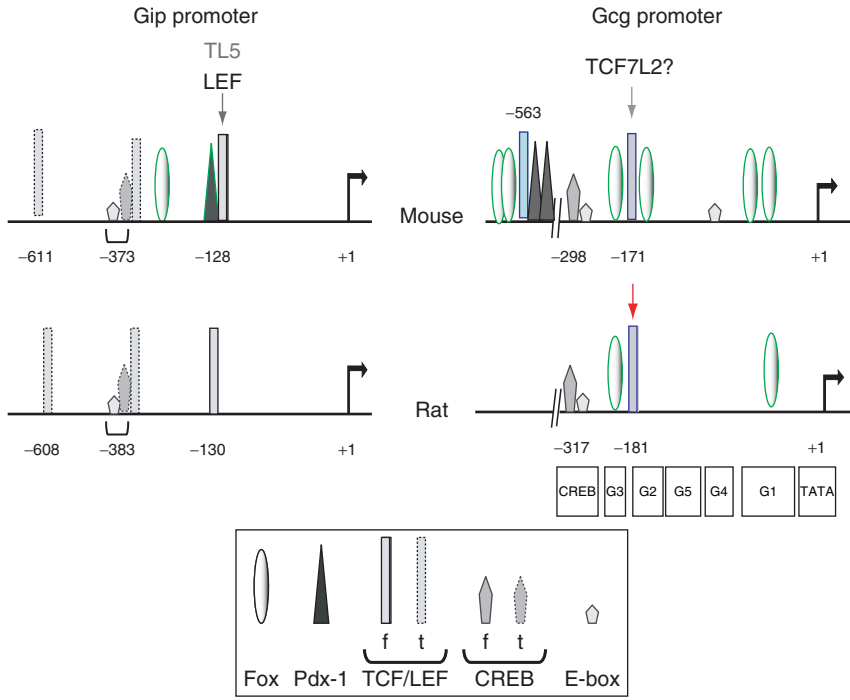

Figure 14.2 Comparison of the 5′flanking regions of incretin genes in rodents. The proximal region of the promoters for mouse and rat *Gcg* and *Gip* is depicted in the scheme. Theoretical transcription factor binding sites for the Wnt effectors TCF7L2 and LEF1, for nutritionally regulated FoxO (elipse) and Pdx-1 (triangle) and the well characterized CREB element in Gcg are presented; an E-box is intriguingly associated to CREB sites in the 4 promoters and is also presented. Dotted contour in TCF/LEF and CREB indicates that functionality of that site has not been proved. The functional LEF1 binding site in mGip promoter (TL5) is marked with an arrow. The equivalent site in the mGcg promoter is marked as TCF7L2? because binding of LEF1 is not discarded. For Gip promoter, the biological significance of conserved CREB theoretical sites (dotted pentagon) is uncertain. The squares underneath the rat Gcg promoter derive from Jin (2008) and serve to refer all the elements cited in this review to the previously characterized elements in the rat Gcg promoter.

transgenic mice bearing glucagon gene-SV40 T antigen (Drucker *et al.*, 1994). Lithium induction in GLUTag cells of a chimeric G2 enhancer fused to the thymidine kinase (TK) minimal promoter demonstrated the functionality of the promoter element in luciferase assays. Lithium-dependent increased binding of β-catenin-TCF7L2 to a chromatin region containing this element was demonstrated by the same group (Yi *et al.*, 2005). Additional studies have been performed in another enteroendocrine cell line named STC-1. STC-1 cells derive from an intestinal tumor of a transgenic mice expressing viral oncogenes (Rindi *et al.*, 1990) and express and secrete

GIP as well as other intestinal hormones (Boylan *et al.*, 1997; Kieffer *et al.*, 1995). This mixed secretory capacity might be viewed as a complication for analysis or as representative of the natural environment of enteroendocrine cells. STC-1 cells are equipped with coreceptors, intermediaries, and final Wnt effectors (β-catenin, TCF7L2, Lef1) that mediate canonical Wnt signaling, and have been successfully used to study control of mGip and mGcg expression (Garcia-Martinez *et al.*, 2009). In STC-1 cells, a mouse Gcg promoter-luciferase reporter lacking the TK sequence (that may mask transcriptional effects) confirmed the ability of β-catenin to activate Gcg expression, and also detected a replacement of the corepressor HDAC1 by the coactivator β-catenin at the defined site. This study also identified an additional upstream higher affinity TCF/LEF-binding site in the mouse Gcg promoter surrounded by elements that theoretically bind nutritionally controlled factors (CGJ unpublished observations). A site for binding of the cyclic AMP-response factor CREB lies approximately equidistant between the two Gcg TCF/LEF elements. In this respect, it is particularly interesting that increased binding of β-catenin to the rat Gcg promoter may also result from cAMP/PKA stimulation which can inhibit GSK3β and stabilize β-catenin in GLUTag cells (Yi *et al.*, 2005). Thus, the CREB and TCF/LEF sites may cooperate in activation of Gcg in response to cAMP signaling, while β-catenin integrates different environmental signals (Wnt, cAMP/PKA inducers) toward an output: increased production of Gcg mRNA to produce GLP-1. Figure 14.2 shows a comparison of the mouse and rat incretin promoters depicting Wnt-responsive TCT/LEF sites, as well as the CREB and other nutritionally regulated sites.

B. WNT/β-catenin induces GIP production

A sequence element, TL5, in the mGip promoter mediates a potent induction by Wnt or lithium in enteroendocrine STC-1 cells (Garcia-Martinez *et al.*, 2009). While both TCF7L2 and LEF1 bind *in vivo* a region containing the TL5 element in unstimulated cells, Wnt or lithium stimulation favors binding of LEF1/β-catenin and replacement of TCF7L2 and HDAC1, leading to transcriptional activation. Even though both, TCF7L2 and LEF1 recognize the same sequence, LEF1 is preferred as an activator at the mGip promoter, implying a highly selective binding requirement given that LEF1 appears to be less abundant than TCF7L2 in enteroendocrine cells (our unpublished observations). Thus, mGip promoter offers an excellent model to study the ill-defined mechanisms that facilitate the selection of a specific TCF/LEF family member at a defined promoter element. These data suggest that LEF1 acts as a transcriptional activator upon binding of its cofactor (β-catenin) while TCF7L2, which unlike LEF1 contains a c-terminal repressor domain that bind Ct-binding proteins 1 and 2, behaves as a repressor. Two additional binding sites for TCF/LEF factors are found

in the first intron of mGip gene named "TL1 and 2." Although these sites display a strong affinity for STC-1 extracts in *in vitro* assays and can mediate a small induction by Lithium, their significance is currently unknown. The intriguingly similar induction of both GIP and GLP-1 incretins by Wnt signaling is suggestive of underlying architectural similarities between their promoters. Functional TCF/LEF sites at incretin promoters are located close to the transcriptional start site. Mouse, rat, and human TCF/LEF binding sites in *Gip* occupy positions -138, -138, and -141, respectively, and positions -170, -181, and -189 in mouse, rat, and human *Gcg*, respectively. Interspecies comparison of the reported TCF/LEF sites at the *Gcg* promoters shows one conserved substitution from mouse to rat and identity from rat to human. TCF/LEF sites at Gip promoters are identical between mouse and rat and two conserved substitutions are found at the proximal human promoter.

Although the uninduced *mGip* and *mGcg* promoters bind both TCF7L2 and LEF1, the *mGip* promoter is highly selective for LEF1 upon activation, something that has not been observed at the site reported for the Gcg promoter. Differences in the TCF/LEF sequence ^{1}AAT/AGTTTCC^{9} at *Gcg* versus that of *Gip* ^{1}GA/TGC/GAAAGG^{9} may account for differential selectivity and *in vitro* EMSA experiments with purified factors may help to clarify this point. Alternatively, promoter context could provide additional cooperative factors/interactions that may differentially stabilize TCF7L2 or LEF1. The human *Gip* promoter bears several theoretical TCF/LEF-binding sites of which a proximal and a distantly located site show high affinity *in vitro* for TCF/LEF from STC-1 cells (our unpublished observations). Perhaps gene-looping favors interactions between proximal and distant factors. Although, further studies are necessary to assign the functional importance of these sites, these data highlight the role of Wnt signaling in the regulation of *Gip* and *Gcg* expression. However, the TCF7L2 to LEF1 switch raises several important issues that may influence the response of enteroendocrine cells to Wnt signaling, particularly the ratio of expression between the different LEF/TCF family members and their isoforms and their relative affinities for the different elements and cofactors in response to either Wnt or cAMP signaling. Preliminary results from our lab suggest that LEF1 and β-catenin may be targeted by different signals to form the bipartite transcriptional activator β-catenin-LEF1. Coincidence of environmental signals targeting β-catenin with those targeting LEF1 will lead to the formation of the transcriptional activator β-catenin-LEF1 that may be viewed as a detector of coincidence, the real signal for Gip expression. The *in vivo* induction of LEF1 in K or L cells in response to Wnt or other signals should be explored further using some of the transgenic models described at the end of this review.

Although it is now clear that incretin expression is regulated by Wnt ligands such as Wnt3a, the origin and nature (agonist or antagonist) of the

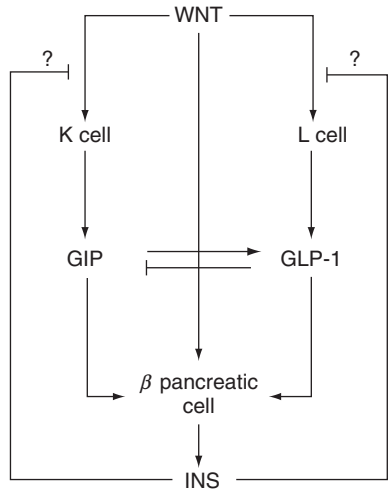

Figure 14.3 Simplified interplay between Wnt and incretins at the enteroinsular axis. Wnt signaling may induce insulin expression and secretion either directly acting on pancreatic β cells which are equipped with Wnt receptors and effectors or indirectly through increased expression of incretins in enteroendocrine cells which target pancreatic β cells to enhance glucose stimulated insulin secretion. Incretin actions on β cell proliferation may be mediated through Wnt effectors. Wnt may also play a role in incretin secretion as it does in insulin secretion. Wnt effectors also mediate incretin signaling in the β-pancreatic cell. We propose that plasma insulin may interfere with Wnt induced incretin production (marked as "?"). In rodents GIP induces GLP-1 secretion and GLP-1 may interfere with GIP secretion because in GLP-1R deficient mice GIP secretion is augmented.

Wnt signals that stimulate *in vivo* enteroendocrine cells as well as the existence and identity of Wnt modulators remain to be elucidated. Figure 14.3 summarize a simple WNT and incretin interplay that might be further complicated by the influence of nutrients. Given that nutrients are the best understood inducers of incretins, it is possible that some nutrients or sub-products of their trafficking/digestion/metabolism mimic WNT ligands or modulate WNT signaling in enteroendocrine cells, for example, lipid binding through lrp receptors. Alternatively, nutrients may induce Wnt secretion by neighboring cells to stimulate enteroendocrine incretin production. Animal models have revealed an association of WNTs with obesity (Christodoulides *et al.*, 2006). For example, Wnt10b transgenic mice (Longo *et al.*, 2004; Wright *et al.*, 2007; Aslanidi *et al.*, 2007) and $lrp5^{-/-}$ mice are resistant to obesity when fed a high fat diet, as are mice with a targeted deficiency for GIPR. However, further work is necessary to unravel how obesity or caloric restriction might impact on Wnt signaling to enteroendocrine cells, or how cosignaling by Wnt–incretins, Wnt–insulin, and Wnt–nutrients may impact adipocytes.

C. Promoter context: The importance of having interesting neighbors

As mentioned above, the β-catenin-TCF7L2-binding site at the *mGcg* promoter is located near a conserved binding site for the cAMP response factor CREB located at position -317 (see Fig. 14.2; Brubaker *et al.*, 1998; Miller *et al.*, 1993). PKA activation induces mouse *Gcg* expression in STC-1 cells most likely via the CREB-binding site in the *mGcg* promoter (our unpublished observations). However, overexpression of the catalytic subunit of PKA did not increased nuclear β-catenin levels in STC-1 cells (unpublished observations), as compared to the increase obtained with lithium, or as it has been described in GLUTag cells (Yi *et al.*, 2005). Furthermore, cooperation between PKA and Wnt could be seen at the *mGcg* promoter only in suboptimal conditions, otherwise each of them led to maximum activity separately (our unpublished results). It is tempting to speculate that activation might be reached by recruitment of the p300 acetyl transferase to the promoter either through CREB or through β-catenin-TCF/LEF binding. Recruitment and positioning leading to transcriptional activation would represent a common target of PKA and Wnt with suboptimal activation of each serving to amplify the effects of the other, thereby ensuring *Gcg* expression. Thus, cooperation between Wnt and PKA seems to be a recurrent theme in endocrine cells, either through inhibition of GSK3β, stabilization of β-catenin, or recruitment of p300.

In silico, analysis of the *mGcg* and *mGip* promoters (see Fig. 14.2) reveals the presence of theoretical binding sites for transcription factors of the FoxO family, known to interact with β-catenin or p300 (unpublished observations). FoxO factors are controlled by insulin and/or glucose, both of which are major regulators of Gcg expression and potentially also of Gip, and may play critical roles integrating environmental signals at these promoters. Similarly, both promoters contain Pdx-1-binding sites in the vicinity of the TCF/LEF sites. FoxO1 and Pdx-1 nuclear localization seem to be mutually exclusive (Kawamori *et al.*, 2006), but the relationship between these factors and TCF/LEF at the promoters of the incretins remains to be elucidated. However, FoxO1 is known to interact with β-catenin and both FoxO1 and Pdx-1 interact with p300, raising the question as to whether a direct or indirect LEF1–β-catenin–FoxO1 interaction on the DNA is possible and whether it would affect the binding of Pdx-1.

IV. Does WNT Influence Incretin Secretion?

Wnt signaling clearly regulates incretin expression, but does it also regulate their secretion? The control of incretin secretion is poorly understood. Nutrient passage through the intestine is the major stimulant of incretin

secretion, but neural or hormonal signals may also mediate the response. Incretin-secreting cells are polarized and may bear different nutrient sensors at the luminal and basal membrane to detect lumen and plasma levels of nutrients. Incretin secretion requires a rise in cytoplasmic calcium concentration, probably involving voltage-gated calcium channels (Ca_V) like insulin secretion by β cells. Ca_V channels sense membrane depolarizations originating from a variety of "nutrient sensors" including ATP-sensitive potassium (K_{ATP}) channel closure (metabolic balance), sodium-glucose cotransporter (SGLT) activity, or activation of sweet taste receptors (see Tolhurst *et al.*, 2009 for a review). All these sensors are present in K cells in primary cultures obtained from transgenic mice that bear fluorescently labeled K cells (Parker *et al.*, 2009). Experiments with nonmetabolizable sugars indicate that SGLT cotransporters are crucial for glucose sensing. Lipid sensing receptors: G protein-coupled lipid receptors Gpr40, Gpr119 and Gpr120 are also abundantly expressed in K cells (Parker *et al.*, 2009) and a number of lrp receptors are also expressed in STC-1 cells (our unpublished observations).

Insulin secretion by pancreatic β cells is stimulated by Wnt, which has been recently shown to control glucokinase (Schinner *et al.*, 2008). Glucokinase is a classical glucose sensor in pancreatic β cells and is also present in enteroendocrine cells, suggesting that Wnts are also part of the nutrient sensor. Moreover, fats are strong secretagogues for incretin-producing cells and have been recently shown to modulate Wnt signaling through binding to lrp receptors. lrp receptors contribute to the nutrient sensor by binding of either lipids or Wnt activating/inhibiting ligands. For example, lrp5 mediates Wnt5 signaling and is essential for glucose-induced insulin secretion (Fujino *et al.*, 2003) in the pancreas. In fact, lrp1 binds more than 30 ligands, interacts with many cytosolic adaptors (Lillis *et al.*, 2008), and modulates Wnt signaling (Terrand *et al.*, 2009), illustrating the enormous versatility of these receptors. Several lrp receptors are abundantly expressed in STC-1 cells (our unpublished observations) and further research should clarify whether modulation of Wnt signaling through Fzl, lrp, or Gpr receptors may also be a signal for incretin secretion.

V. Does WNT Influence Incretin Receptors and/or Their Signaling?

GLP-1R bound by GLP-1 increases cAMP levels through Gαs-adenylyl-cyclase coupling and this leads to accumulation of the WNT effector β-catenin to promote β cell survival and proliferation. GIPR signaling in pancreatic cells also raises cAMP levels (Wheeler *et al.*, 1999) and promotes proliferation and could, therefore, potentially activate Wnt effectors in a

similar way to GLP-1R. How activation of these receptors is modulated by the presence of Wnt ligands has to be elucidated. Hyperglycemia and hyperGIPemia induce homologous desensitization (Lynn *et al.*, 2003; Tseng and Zhang, 2000) by internalization of the receptor, probably uncoupling it from possible Wnt intermediaries. Lipids also desensitize GIPR in hyperglycemia and it would be interesting to examine the interactions between lipid and GIP signaling with that of Wnt signaling. To date, WNT ligands that affect expression or signaling by incretin receptors either in pancreas or in extrapancreatic tissues have not been reported. TCF7L2 risk alleles associated with diabetes have been reported to correlate with low expression of the factor in pancreatic islets and low expression of GIPR and GLP-1R (Shu *et al.*, 2009), and silencing Tcf7l2 also diminishes the expression of both GIPR and GLP-1R. Moreover, TCF7L2 is associated to hyperglycemia (diabetes), which in turn downregulates GIPR and GLP-1R. Hyperglycemia then may reduce the activity of TCF7L2 and as a consequence result in low incretin receptor expression or may cause independently a reduction of expression levels and desensitization. Beyond these correlations, the mechanism by which TCF7L2 governs the expression of incretins given their importance as therapeutic targets needs to be explored further.

VI. Do Incretins Influence Wnt Signaling? GLP-1 Uses WNT Effectors in Pancreas

The stimulation of pancreatic β cell proliferation by GLP-1 requires Wnt effectors (Liu and Habener, 2008). GLP-1 binding to its receptor in pancreatic β cells increases cAMP production, resulting in PKA stimulation, and phophorylation and consequent stabilization of β-catenin that then enters the nucleus to activate TCF7L2 targeted gene transcription. Since the *Gcg* promoter is targeted by Wnt signaling, GLP-1 might be considered both inducer and effector of Wnt signaling (Gustafson and Smith, 2008). However, whether GIP can similarly impact on the Wnt signaling pathway is unknown. Wnt induction of incretin expression (and possibly secretion), receptor exhibition and signaling is summarized in Fig. 14.3. The extrapancreatic actions of GIP and/or GLP-1 include target cells such as adipocytes and osteoblasts that are emerging as targets of Wnt signaling, and it will be important to determine whether in these tissues GIP and/or GLP-1 signaling may be mediated by the same or different Receptors/mechanisms. The possibility that cell type-specific Wnt ligands or signal transduction intermediaries may also significantly affect the Wnt–incretin interplay is also a key area for future interrogation.

A. Indirect ways to influence Wnt signaling: glucose, lipids, adipokines?

GIP and GLP-1 stimulate glucose-dependent insulin secretion from pancreatic β cells. This may imply that glucose signaling is enhanced by incretin cosignaling. Since incretin (at least GLP-1) signal in β cells through Wnt effectors, the possibility that Wnt intermediaries convey signals from glucose or lipids deserves exploration. Thus, modulation of Wnt signaling by incretin could occur by incretin binding to their receptors or by glucose or lipids signaling in the cell. Nutrient and incretin interactions at the pancreatic β cell may extend far beyond costimulation of insulin expression and secretion since glucose also regulates incretin receptor exhibition on the cell surface, with hyperglycemia resulting in receptor de-localization (Xu *et al.*, 2007; Zhou *et al.*, 2007). Complex interactions between different nutrients, incretins and particular WNT ligands might take place at target cells. For example, fatty acid stimulation of pancreatic β cells results in increased or decreased incretin receptors in normoglycemia or hyperglycemia, respectively (Lynn *et al.*, 2003). Similarly the outcome of Wnt signaling might be different in normo- or hyperglycemia, etc. Figure 14.4 represents a model in which cosignaling by nutrients and Wnt is required for incretin production at the enteroendocrine cell. Cosignaling by Wnt and nutrients or Wnt and incretins or Wnt and incretins and nutrients might be necessary to promote insulin secretion or to accomplish other physiological functions and insulin may feed back to Wnt signaling in a different way depending on cosignaling background.

VII. What is the Meaning of the Wnt–Incretin Interplay for Health and Disease?

As mentioned above, the strongest genetic linkage established to date in independent cohorts for diabetes corresponds to certain polymorphisms in the *Tcf7l2* gene (Saxena *et al.*, 2006; Grant *et al.*, 2006). The *Tcf7l2* risk alleles are associated with impaired insulin secretion and incretin effects as well as with increased hepatic glucose production (Pilgaard *et al.*, 2009). The *Tcf7l2* polymorphisms are located in introns 3 and 4, and cannot be attributed to amino acid changes in the mature protein, though the mechanism underlying the observed effects remain obscure. Transcript stability (reflected on protein levels) or splicing may be affected. To date it is not clear whether splice variants bind the same target genes with the same affinity and output. Alternatively spliced forms may interact differently with cofactors or neighboring transcription factors and may be subject to differential posttranslational modifications, all of which could affect

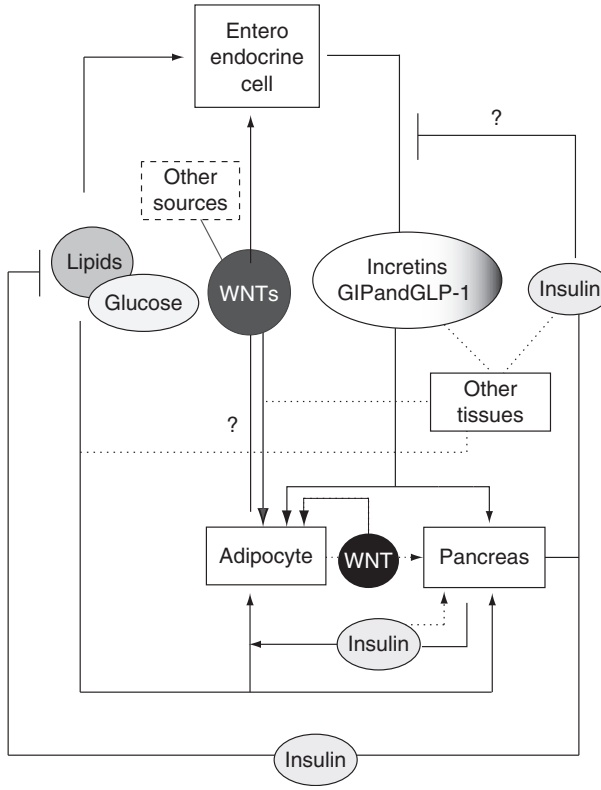

Figure 14.4 The Wnt–nutrient and incretin interplay for nutrient detection and insulin production. Glucose and lipids passage through the intestine stimulate incretin production, and perhaps Wnt production/modifications. Glucose and lipids are absorbed and incorporated into plasma from where together with incretins stimulate insulin production/secretion and perhaps again also stimulate Wnt production either by adipocytes or by other neighboring cells. Wnt ligands may modulate the actions of nutrients alone, or incretin and nutrients on pancreatic cells. Wnt ligands or intermediaries may be required for incretin- and insulin-nutrient cosignaling on different target tissues. Insulin may need Wnt ligands or effectors to accomplish glucose uptake and to abolish high glucose levels that initiated its secretion. Continuous lines denote known inductions (arrows) or repressions (line), dotted lines denote proposed cosignaling. Interrogants mark possible negative feedback points.

function. However, alternative splicing and risk alleles are not associated in pancreatic cells (Prokunina-Olsson *et al.*, 2009). Yet, while selection of a specific splicing isoform in enteroendocrine cells producing incretins could underlie the increased risk of diabetes, GIP or GLP-1 levels do not appear to be affected in risk allele carriers (Lyssenko *et al.*, 2007). Nevertheless, tissue-specific differences in the distribution of Tcf7l2 splicing variants do exist, and even a pancreas-specific splicing form has been defined, but association

with diabetes is again not apparent (Prokunina-Olsson *et al.*, 2009). To date no specific splicing variants have been identified associated to diabetes suggesting that risk alleles might impact on RNA stability. *Tcf7l2* risk alleles were associated with 5-fold increased in pancreatic mRNA levels (Lyssenko *et al.*, 2007). However, this may reflect an adaptation due to decreased protein levels (Shu *et al.*, 2009). As mentioned above, TCF7L2 is a transcriptional repressor convertible into an activator upon binding of β-catenin. As a repressor TCF7L2 bears 2 independent corepressor interacting domains, and the C-terminal extrarepressor domain is present only in the E variants of TCF7L2 and absent from LEF1 (Tang *et al.*, 2008), suggesting that TCF7L2 may be a stronger repressor than LEF1. Further work is undoubtedly needed to characterize (i) Variations in TCF7L2 protein levels and to define splicing form specific functionality in enteroendocrine and pancreatic α and β cells; (ii) the interplay between nutrients and TCF7L2 variants and how affect incretin expression, (iii) the TCF7L2 variant contribution to insulin expression/secretion in response to glucose and incretins; (iv) their contribution to basal and GIP stimulated glucagon production and secretion by α-pancreatic cells; (v) the impact of different risk alleles on circulating levels of active GIP and GLP-1. Dominant negatives of TCF7L2, unable to bind β-catenin, have been helpful to define its functions but these experiments need to be complemented with siRNA-mediated knock down of TCF7L2 because the dominant-negatives only suppress activator function without interfering with the repressor function reported for TCF7L2 at the mGip promoter (Garcia-Martinez *et al.*, 2009) while siRNA interferes with both (Tang *et al.*, 2008). Thus, the Wnt/β-catenin signaling (through TCF7L2 or LEF1) is required at pancreatic islets for β cell survival (Shu *et al.*, 2008), for production and secretion of insulin (Loder *et al.*, 2008), for expression of incretin receptors (Shu *et al.*, 2009) and to mediate GLP-1 effects (Liu and Habener, 2008); at the enteroendocrine cells for expression of incretins: GIP (Garcia-Martinez *et al.*, 2009) and GLP-1(Yi *et al.*, 2005); at the adipocyte to control differentiation (Ross *et al.*, 2000). There is no doubt that risk Tcf7l2 alleles alter incretin effects; however, the Wnt-dependence of key metabolic tissue function is based mostly on correlations and the underlying molecular mechanisms need to be investigated more fully.

In addition to the Wnt effector TCF7L2, Wnt ligands and receptors have also been associated with metabolic disorders related to diabetes and there is consequently intense research on the biology of different Wnt ligands. Wnt ligands that inhibit canonical signaling such as Wnt10b (Wright *et al.*, 2007) or DKK1 (Christodoulides *et al.*, 2006) have been found, and adipocytes produce a variety of Wnt ligands that enhance insulin secretion at the pancreas (Schinner *et al.*, 2008) and might act as adipokines (Schinner *et al.*, 2009). Wnt coreceptors like Frizzled 1 (Gao and Wang, 2007) or lrp1 (Terrand *et al.*, 2009) are also involved in metabolic pathologies. The *lrp5* locus previously linked to type I diabetes (Guo *et al.*, 2006;

Nakagawa *et al.*, 1998; Twells *et al.*, 2001) is arousing much interest at present since Lpr5$^{-/-}$ mice present impaired glucose tolerance and blunted plasma insulin concentrations in response to glucose. In lrp5-deficient islets, Wnt-ligand treatment does not lead to glucose-induced insulin-secretion, although it does it in wild type islets. This suggests that glucose requires Wnt signaling to induce insulin secretion. It would be interesting to test nutrient-induced incretin production in these mice and incretin stimulation of pancreatic β cells to ascertain if cosignaling between Wnt and GIP and GLP-1 is also affected.

In summary, Tcf7l2 risk alleles alter incretin function and incretins may use TCF7L2 to exert their effects. We propose a model in which cosignaling by Wnt–nutrients is important for incretin and insulin production (see Fig. 14.4). Wnt–nutrient signaling may be further modulated at the enteroendocrine or pancreatic cells (α and β) as well as at the adipocyte. Complex interactions between Wnt ligands, nutrients, incretins, and/or insulin might tune the metabolic response. The final incretin outcome is the control of nutrient concentration such as glucose or lipids in plasma. High plasma glucose and lipids induce incretin and insulin production and alter expression and function of receptors for incretins and insulin; whether the receptors for Wnt ligands or the type and concentration of Wnt ligands is affected by high glucose or lipids should be investigated. Together, these studies point toward a role for Wnt signaling in the pathogenesis of metabolic diseases especially diabetes.

VIII. Perspectives

The study of the control of incretin production has been hampered by the fact that enteroendocrine cells are rare and scattered along the intestinal epithelium, and that no morphological criteria can be used to distinguish enteroendocrine cells from other cell types. Progress is therefore critically dependent on the development of tools to facilitate the study of enteroinsular axis. A number of models have been developed to study incretin production and secretion *in vitro*, in particular the GLUTag and STC-1 cells described and a human enteroendocrine cell line, NCI-H716 also exists although it is less well characterized (Reimer *et al.*, 2001; Theodorakis *et al.*, 2006).

Besides these cell lines, transgenic mice that bear fluorescently labeled K cells (Blitzer and Nusse, 2006) promise to be an invaluable tool to study the biology of K and L cells. The expression of specific Wnt receptors and their *in vivo* regulation by nutrients, insulin or other signals should be studied in these animals, as well as the potential regulation of incretin expression by natural Wnt ligands and the expression of incretin receptors. The regulation

of LEF1 expression must also be examined given the fact that LEF1 selectively activates *Gip* expression in STC-1 cells. Endosomal Wnt signaling components are required for canonical signaling (Blitzer and Nusse, 2006) and the role of endosomes in enteroendocrine cells might be important for cosignaling, and in particular the coincidence of endosomal Wnt intermediaries with internalized incretin receptors in β-pancreatic cells should be explored.

Isolated mouse pancreatic islets can be cultured and will be very useful in understanding how are incretin receptors controlled by Wnt signaling, which Wnt ligands are sensed, and whether or not cosignaling Wnt–nutrients or Wnt–incretin has a biological meaning.

Transgenic mice bearing EGFP or β-galactosidase under the control of β-catenin (Moriyama *et al.*, 2007) will exhibit fluorescence or color when canonical Wnt signaling is activated and may prove to be very valuable to understand the role of Wnt signaling in enteroendocrine cells as well as in tissues such as the pancreas, adipocytes, muscle, osteoblast, and brain where it could be cosignaling with incretins. These mice can be used to explore whether nutrients or their digestion products use Wnt signaling in enteroendocrine cells to induce expression of incretins.

A number of knockout mice have proven to be extremely valuable to understand the pleiotropic roles of incretins, specifically the GLP-1R$^{-/-}$ (Scrocchi *et al.*, 1996), GIPR$^{-/-}$ (Miyawaki *et al.*, 1999) and a double incretin receptor knockout (DIRKO) mice (Hansotia *et al.*, 2004; Preitner *et al.*, 2004). Targeted disruption of *K* cells (Althage *et al.*, 2008) has also been extremely informative. Differences in Wnt signaling outcome from the enteroendocrine and pancreatic cells from these mice and their wild type littermates as well as other tissues targeted by Wnt and incretins should be explored. In addition, a number of synthetic peptide analogues of the incretins or antagonists of their receptors are also available to test interference with Wnt signaling at pancreatic β cells.

Other mice deficient in different Wnt signaling components such as lrp5$^{-/-}$ (Fujino *et al.*, 2003), or β-catenin-deficient mice (Murtaugh *et al.*, 2005) or even a Wnt10b transgenic (Wright *et al.*, 2007) should be directly compared for glucose tolerance in a wild-type background or in incretin receptor(s) deficiency background.

The ob/ob mice (Gray *et al.*, 2006; Ingalls *et al.*, 1950; Kanda *et al.*, 2009), an obese mutant with the leptin gene silenced, the reciprocal diabetic model db/db, which bears a mutant leptin receptor and the precociously diabetic POKO mice, a PPAR gamma and ob/ob double knock out may be useful to study altered Wnt signaling from infiltrated adipocytes or macrophages or present in serum. Pancreatic and enteroendocrine effects should be tested and the incretin receptor knock outs should be tested in these backgrounds. See Table 14.2 for an overview on useful animal models to study Wnt and incretin connections.

Table 14.2 Useful models to study Wnt and incretin interactions

Model	References
Fluorescent K cells	Parker *et al.* (2009)
GIPR-knockout	Miyawaki *et al.* (2002)
GLP-1R-knockout	Scrocchi *et al.* (1996)
DIRKO: double incretin receptor knockout mice	Preitner *et al.* (2004) Hansotia *et al.* (2004)
Chemically ablated GIPR in ob/ob background	Parker *et al.* (2007)
K cell ablation	Althage *et al.* (2008)
$Lrp5^{-/-}$	Fujino *et al.* (2003)
β-$Catenin^{-/-}$	Murtaugh *et al.* (2005)
STC-1: mouse enteroendocrine cell line producing GIP, GLP-1, and other hormones	Rindi *et al.* (1990)
GLUTag: mouse enteroendocrine cell line producing only GLP-1	Drucker *et al.* (1994)
NCI-H716: human enteroendocrine cell line	Reimer *et al.* (2001) Theodorakis *et al.* (2006)
ob/ob: obese mouse with a mutation in the leptin gene	Ingalls *et al.* (1950)
db/db: diabetic mouse with a mutation in the leptin receptor gene	Reviewed in Kanda *et al.* (2009)
POKO: mouse double knockout, PPAR gamma deficient in the ob/ob background	Gray *et al.* (2006)
Reporter transgenic mouse lines, ins-TOPEGFP ins-TOPGAL	Moriyama *et al.* (2007)
FABP-Wnt10b	Wright *et al.* (2007)

In summary, there is ample evidence that Wnt and incretin signaling are closely coupled and play a key role in the maintenance of energy balance and nutrient response. The development of a wide range of tools will facilitate the study of the enteroinsular axis and provide critical insights into how organisms manage their response to nutrient availability.

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CHAPTER FIFTEEN

Incretin-Based Therapy and Type 2 Diabetes

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Contents

Abstract

This chapter focuses on the incretin hormones, glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP), and their therapeutic potential in treating patients with type 2 diabetes. Type 2 diabetes is characterized by insulin resistance, impaired glucose-induced insulin secretion, and inappropriately regulated glucagon secretion which in combination eventually result in hyperglycemia and in the longer term microvascular and macrovascular diabetic complications. Traditional treatment modalities—even multidrug approaches—for type 2 diabetes are often unsatisfactory at getting patients to glycemic goals as the disease progresses due to a steady, relentless decline in pancreatic beta-cell function. Furthermore, current treatment modalities are often limited by inconvenient dosing regimens, safety, and tolerability issues, the latter including hypoglycemia, body weight gain, edema, and gastrointestinal side effects. Therefore, the actions of GLP-1 and GIP, which include

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potentiation of meal-induced insulin secretion and trophic effects on the beta-cell, have attracted a lot of interest. GLP-1 also inhibits glucagon secretion and suppresses food intake and appetite. Two new drug classes based on the actions of the incretin hormones have been approved for therapy of type 2 diabetes: injectable long-acting stable analogs of GLP-1, *incretin mimetics*, and orally available inhibitors of dipeptidyl peptidase 4 (DPP4; the enzyme responsible for the rapid degradation of GLP-1 and GIP), the so-called *incretin enhancers*. In this chapter, we will describe the physiological effect of the incretin hormones—the incretin effect—in a historical perspective and focus on the two new classes of antidiabetic agents and will outline the scientific basis for the development of incretin mimetics and incretin enhancers, review clinical experience gathered so far, and discuss future expectations for incretin-based therapy.

I. Introduction

In this chapter, we will try to outline the pathophysiology of type 2 diabetes with particular focus on incretin-related deficiencies and, combined with a historical perspective on the findings leading to the establishment of the incretin effect, we will try to delineate the development of incretin-based treatment modalities. Additionally, we will review the major clinical findings on incretin-based treatments and comment on future expectations for incretin-based therapy.

A. Type 2 diabetes

Type 2 diabetes is the result of genetic disposition combined with sedentary lifestyle and obesity (The expert committee on the diagnosis and classification of diabetes mellitus, 2002). Type 2 diabetes comprises 90% of people with diabetes around the world. The World Health Organization estimates that more than 180 million people worldwide have diabetes, and, as the western lifestyle is making its entry into the developing countries, this number is likely to more than double within the next decades (Wild *et al.*, 2004). It is now well established that insulin resistance and beta-cell dysfunction (inability of beta-cells to respond adequately to the increasing insulin need imposed by insulin resistance) are central defects in the pathophysiology of type 2 diabetes (Kahn, 2003; Pratley and Weyer, 2001). Furthermore, evidence for inappropriate secretion of glucagon playing an important role in the pathogenesis of type 2 diabetes is accumulating (Kahn, 2000); fasting and postprandial hyperglucagonemia in type 2 diabetes have been shown to result in increased glucagon-induced hepatic glucose production, which in turn contributes to fasting hyperglycemia and exaggerated

postprandial glucose excursions (Baron *et al.*, 1987; Muller *et al.*, 1970; Reaven *et al.*, 1987; Shah *et al.*, 2000). Last, the pathophysiology of type 2 diabetes has been shown to be characterized by a severely reduced incretin effect (Nauck *et al.*, 1986).

B. The incretin effect

The incretin effect refers to the amplification of insulin secretion that occurs when glucose is ingested orally as opposed to infused intravenously in amounts that result in identical glucose excursions (Vilsbøll and Holst, 2004). The scientific history of the incretin effect extends back more than 100 years, and the scientific interest surrounding it has only intensified over time. In 1906, extracts of mucosa from porcine small intestine were used by Moore *et al.* as a treatment for diabetes, hoping that "the pancreas secretion might be stimulated by the substance of the nature of a hormone yielded by the duodenal mucosa membrane" (Moore, 1906). In 1964, McIntyre *et al.* and Elrick *et al.* demonstrated that orally administered glucose evokes a greater insulin response than does intravenously administered glucose, and both groups hypothesized that gut-derived factors could have potentiating effects on insulin secretion after oral ingestion of glucose (Elrick *et al.*, 1964; McIntyre *et al.*, 1964). A few years later, in 1967, this finding was confirmed by Perley and Kipnis, who administered oral glucose; and, on a separate day, copied the oral glucose curve with an isoglycemic intravenous (i.v.) glucose infusion in obese and normal weight patients with diabetes and in healthy control subjects (Perley and Kipnis, 1967). They concluded that the insulin response to isoglycemic i.v. glucose administration only amounted to 30–40% of that seen after oral glucose. Today, the isoglycemic method used by Perley and Kipnis is widely accepted as the method of choice to measure the incretin effect. The effect is defined as the beta-cell secretory response evoked by factors other than glucose itself and is represented by the difference in integrated responses of plasma insulin, plasma C-peptide, or insulin secretion rate, measured in response to oral glucose ingestion versus isoglycemic i.v. glucose infusion. In healthy subjects, the incretin effect accounts for up to 70% of the total amount of insulin released in response to an oral glucose load (Nauck *et al.*, 1986). This amplification of glucose-induced insulin secretion is the result of the actions of incretin hormones, which are released from the gut in the presence of intraluminal nutritional components. Incretin hormones potentiate glucose-induced insulin secretion and, therefore, play an essential role in the regulation of glucose homeostasis—in particular, postprandial glucose levels.

II. The Incretin Hormones

A. GLP-1 and GIP

Many hormones have been suspected to contribute to the incretin effect, but today, there is ample evidence to suggest that the incretin effect mainly is conveyed by the two incretin hormones: glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 is a 30-amino acid polypeptide produced in the endocrine L-cells of the intestinal epithelium as a product of glucagon gene expression (Holst, 2007). The glucagon gene is expressed in both pancreatic alpha-cells and mucosal endocrine L-cells in the small intestine. The primary transcripts and translation products of the gene in the two types of cells are identical but, as illustrated in Fig. 15.1, the posttranslational processing differs in the two tissues (Mojsov *et al.*, 1986). In the pancreas, proglucagon is cleaved by prohormone convertase 2 to glucagon, glicentin-related pancreatic peptide and the so-called major proglucagon fragment (Holst *et al.*, 1994; Mojsov *et al.*, 1986; Orskov *et al.*, 1986). Apart from glucagon, these fragments seem to be biologically inactive (Orskov *et al.*, 1992). In contrast, in the intestinal L-cells, proglucagon is processed by prohormone convertase 1 to GLP-1, glucagon-like peptide-2 (GLP-2; Orskov *et al.*, 1989) and glicentin (Thim and Moody, 1981). GLP-1 is—as mentioned—secreted in response to ingestion of nutrients and is strongly insulinotropic (Holst *et al.*, 1987; Mojsov *et al.*, 1987)—a true incretin hormone—and GLP-2, also secreted in response to ingestion of nutrients, is a key regulator of small intestinal

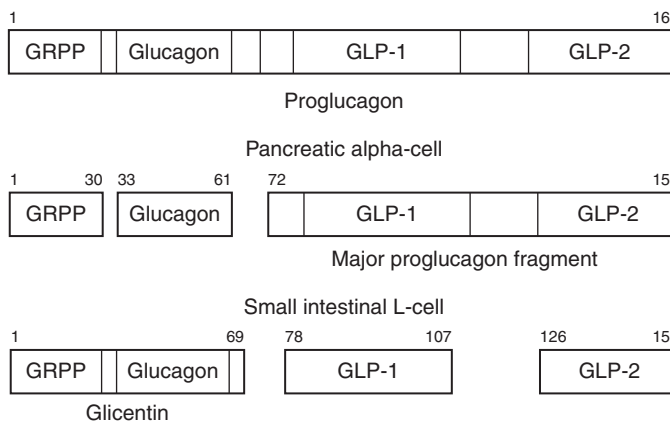

Figure 15.1 Proglucagon processing in human pancreatic alpha-cells and in mucosal endocrine L-cells in the small intestine. GRPP, glicentin-related pancreatic peptide; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2.

growth (Drucker, 2001). The bioactive forms of GLP-1, amidated and glycine extended GLP-1, are designated GLP-1 7-36 amide and GLP-1 7-37. GLP-1-secreting L-cells are found throughout the intestinal tract but their density is highest in the ileum and parts of the colon. GIP is a 42-amino acid polypeptide produced in the endocrine K-cells (utilizing prohormone convertase 1 to process preproGIP to GIP), which are more frequent in the proximal small intestine (Vilsbøll and Holst, 2004).

B. Secretion of GLP-1 and GIP

In the fasting state, the plasma concentrations of the incretin hormones are very low, although they are not immeasurable, suggesting that there is a certain basal rate of secretion (Holst, 2007). Both incretin hormones are secreted rapidly (within 10–20 min) in response to ingestion of nutrients, with lipids and simple carbohydrates being potent stimulators of secretion (Holst, 2007) and protein eliciting less pronounced GLP-1 responses (Carr *et al.*, 2008). Peak concentrations of GIP and GLP-1 are reached as soon as 15–30 and 30–45 min, respectively, after ingestion of, for example, glucose. The rapid secretion following ingestion of nutrients—long before the substrates ingested are present in the small intestine—has led to the notion of vagus-mediated stimulation of secretion (Imeryuz *et al.*, 1997; Meier *et al.*, 2002). However, identification of glucokinase expression in the K-cells (Cheung *et al.*, 2000) and glucose-stimulated GLP-1 secretion and firing of action potentials, via mechanisms involving closure of adenosine 5′-triphosphate (ATP)-sensitive K^+ channels, in GLUTag cells (an L-cell model) (Reimann and Gribble, 2002) provide evidence for a direct relationship between absorption of nutrients and secretion of GIP and GLP-1. Furthermore, secretion of GLP-1 after uptake of the nonmetabolizable monosaccharide methyl-α-glucopyranoside through sodium-glucose cotransporters in GLUTag cells has been observed (Gribble *et al.*, 2003). The direct relationship between absorption of nutrients and secretion of GLP-1 is further supported by the observation of intact GLP-1 responses following ileal instillation of carbohydrates and lipids (Layer *et al.*, 1995). In addition, a study performed on anesthetized pigs showed no effect of electrical stimulation of the vagal trunks at the level of the diaphragm (Hansen *et al.*, 2004). Last, observations indicate that GLP-1 and GIP are colocalized in a subset of endocrine cells throughout the gastrointestinal tract (Mortensen *et al.*, 2000, 2003; Theodorakis *et al.*, 2006). This finding may explain the fast secretory responses following ingestion of nutrients, but other mechanisms—for instance, paracrine interaction between the two incretin hormones as indicated by data in dogs (Damholt *et al.*, 1999), and intrinsic neuroendocrine mechanisms (Dube and Brubaker, 2004)—may be involved.

C. Degradation of GLP-1 and GIP

After the secretion of GIP and GLP-1, both hormones are degraded by the enzyme dipeptidyl peptidase 4 (DPP4; Deacon *et al.*, 1995a, 2000; Kieffer *et al.*, 1995; Pederson *et al.*, 1996). This enzyme, also known as the T-cell antigen CD26, is a serine peptidase found in numerous sites such as the intestinal and renal brush border membranes, hepatocytes, and vascular endothelium, as well as in a soluble form in plasma (Mentlein, 1999). It cleaves off the two N-terminal amino acids of peptides with a penultimate proline or alanine residue, and for the incretin hormones, this abolishes their insulinotropic activity (Deacon *et al.*, 1995a, 2000; Kieffer *et al.*, 1995; Pederson *et al.*, 1996). While GLP-1 is rapidly degraded in the circulation, resulting in a clearance which exceeds cardiac output and an apparent half-life of 1–1.5 min (Deacon *et al.*, 1995b; Vilsbøll *et al.*, 2003), GIP is degraded more slowly, with a half-life of 7 min for the intact hormone (Deacon *et al.*, 2000; Vilsbøll *et al.*, 2006). The truncated metabolites are eliminated more slowly through the kidneys, with half-lives of 4–5 and 17 min, respectively (Deacon *et al.*, 1995a, 2000; Vilsbøll *et al.*, 2003, 2006).

D. Actions of GLP-1 and GIP

Specific receptors for GLP-1 and GIP are found in the pancreatic beta-cell plasma membrane. Both receptors belong to the glucagon subfamily of G protein-coupled receptors. Following binding and subsequent activation of adenylate cyclase, intracellular accumulation of cyclic adenosine monophosphate, closure of ATP-sensitive K^+ channels and elevation of cytosolic Ca^{++} concentrations, mobilization and exocytosis of insulin-containing granules occur (Ding *et al.*, 1997; Gromada *et al.*, 1998). The insulinotropic action of both hormones is strictly glucose-dependent and consists of potentiation of glucose-induced insulin secretion. Therefore, neither hormone has insulinotropic activity at lower glucose concentrations (less than 4 mM). The insulinotropic potential of GLP-1 and GIP was investigated in recent human experiments involving clamping of plasma glucose at fasting and postprandial levels and exact copying of the meal-induced concentrations of both GLP-1 and GIP by i.v. infusions (Vilsbøll *et al.*, 2003). The results showed that both hormones are active with respect to enhancing insulin secretion from the beginning of a meal (even at fasting glucose levels), and that they contribute almost equally, but with the effect of GLP-1 predominating at higher glucose levels (Vilsbøll *et al.*, 2003). Even though GLP-1 is more potent than GIP, higher circulating concentrations of GIP result in the net effect that both hormones contribute almost equally to the incretin effect in healthy subjects (Vilsbøll *et al.*, 2003). From studies in mice with targeted lesions of both GIP and GLP-1 receptors, it was concluded that the hormones are essential for a normal glucose tolerance

and that the effect of deletion of one receptor was "additive" to the effect of deleting the other (Hansotia *et al.*, 2004). Thus, there is little doubt that the GIP and GLP-1 play important roles in postprandial insulin secretion and, therefore, glucose tolerance in humans and animals.

Both hormones have—in addition to their glucose-dependent insulinotropic effect (the incretin effect)—other actions. GLP-1 has been shown to enhance all steps of insulin biosynthesis as well as insulin gene transcription (Fehmann *et al.*, 1995). Activation of the transcription factor PDX-1, a key regulator of islet growth and insulin gene transcription may be involved (Buteau *et al.*, 1999). In addition, GLP-1 upregulates the genes for the cellular machinery involved in insulin secretion, such as the glucokinase and GLUT-2 genes (Wang *et al.*, 1997). Importantly, GLP-1 also has trophic effects on beta-cells (Egan *et al.*, 2003). It stimulates beta-cell proliferation (Stoffers *et al.*, 2000; Xu *et al.*, 1999) and enhances the differentiation of new beta-cells from progenitor cells in the pancreatic duct epithelium (Zhou *et al.*, 1999). Importantly, GLP-1 has been shown to be capable of inhibiting apoptosis of beta-cells, including human beta-cells (Buteau *et al.*, 2004). Furthermore, GLP-1 robustly inhibits glucagon secretion, and the combined effects on insulin and glucagon secretion result in inhibition of hepatic glucose production (Larsson *et al.*, 1997), which contributes significantly to the glucose-lowering effect of GLP-1 (Hvidberg *et al.*, 1994). Additionally, GLP-1 decreases gastrointestinal motility, thereby curtailing postprandial glucose excursions (Willms *et al.*, 1996), and promotes satiety (Flint *et al.*, 1998), probably via activation of GLP-1 receptors in the brain in combination with decreased gastrointestinal motility. Therefore, chronic administration of GLP-1 leads to weight loss (Zander *et al.*, 2002). When exogenous GLP-1 is administered in supraphysiological amounts, it is dose-dependently associated with gastrointestinal side effects, with nausea being the most frequently reported complaint. GLP-1 receptors are also found in the heart; and, a physiological role for these was shown in mice lacking the GLP-1 receptor (Gros *et al.*, 2003). These mice exhibit impaired left ventricular contractility and diastolic functions, as well as impaired responses to exogenous epinephrine. Furthermore, studies indicate that GLP-1 protects the ischemic and reperfused myocardium in rats (Bose *et al.*, 2005), improves the ejection fraction in patients treated with angioplasty after acute myocardial infarction (Nikolaidis *et al.*, 2004a), and improves left ventricular function and systemic hemodynamics in dogs with exercise-induced dilated cardiomyopathy (Nikolaidis *et al.*, 2004b). Furthermore, GLP-1 has been found to reduce the postprandial rise in triglycerides and lower the concentration of free fatty acids in healthy subjects (Meier *et al.*, 2006), and improve endothelial dysfunction in patients with T2DM and coronary heart disease (Nystrom *et al.*, 2004). Finally, GLP-1 has been associated with improved learning in rats and has also displayed neuroprotective effects (During *et al.*, 2003; Perry *et al.*, 2002).

Regarding the actions of the other incretin hormone, GIP, a number of studies provide evidence for a role of the hormone in lipid metabolism: Lipids are strong stimulators of GIP secretion; 24-h GIP profiles parallel plasma concentrations of triglycerides (Elliott *et al.*, 1993); and functional GIP receptors are found on adipocytes (Yip *et al.*, 1998). Furthermore, administration of GIP has been reported to increase chylomicron clearance in dogs (Wasada *et al.*, 1981), lower postprandial triglyceride levels in rats (Ebert *et al.*, 1991), increase glucose transport in rat adipocytes (Oben *et al.*, 1991), increase fatty acid synthesis in adipocytes (Baba *et al.*, 2000; Beck and Max, 1983; Oben *et al.*, 1991), and to increase lipoprotein lipase activity in rat adipose tissue explants (Knapper *et al.*, 1995). Interestingly, mice with a deletion of the GIP receptor gene become slightly glucose intolerant (Miyawaki *et al.*, 1999); and, unlike wild type controls, they do *not* become obese when given a high fat diet (Miyawaki *et al.*, 2002). In human studies GIP in combination with insulin and light hyperglycemia was reported to increase adipose tissue blood flow and glucose uptake, and increase free fatty acid reesterification; suggesting a role for GIP in adipose tissue triglyceride deposition (Asmar *et al.*, 2010). Like GLP-1, GIP has been shown to play a role in the maintenance of beta-cell mass by stimulating cellular proliferation and decreasing apoptotic activity in beta-cell lines (Trumper *et al.*, 2002). In contrast to GLP-1 (Larsson *et al.*, 1997), GIP has been shown to stimulate pancreatic glucagon secretion (Meier *et al.*, 2003; Pederson and Brown, 1978). So far data on GIP do not provide evidence for effects on the gastrointestinal system or on food intake.

III. Incretin Hormones in Type 2 Diabetes

In 1986, Nauck *et al.* showed that the incretin effect was severely reduced in patients with type 2 diabetes (Nauck *et al.*, 1986) and subsequent investigations have yielded a more detailed analysis of this pathophysiological phenomenon (Vilsbøll and Holst, 2004). Vilsbøll *et al.*, Toft-Nielsen *et al.*, and other groups have found the postprandial (mixed meal) secretion of GLP-1 to be significantly reduced in these patients (Toft-Nielsen *et al.*, 2001; Vilsbøll *et al.*, 2001), while the postprandial secretion of GIP was found to be less affected (Vilsbøll *et al.*, 2001). With regard to the insulinotropic effects of the two hormones in patients with T2DM, Krarup *et al.* reported a negligible beta-cell response to GIP (Krarup *et al.*, 1987), and Vilsbøll *et al.* showed that while GLP-1 may almost normalize glucose-induced insulin secretion, the insulinotropic effect of GIP has virtually disappeared (Vilsbøll *et al.*, 2002). Clinical studies investigating GLP-1, however, have indicated that the diabetic beta-cell has a decreased sensitivity toward GLP-1, though a slightly supraphysiological infusion is able to

normalize the beta-cell glucose sensitivity—and thereby the beta-cell response to glucose (Kjems *et al.*, 2003). In contrast, the diabetic alpha-cell response to GLP-1 seems to be conserved in patients with type 2 diabetes (Hare *et al.*, 2009). Disappointingly, due to the rapid inactivation of GLP-1 by DPP4, subcutaneous injection of the maximally tolerable dose of native GLP-1 (1.5 nmol/kg—higher doses cause nausea and vomiting) in type 2 diabetes patients, only resulted in a small short-lasting effect on insulin secretion and a correspondingly small effect on plasma glucose (Nauck *et al.*, 1996). Thus, native GLP-1 administered subcutaneously is of little use clinically due to the extraordinarily rapid degradation of the peptide. Nevertheless, the finding that the insulinotropic effect of GLP-1 is preserved and that infusions with GLP-1 may normalize glucose-induced insulin secretion (Kjems *et al.*, 2003), combined with its apparently trophic and protective effects on the beta-cells, have led to extensive research with the aim of exploiting the actions of GLP-1 as a new treatment for type 2 diabetes.

IV. Incretin-Based Therapy

In 1993, Nauck *et al.* demonstrated that continuous i.v. infusion of native GLP-1 is capable of normalizing blood glucose concentrations in fasting patients with type 2 diabetes (Nauck *et al.*, 1993). However, i.v. infusions are clearly not of any clinical utility. In 2002, Zander *et al.* (2002) demonstrated that 6 weeks of continuous subcutaneous (s.c.) infusion of native GLP-1 (using an insulin pump) significantly decreased hemoglobin A_{1c} (HbA_{1c}) and body weight, and greatly improved the first-phase insulin response and maximal secretory capacity. Although native GLP-1 seems to be efficient in short-term treatment, the necessity of administration by continuous infusion leaves this treatment modality unfeasible for long-term use. Two different strategies of circumventing this problem have been successful so far. *Incretin mimetics* refer to GLP-1 receptor agonists that are resistant to inactivation by DPP4 and are modified in a way that prolongs and enhances the effect of the hormone; and the other strategy involves inhibition of DPP4, hereby enhancing the survival and therefore the effect of endogenously released GLP-1 (and GIP), the so-called *incretin enhancers*. In the following these two strategies will be reviewed.

A. GLP-1 receptor agonists

Two GLP-1 receptor agonists have been approved for the treatment of type 2 diabetes; exenatide (Byetta) for twice-daily s.c. injection and liraglutide (Victoza) for once-daily s.c. injection. Additionally, formulations of GLP-1

receptor agonists with extended actions suited for once-weekly dosing are in clinical development. In the following, we will summarize the current clinical data regarding GLP-1 receptor agonists.

Exenatide was isolated from the saliva of the lizard *Heloderma suspectum* in a search for biologically active peptides (Eng *et al.*, 1992). Exenatide shares only 53% sequence homology with native GLP-1, but is equipotent with regard to binding and activation of GLP-1Rs on pancreatic beta-cells (Thorens *et al.*, 1993). Exenatide is rapidly absorbed and peak plasma concentrations are reached 2 h after injection. It is cleared primarily in the kidneys by glomerular filtration and, as a result, the half-life of s.c. administered exenatide is 2 h. Consequently, significant plasma levels of exenatide in plasma are only evident for 5–6 h after s.c. administration of the maximally tolerated dose (Kolterman *et al.*, 2005; Simonsen *et al.*, 2006). The clinical effects of exenatide treatment were originally investigated in a total of 2731 patients (Bosi *et al.*, 2008). Exenatide as add-on therapy to metformin, sulphonylurea (SU), or both conferred statistically significant improvement in glycemic control (hemoglobin A_{1c} [HbA_{1c}] change of -1.0% from a baseline of 8.2% vs. an increase of $\sim 1.5\%$ in the placebo groups) and reduction in fasting plasma glucose (-0.5 mM in the exenatide groups vs. an increase of ~ 1 mM in the placebo groups). Exenatide induces similar changes in HbA_{1c} (about -1%) as insulin glargine (Heine *et al.*, 2005) and insulin aspart (Nauck *et al.*, 2007). On average, the weight loss in the three studies comparing exenatide to oral antidiabetics amounted to 1.6 kg in the exenatide-treated patients (Madsbad *et al.*, 2008). In an open-label extension of the three studies (with the limitations of a substantial dropout rate), 3-year sustained effects were demonstrated with respect to glycemic control and body weight (change in HbA_{1c} of -1%, body weight of -5.3 kg) (Klonoff *et al.*, 2008). Minor, but significant improvements in triglycerides, total cholesterol, low-density lipoprotein, and high-density lipoprotein levels in favor of a reduced cardiovascular risk profile with exenatide have been reported (Klonoff *et al.*, 2008). Additionally, 6 months of exenatide treatment is associated with significant reduction in systolic blood pressure compared with placebo (-2.8 mm Hg) or insulin (-3.7 mm Hg) (Okerson *et al.*, 2010).

Liraglutide is an acylated analog of human GLP-1. It has a 97% sequence homology to native GLP-1 and similar interactions with the GLP-1R as GLP-1. A high degree of plasma protein binding causes decreased susceptibility to metabolism by DPP4, and the half-life after s.c. administration of liraglutide is ~ 13 h (Thim and Moody, 1981), which makes it suitable for once-daily dosing. The clinical effects of liraglutide treatment have been investigated in the LEAD (Liraglutide Effect and Action in Diabetes) series of phase 3 studies. Liraglutide in monotherapy (52 weeks of treatment) lowered HbA_{1c} by 0.8% and 1.1% in doses of 1.2 and 1.8 mg, respectively, which was significantly more than the -0.5% change reached with the SU

glimepiride (Garber *et al.*, 2009). In LEAD 2 (Nauck *et al.*, 2009a), liraglutide (1.8 mg) added to existing metformin treatment reduced HbA_{1c} by 1.0%, significantly more than placebo, which increased HbA_{1c} by 0.09%, and was comparable to the reduction observed with glimepiride (0.7%). In LEAD 1 (Marre *et al.*, 2009), liraglutide (1.8 mg) reduced HbA_{1c} by 1.1% when added to glimepiride treatment; this was significantly more than the 0.4% reduction with the addition of rosiglitazone and the 0.2% increase with placebo. In LEAD 4 (Zinman *et al.*, 2009), liraglutide (1.8 mg) added to metformin in combination with rosiglitazone achieved a reduction in HbA_{1c} of 1.5% (significantly better than the 0.5% reduction with placebo). A reduction of 1.3% was reached when liraglutide was added to metformin in combination with glimepiride in the LEAD 5 study (significantly better than placebo [−0.2%] and the active comparator insulin glargine [−1.1%]). In LEAD 6 (Buse *et al.*, 2009) HbA_{1c} reduction was significantly greater with liraglutide treatment than with exenatide (1.1% vs. 0.8%). As for exenatide, liraglutide has significant effect on body weight; the effect seems slightly but not significantly superior to exenatide (Buse *et al.*, 2009). In LEAD 3, body weight reduction of 2.5 kg (baseline 93 kg) was significantly different compared with a 1.1-kg increase with glimepiride (Garber *et al.*, 2009). In LEAD 2, there was a 2.8-kg weight reduction (significantly different from a 1.0-kg increase with glimepiride) (Nauck *et al.*, 2009b). In LEAD 1, a decrease of 0.2 kg was significantly different compared with a 2.1-kg increase with rosiglitazone (Marre *et al.*, 2009). In LEAD 6 (Buse *et al.*, 2009), greater reductions in triglycerides (−0.4 vs. −0.2 mM) and free fatty acids (−0.17 vs. −0.10) in the liraglutide group were observed; and both liraglutide and exenatide caused significant decreases in blood pressure.

The major side effects of exenatide and liraglutide are mild to moderate nausea and vomiting. These side effects are dose dependent and decline over time (Amori *et al.*, 2007). The incidence of treatment-associated hypoglycemia is reported to be low (www.ema.europa.eu). However, combined with SU the risk of minor hypoglycemic episodes is reported to be in the range of 15–36% for exenatide (Amori *et al.*, 2007) and 8–25% for liraglutide (Blonde and Russell-Jones, 2009). Approximately 40% of exenatide-treated patients in long-term, placebo-controlled studies developed antibodies against exenatide (Amori *et al.*, 2007)—it is uncertain whether this has any implications for the effect of the treatment. Among liraglutide-treated patients, 8% exhibited antibodies (Garber *et al.*, 2009; Marre *et al.*, 2009; Nauck *et al.*, 2009b; Russell-Jones *et al.*, 2009; Zinman *et al.*, 2009). At least 200 reports on pancreatitis in exenatide-treated patients has been reported; however, no causal relationship between pancreatitis and GLP-1-based therapy exists. In carcinogenicity studies with liraglutide, C-cell tumors were observed in thyroid tissue of mice and rats. Potential

mechanisms behind C-cell tumor development and possible clinical implications remain to be established (www.ema.europa.eu).

Several GLP-1 analogs in formulations making them suitable for once-weekly administration are in clinical development.

CJC-1134-PC is a modified exendin-4 analog conjugated to human recombinant albumin (HRA) *in vitro* to form a long-acting DPP4-resistant GLP-1R agonist. Despite being a much larger molecule than exendin-4, it retains the ability to bind to and activate the GLP-1R (Baggio *et al.*, 2008). The efficacy of CJC-1134-PC of HbA_{1c} compared with placebo was evaluated in a 3-month phase 2 clinical trial including 224 patients with type 2 diabetes inadequately controlled on metformin. HbA_{1c} was reduced by CJC-1134-PC with 0.8% (1.5–2 mg once weekly) and 1.4% (1.5 mg twice weekly), compared to 0.4% with placebo. Body weight decreased during the 3-month treatment period with reductions of −1.2 kg (−1.5 mg twice weekly); however, this was nonsignificant compared with placebo (−0.4 kg) (Wang *et al.* ADA, 2009). Drug-related nausea (23%), vomiting (11%), and diarrhea (10%) were observed with corresponding values of 10%, 6%, and 8% with placebo treatment. These gastrointestinal side effects were reported to diminish over time.

Albiglutide is generated by fusion of a DPP4-resistant GLP-1 analog dimer to human albumin. The compound has a long half-life as a result of its fusion with albumin and its resistance to degradation by DPP4 brought about by connecting two copies of GLP-1, each with an amino acid substitution (ala to glu) at the DPP4-sensitive hydrolysis site. The efficacy of albiglutide on HbA_{1c} was evaluated in a phase 2 clinical trial in which 356 patients with type 2 diabetes on metformin or drug naïve (baseline HbA_{1c}, 8.0%) were randomized to receive 16 weeks of double-blind s.c. treatment with placebo, open-label exenatide (5 μg twice daily for 4 weeks followed by 10 μg twice daily), or albiglutide in three dosing schedules: weekly (4, 15, or 30 mg), biweekly (15, 30, or 50 mg), or monthly (50 or 100 mg) (Rosenstock *et al.*, 2009). Placebo reduced HbA_{1c} by 0.2%, exenatide by 0.5%, and albiglutide reduced HbA_{1c} dose dependently in each dosing schedule by maximally 0.9% (30 mg once weekly), 0.8% (50 mg biweekly), and 0.9% (100 mg monthly) (Rosenstock *et al.*, 2009). Gastrointestinal related side effects as nausea and vomiting were the most common adverse events, but diminished over the course of the study (Rosenstock *et al.*, 2009).

Taspoglutide (R1583/BIM51077) is also a human GLP-1 analog. It is similar in structure to native GLP-1 except for two amino acid substitutions in positions 8 and 35 with aminoisobutyric acid, which render the molecule resistant to degradation by DPP4. The efficacy of taspoglutide on HbA_{1c} was evaluated in a phase 2 clinical trial in 360 patients with type 2 diabetes inadequately controlled on metformin (baseline HbA_{1c}, 7.9%) (Nauck *et al.*, 2009c). Patients were randomized to receive 8 weeks of double-blind s.c.

treatment with placebo or taspoglutide (5, 10, or 20 mg once weekly or 10 or 20 mg once every 2 weeks) as add-on therapy. Placebo reduced HbA_{1c} by 0.2% and taspoglutide reduced HbA_{1c} by 1.0% (5 mg once weekly, 10 and 20 mg once every 2 weeks) and 1.2% (10 and 20 mg once weekly). Body weight decreased in a dose-dependent manner throughout the 8-week treatment period with significant reductions of 2.1 (10 mg once weekly), 2.8 (20 mg once weekly), and 1.9 kg (20 mg once every 2 weeks) compared to 0.8 kg with placebo (Nauck *et al.*, 2009c). Nausea and vomiting were the most common adverse events to taspoglutide-treatment (52% and 22%, respectively, in the 20-mg once-weekly group). These side effects tended to occur during the first day after drug administration, were associated with peak plasma drug concentration, and resolved within 1 day. With taspoglutide 6 out of the 297 patients experienced mild to moderate hypoglycemia (two of these were asymptomatic). Recently taspoglutide was reported to provide comparable glycemic control, superior weight loss and less hypoglycemia compared to insulin glargine as add-on to inadequate treatment with metformin and SU (Nauck *et al.* ADA, 2010). Also, taspoglutide was reported to provide superior glycemic control compared to sitagliptin (Bergenstal *et al.* ADA, 2010) and twice-daily exenatide (Rosenstock *et al.* ADA, 2010).

Exenatide Once-Weekly is based on a common biodegradable medical polymer (Tracy *et al.*, 1999; Verspohl, 2009). In patients with type 2 diabetes inadequately controlled on metformin and/or diet, 0.8 and 2.0 mg of exenatide once weekly for 15 weeks resulted in HbA_{1c} reductions of 1.4% and 1.7% compared with an increase of 0.4% in the placebo group. The 2.0 mg-group experienced a weight loss of 3.6 kg versus no weight change in the other groups (Kim *et al.*, 2003). A study comparing the effect of 10 μg of exenatide twice daily and 2.0 mg of exenatide once weekly in 295 patients with type 2 diabetes, exenatide once weekly was superior (HbA_{1c} reduction of 1.9% vs. 1.5%). No difference in body weight reduction was observed (−3.7 vs. −3.6 kg) (Drucker *et al.*, 2008). Nausea (predominantly mild in intensity) is also the most frequently reported adverse event among exenatide once weekly-treated patients (19% [0.8 mg] and 27% [2.0 mg] vs. 15% [placebo; Kim *et al.*, 2003]; 39% [2.0 mg] vs. 50% [10 μg of exenatide twice daily; Drucker *et al.*, 2008]; and 33% [2.0 mg; Iwamoto *et al.*, 2009]).

B. DPP4 inhibitors

The antidiabetic effects of GLP-1 can also be exploited by protecting endogenous GLP-1 from degradation by the enzyme DPP4 (Holst and Deacon, 1998). Administration of inhibitors of this enzyme increase the circulating levels of both active GLP-1 and GIP, and this is associated with the expected antidiabetic effects including stimulation of glucose-induced insulin secretion, inhibition of glucagon secretion and possibly preservation

of beta-cell mass (Deacon and Holst, 2006). The inhibitors are small molecules that are active upon oral administration, and, as mentioned, they appear to have antidiabetic effects that are quite similar to those obtained with the incretin mimetics, which all require parenteral administration (Ahren *et al.*, 2004). However, the inhibitors have little effects on body weight, presumably because the plasma concentrations of active GLP-1 are not elevated sufficiently to exert this effect (Deacon, 2005)]. Several specific inhibitors are currently undergoing clinical development, but currently only three (sitagliptin (Januvia®, Merck Sharp and Dohme), vildagliptin (Galvus®, Novartis), and saxagliptin (Onglyza®, AstraZeneca/Bristol-Myers Squibb)) have been approved as treatments of type 2 diabetes. Clinical data on these will be discussed in the following.

Sitagliptin is rapidly absorbed, and steady-state plasma concentrations are attained within 2 days after once-daily dosing (Deacon, 2005). Sitagliptin is not appreciably metabolized *in vivo*, and ~90% of the dose is excreted renally, unchanged as the parent drug. Sitagliptin as monotherapy (100 or 200 mg q.d.) demonstrated a placebo-adjusted reduction in HbA_{1c} of 0.79% and 0.94%, respectively (Aschner *et al.*, 2006). Evaluation of sitagliptin treatment as add-on to ongoing metformin therapy in patients with type 2 diabetes (baseline HbA_{1c} of 8.0%), demonstrated a placebo-subtracted reduction of HbA_{1c} from baseline of 0.65% after 24 weeks of treatment with sitagliptin 100 mg q.d. (Charbonnel *et al.*, 2006). Interestingly, data from another study using sitagliptin or glipizide as add-on to existing metformin therapy (baseline HbA_{1c} of 7.5%) showed that the same levels of glycemic control were obtained in the two groups. However, the incidence of hypoglycemia was markedly higher in the glipizide group compared to the sitagliptin group (32% vs. 5%) and furthermore, a difference in body weight of 2.5 kg in favor of the DPP4 inhibitor was reported. Addition of sitagliptin to patients inadequately controlled on pioglitazone (baseline HbA_{1c} of 8.0%), also significantly improved HbA_{1c} (0.7%) during a 24-week treatment period (Rosenstock *et al.*, 2006).

Vildagliptin is a tight-binding inhibitor with a slow off-rate. It is rapidly absorbed and has low protein binding. The predominant route of metabolism is hydrolysis to produce a pharmacologically inactive metabolite, with 85% of the dose being excreted as this metabolite in the urine. When given for 12-weeks, vildagliptin showed a sustained effect on HbA_{1c} by 0.6% (baseline HbA_{1c} of 8.0%) (Pratley *et al.*, 2006). When vildagliptin (50 mg q.d.) was compared to rosiglitazone (8 mg q.d.) in drug-naïve patients (baseline HbA_{1c} of 8.7%), similar improvements in glycemic control were obtained, but lipid profiles were improved to a greater extent with vildagliptin, which was also body weight neutral (compared to an increase in body weight and risk of edema in the rosiglitazone treated group) (Rosenstock *et al.*, 2007). In a 1-year trial, vildagliptin (50 mg q.d.) was added to patient who were inadequately controlled with metformin

(baseline HbA_{1c} of 7.9%). A decrease in HbA_{1c} of 0.7 percentage points was observed within the initial 12 weeks of treatment and a sustained effect on the glycemic control (a decrease in HbA_{1c} of 1.1 percentage points when compared to placebo) was seen throughout the 1-year study period (open extension). Another study demonstrated that vildagliptin even as add-on to patients with type 2 diabetes (baseline HbA_{1c} of 8.5%) inadequately controlled by insulin (>30 U/day), was able to significantly reduce HbA_{1c} by 0.7 percentage points (Fonseca *et al.*, 2007). In spite of an improved glycemic control, significantly fewer hypoglycemic events were seen in the DPP4-treated group (Fonseca *et al.*, 2007). Interestingly, recent 2-year data comparing vildagliptin and glimepiride in patients with type 2 diabetes inadequately controlled with metformin alone show increased glucagon in glimepiride-treated patients versus decreased glucagon in vildagliptin-treated patients (Ahren *et al.*, 2010).

Saxagliptin is the latest DPP4 inhibitor on the market. It has high potency, selectivity toward DPP4, good oral bioavailability and duration of action ideal for dosing once daily (Augeri *et al.*, 2005; Gallwitz, 2008). In 1306 drug-naïve type 2 diabetes patients saxagliptin as add-on to metformin resulted in 60% of patients reaching an HbA_{1c} goal <7% (Jadzinsky *et al.*, 2009). In a study comparing saxagliptin 2.5 and 5 mg plus glyburide 7.5 mg to glyburide monotherapy over 24 weeks, HbA_{1c} decreases from baseline of −0.5% and −0.6% versus +0.1% were observed (Chacra *et al.*, 2009). As add-on to existing glitazone treatment saxagliptin led to significant decreases in HbA_{1c} versus placebo (−0.7% (2.5 mg) and −0.9% (5 mg) vs. −0.3% (placebo)). Fasting plasma glucose, postprandial glucose and the proportion of patients reaching an HbA_{1c} goal of <7% were significantly improved in the saxagliptin-treated patients (Hollander *et al.*, 2009).

Both preclinical and clinical experience with DPP4 inhibitors show that they have good tolerability and very few side effects. Improving the glycemic control in patients with diabetes is usually associated with an increased risk of developing hypoglycemia. Due to the strictly glucose-dependency of the incretin hormones, hypoglycemia is very rare during incretin-based therapies, even in the fasting state or if a meal is missed. Potential side effects of DPP4 inhibition may result from the inadvertent inhibition of related enzymes. Some debate has been raised concerning the role of DPP4 activity for normal immune function, but it appears that its absence can be compensated for (Marguet *et al.*, 2000; Pederson *et al.*, 1996) and that treatment with DPP4 inhibitors is not associated with adverse events of any concern (Deacon and Holst, 2006; Lankas *et al.*, 2005). Regarding patients with renal impairment, studies are ongoing (Deacon and Holst, 2009; Gallwitz, 2008; Tahrani *et al.*, 2009); however, saxagliptin was well-tolerated in subjects with mild, moderate, or severe hepatic impairment (Gallwitz, 2008). Final evaluation particularly with respect to rare unexpected side effects must await results from additional longer-term

clinical studies, but the currently available data show an excellent safety profile and tolerability of the DPP4 inhibitors.

V. Conclusion and Perspectives

Over the past more than 100 years the scientific description of the incretin hormones and their effects has revealed incretin physiology to be of utmost importance in the regulation of glucose homeostasis. Even though the notion that gut-derived mechanisms activated following meal ingestion result in insulin release stretches a long way back, GLP-1 was not described until three decades; and the first GLP-1-based antidiabetic treatment modality has now been on the market for six years. To call the effects of GLP-1 antidiabetic is almost an understatement—antidysmetabolic might be more suiting as GLP-1-based treatment potentially can reduce overweight, attenuate hypertension and improve dyslipidemia. Preexisting antidiabetic agents target only one aspect of the pathophysiology of type 2 diabetes, and notably neither tackle the progressive deterioration in beta-cell mass and function nor the hyperglucagonemia that accompanies the disease. In contrast, incretin-based approaches are pleiotropic, and unlike existing therapies, both alpha- and beta-cell dysfunction are targeted. Actually, the glucagonostatic effect of the "GLP-1" levels brought about by injection of GLP-1 analogs seems to account for as much as half of the total glucose-lowering effect of GLP-1 in patients with type 2 diabetes (Hare *et al.*, 2010). This potent alpha-cell inhibition, together with the effect on gastric emptying, opens the possibility of clinical benefits of incretin-based treatment in patients with type 1 diabetes (Kielgast *et al.*, 2009). Studies indicate that incretin-based therapies—perhaps especially because of the potential trophic effects on the pancreatic beta-cells—may halt the progression of disease that inevitably seems to accompany conventional treatment. So far this has not been established in long-term clinical trials, but animal studies show that administration of GLP-1 analogs or DPP4 inhibitors is associated with beta-cell proliferation and beta-cell protection. If the beta-cell-preserving potential of these drug classes can be demonstrated in humans, incretin-based therapies may be able to counteract one of the underlying causes of progression of type 2 diabetes; the gradual loss of beta-cell function and mass. Furthermore, clinical studies of the already marketed drugs have also shown promising effects on lipid profile and blood pressure, which could imply substantial benefit on macrovascular outcomes, a critical therapeutic goal in the treatment of type 2 diabetes. Within the next years many new and promising GLP-1 analogs and also new and promising DPP4 inhibitors will be introduced to the market, and, the future will elucidate whether these

will have the potential of being disease-modifying drugs and reduce the risk of cardiovascular disease in patients with type 2 diabetes.

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CHAPTER SIXTEEN

GPR119 Agonists for the Potential Treatment of Type 2 Diabetes and Related Metabolic Disorders

Unmesh Shah* *and* Timothy J. Kowalski†

Contents

Abstract

Type 2 diabetes (T2D) has reached epidemic proportions, and there is an unmet medical need for orally effective agents that regulate glucose homeostasis. GPR119, a class-A (rhodopsin-like) G protein-coupled receptor expressed primarily in the pancreas and gastrointestinal tract, has attracted considerable interest as a T2D drug target in recent years. The activation of GPR119 increases the intracellular accumulation of cAMP, leading to enhanced glucose-dependent insulin secretion from pancreatic β-cells and increased release of the gut peptides GLP-1 (glucagon-like peptide 1), GIP (glucose-dependent insulinotropic

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peptide) and PYY (polypeptide YY). Oral administration of small molecule GPR119 agonists has been shown to improve glucose tolerance in both rodents and humans. This review summarizes the research leading to the identification of GPR119 as a potential drug target for T2D and related metabolic disorders, and provides an overview of the recent progress made in the discovery of orally active GPR119 agonists.

I. Introduction

The prevalence of diabetes mellitus has increased at an alarming rate in recent years, with an estimated 285 million people worldwide suffering from this serious chronic disease (International Diabetes Federation Diabetes Atlas, 2009). By 2030, this number is projected to exceed 400 million, costing the world economy more than US$490 billion in health-care expenditure. Sedentary lifestyles and the increased occurrence in obesity have additionally contributed to the predicted diabetes epidemic (Diamond, 2003; Hossain *et al.*, 2007). Although diabetes is underreported as a cause of death, the associated secondary complications related to cardiovascular disorders, retinopathy, nephropathy, and neuropathy are often the leading cause of mortality.

The majority of diabetic patients (90–95%) have type 2 diabetes (T2D), which is characterized by high blood glucose levels (hyperglycemia) resulting from insulin resistance, defective insulin secretion (β-cell dysfunction), or hepatic glucose overproduction (Leahy, 2008; Stumvoll *et al.*, 2005). Current pharmacological treatments fall into three major categories: drugs that improve insulin sensitivity, drugs that increase insulin secretion from β-cells in a glucose-independent or glucose-dependent fashion, and insulin replacement (Ashiya and Smith, 2007; Levetan, 2007; Stumvoll *et al.*, 2005). The first-line drugs include the insulin-sensitizing agent metformin and the glucose-independent insulin-releasing agents such as sulfonylureas and meglitinide analogs. Although both the classes of drugs effectively lower HbA_{1c} and are heavily prescribed, they do not produce durable improvements in disease progression. In addition, sulfonylureas carry the side effects of hypoglycemia and weight gain. The peroxisome proliferator activator receptor gamma (PPARγ) agonist class of insulin sensitizers (thiazolidinediones; TZDs) is the most prescribed after metformin and sulfonylureas. TZDs display robust efficacy but are associated with weight gain, congestive heart failure, and fractures; death due to cardiovascular events has been suggested with rosiglitazone, but the data are currently inconclusive (Nissen and Woslki, 2007). Alpha-glucosidase inhibitors, agents that work by inhibiting the breakdown of carbohydrate to glucose in the intestine, are only modestly effective and produce GI side effects. Although insulin is

associated with weight gain and hypoglycemia, there is increasing acceptance of its use as a second medication in addition to metformin in subjects with an A1c level $>8.5\%$ (Nathan *et al.*, 2009).

The gastrointestinal hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are released in response to ingestion of food, stimulate insulin secretion in a glucose-dependent manner (Drucker, 2007). Strategies involving GIP have received relatively less attention, primarily due to the decreased GIP responsiveness in T2D patients (Vilsbøll *et al.*, 2002). On the other hand, approaches focused on exerting a pharmacological GLP-1 effect have resulted in the recent introduction of a new class of drugs that targets GLP-1 or its receptor (Drucker *et al.*, 2010). These agents enhance glucose-dependent insulin secretion (GDIS) by direct agonism of the GLP-1 receptor (GLP-1 mimetics; e.g., exenatide, liraglutide) (Drucker and Nauck, 2006) or by preventing the breakdown of endogenously produced GLP-1 through selective inhibition of dipeptidyl peptidase IV (DPP-IV inhibitors; e.g., sitagliptin, vildagliptin, saxagliptin) (Ahrén, 2008; Brubaker, 2007). Both GLP-1 mimetics and DPP-IV inhibitors provide effective lowering of HbA1c and present a significantly low risk of hypoglycemia.

In addition to stimulating GDIS, GLP-1 has been shown to suppress glucagon secretion, delay gastric emptying, and decrease appetite (Drucker and Nauck, 2006; Gutniak *et al.*, 1992). Activation of GLP-1 receptors has also been shown to enhance β-cell proliferation in rodents and to inhibit β-cell apoptosis in rodent and human islets (Baggio and Drucker, 2006; Brubaker and Drucker, 2004; Farilla *et al.*, 2003). While the identification of orally active small-molecule GLP-1 agonists continues to remain challenging, strategies complementary to DPP-IV inhibitors that are based upon enhancing GDIS via stimulation of incretin hormone release have emerged as a promising approach in the treatment of diabetes. Furthermore, the use of a GLP-1 secretagogue in combination with DPP-IV inhibition may not only provide improved glycemic control, but also induce weight loss, a feature observed with GLP-1 mimetics but not with DPP-IV inhibitors. Several nonpeptide binding G protein-coupled receptors (GPCRs) have been deorphanized recently and are currently being evaluated as candidate GLP-1 secretagogues for T2D (Ahrén, 2009; Fyfe *et al.*, 2007a; Mohler *et al.*, 2009). Among these, the G protein-coupled receptor 119 (GPR119) has received considerable attention from the pharmaceutical industry in recent years (Fyfe *et al.*, 2008a; Jones and Leonard, 2009; Jones *et al.*, 2009; Overton *et al.*, 2008; Shah, 2009). This review summarizes the research leading to the identification of GPR119 as a potential target for T2D and related metabolic disorders, and provides an overview of the recent progress made in the discovery of orally active GPR119 agonists.

II. GPR119 Receptor Expression

GPR119 is a class A (rhodopsin-like) GPCR originally identified through a bioinformatics approach (Fredriksson *et al.*, 2003). The initial sequencing and signaling characteristics of this receptor were independently described by several research groups in the scientific and patent literature under various names (see Fyfe *et al.*, 2008a). Although GPR119 belongs to the class A GPCR family, there is little overall sequence homology to other receptors. A phylogenetic analysis assigned GPR119 to the subfamily of biogenic amine and MECA (*m*elanocortin, *e*ndothelial, *c*annabinoid, and *a*denosine) receptors (Costanzi *et al.*, 2008; Fredriksson *et al.*, 2003), and its closest homologs are the adenosine (A1 and A3) and cannabinoid receptors (Brown, 2007; Costanzi *et al.*, 2008). The human receptor is encoded by a single exon gene located on the short arm of chromosome X (Xp26.1) and is comprised of 335 amino acids. GPR119 homologs have been identified in several vertebrate species, including the mouse, rat, zebrafish, monkey, and dog (see Overton *et al.*, 2008).

The GPR119 receptor displays a restricted expression pattern, with pancreas, fetal liver, and GI tract identified as the major sites of expression in human tissues (Bonini *et al.*, 2001, 2002; Griffin, 2006; Jones *et al.*, 2004). In rodents, however, the receptor is also expressed in many regions of the brain in addition to the pancreas and GI tract (Bonini *et al.*, 2001, 2002; Jones *et al.*, 2004). Expression analysis within human and rodent pancreas has shown that GPR119 mRNA is localized to the islets of Langerhans (Chu *et al.*, 2007; Jones *et al.*, 2004; Soga *et al.*, 2005). No definitive characterization of islet cell GPR119 expression has been performed; however, *in situ* hybridization (Chu *et al.*, 2007; Lan *et al.*, 2009) and immunohistochemical studies (Chu *et al.*, 2007) in rat and mouse pancreas strongly suggest that the receptor is expressed in insulin-producing β-cells. Expression of GPR119 mRNA in insulinoma-derived β-cell lines (MIN6, HIT-T15, NIT-1, RIN5) provides further evidence for the β-cells as a major site of GPR119 expression within the pancreatic islets (Chu *et al.*, 2007; Jones *et al.*, 2004; Soga *et al.*, 2005). Currently, it is not known whether GPR119 is also expressed in the glucagon-secreting α-cells. Immunohistochemical and double-immunofluorescence studies performed by Sakamoto *et al.* (2006) have shown predominant localization of GPR119 in pancreatic polypeptide-secreting rat and mouse islet cells, with no definitive immunoreactivity observed in β-cells; however, these findings have not been replicated.

In addition to the pancreas, significant expression of GPR119 has been detected in the duodenal, jejunal, ileal, and colonic regions of the human and rodent GI tract (Bonini *et al.*, 2001, 2002; Chu *et al.*, 2008). *In situ* hybridization studies have demonstrated colocalization of GPR119 with preproglucagon-expressing L-cells in rat ileum and in mouse colon (Chu *et al.*, 2008). Additionally, recent studies have demonstrated GPR119 expression in microdissected murine intestinal L-cells and GIP-secreting

K-cells (Parker *et al.*, 2009). Consistent with these observations, Northern Blot analysis has shown that GPR119 is expressed in GLP-1 producing GLUTag and STC-1 cell lines (Chu *et al.*, 2008).

III. GPR119 Signaling and Deorphanization

High-level expression of GPR119 in transfected HEK293 cells led to an increase in intracellular cAMP levels via activation of adenylate cyclase (Bonini *et al.*, 2001, 2002; Chu *et al.*, 2007; Overton *et al.*, 2006), indicating that this receptor couples efficiently to $G\alpha_s$. Using this constitutively active GPR119-expressing cell line, Chu *et al.* (2007) failed to observe increases in inositol phosphate with or without coexpression of a $G\alpha_q/G\alpha_i$ chimera, suggesting that GPR119 exhibits poor coupling efficiency to $G\alpha_i$ and $G\alpha_q$.

Efforts to deorphanize GPR119 have identified two classes of potential endogenous ligands: phospholipids and fatty-acid amides (Overton *et al.*, 2006; Soga *et al.*, 2005). Soga *et al.* (2005) reported that palmitoyl-, stearoyl-, and oleoyl-lysophosphatidylcholine (LPC), as well as lysophosphatidylethanolamine, oleoyl-lysophosphatidic acid, and lysophosphatidylinositol, increase cAMP accumulation in human GPR119-transfected RH7777 cells, with EC_{50}s ranging from 1.5 to 5.7 μM. The same group showed that oleoyl-LPC (Fig. 16.1) increases glucose-dependent insulin release in perfused rat pancreas and enhances insulin release and cAMP accumulation in NIT-1 cells. A partial knockdown of GPR119 expression using a GPR119-specific siRNA attenuated the LPC-induced insulin release from NIT-1 cells, suggesting that this lysophospholipid promotes insulin release via GPR119 signaling. Recent reports, however, have failed to demonstrate the GPR119-dependent nature of LPC-induced insulin release in RIN-5F cell lines (Chu *et al.*, 2010) or in isolated murine islets (Lan *et al.*, 2009).

Overton *et al.* (2006) have reported that the fatty-acid amide oleoylethanolamide (OEA; Fig. 16.1) promotes a concentration-dependent increase in

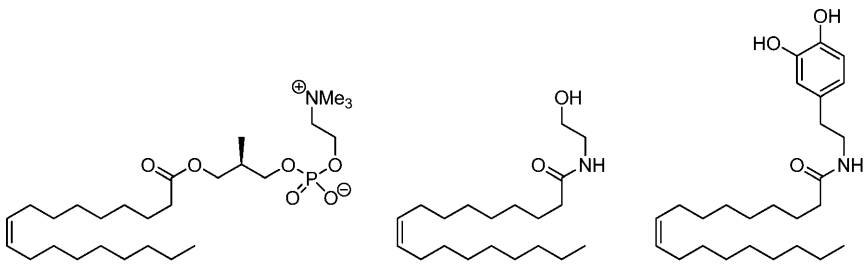


Figure 16.1 Proposed endogenous ligands of GPR119.

cAMP levels in stably transfected and endogenous GPR119-expressing cell lines with potency that was greater than LPC. Subsequently, Ning *et al.* (2008) showed that OEA increases GDIS in MIN6 cells, and this was found to be associated with increased cAMP and potentiation of glucose-stimulated increases in intracellular calcium. Recently, Lauffer *et al.* (2009) demonstrated OEA-induced increases in GLP-1 secretion in human- and mouse-derived intestinal L-cells (hNCI-H716 and mGLUTag cells, respectively). This finding was shown to be a GPR119-dependent phenomenon since the effect was significantly reduced in mGLUTag cells transfected with GPR119-specific siRNA. Interestingly, the effects of OEA on GLP-1 release were diminished at higher concentrations. The authors attributed this to possible GPR119 desensitization as $G\alpha_s$-coupled receptors are known to undergo homologous desensitization (Kelly *et al.* 2008; Lauffer *et al.*, 2009). OEA has also been described to reduce food intake and body weight gain in rodent feeding models (Rodríguez de Fonseca *et al.*, 2001). Overton *et al.* (2006) have hypothesized that these effects of OEA may be mediated, at least in part, via the GPR119 receptor. On the other hand, studies using transgenic mouse models have shown that the hypophagic effect of OEA is maintained in GPR119 null mice (Lan *et al.*, 2009) but is absent in PPARα null animals (Fu *et al.*, 2003), questioning the importance of GPR119 signaling in the feeding effects of OEA.

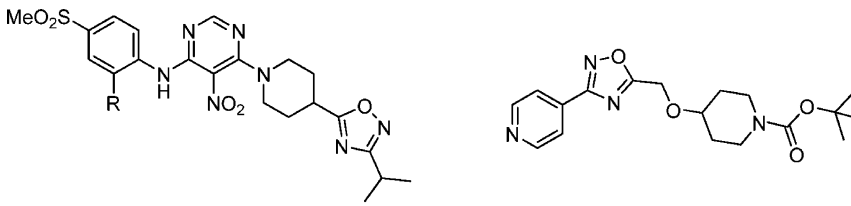
More recently, Chu *et al.* (2010) have identified *N*-oleoyldopamine (OLDA; Fig. 16.1) and other hydroxybenzyl lipid amides as activators of GPR119. OLDA was demonstrated to increase insulin release *in vitro* using HIT-T15 cells, which endogenously express GPR119, and in RIN-F5 cells stably transfected with GPR119. Although OLDA is also a transient receptor potential vanilloid 1 (TRPV1) agonist, the effect of OLDA on insulin release in HIT-T15 cells was similar in the presence and absence of the TRPV1 antagonist 6′-iodononivamide. *In vivo* studies demonstrated that oral administration of OLDA (100 mg/kg) increased GIP release and improved oral glucose tolerance in mice; these effects were absent or attenuated in GPR119 null mice. Although enzymes responsible for the synthesis and degradation of OLDA were shown to be present in pancreatic islets, data supporting a role for OLDA as an endogenous GPR119 ligand are lacking. Nonetheless, this work raises the possibility that other lipid amides may play a physiological role via GPR119 signaling.

IV. GPR119 Agonism and Glucose Homeostasis

The distribution of GPR119 has implicated a role in glucose homeostasis and feeding behavior/satiety. Mice null for GPR119 are viable and appear to develop and reproduce normally. Consistent with the effects of GPR119

signaling on GLP-1 release *in vitro*, $gpr119^{-/-}$ mice have been shown to display lower plasma GLP-1 [7–36]amide levels in the postprandial state and after an oral glucose load (Chu *et al.*, 2008; Lan *et al.*, 2009). Additionally, GPR119 null mice maintained on a low fat (10% of kcal) semipurified diet were reported to have lower body weight and fat mass relative to wild-type mice; this was associated with a trend toward lower food intake (Lan *et al.*, 2009). Interestingly, despite these differences, glucose tolerance and insulin sensitivity of $gpr119^{-/-}$ mice, as judged by an oral glucose tolerance test (oGTT), an insulin tolerance test, and plasma glucose and insulin levels, were similar to wild-type mice in the transgenic line generated by Lan *et al.* (2009).

The discovery of synthetic GPR119 agonists has enabled studies in rodent models of diabetes and obesity, providing further insight into the role of this receptor on metabolic homeostasis and the use of agonists for therapeutic benefit in T2D and obesity. The best described agonist to date is AR231453 (Fig. 16.2), a small-molecule GPR119 agonist discovered at Arena Pharmaceuticals, Inc. (Semple *et al.*, 2008), which has been utilized extensively to study the role of GPR119 in glucose homeostasis (Chu *et al.*, 2007, 2008). AR231453 is a highly potent and selective GPR119 agonist with no off-target activity when screened against a panel of receptors and enzymes, including pancreatic islet receptors, known and orphan GPCRs, and enzymes such as DPP-IV (Chu *et al.*, 2007). AR231453 significantly increased cAMP levels in transfected HEK293 cells (EC_{50} = 5.7 nM) and in HIT-T15 cells (EC_{50} = 4.7 nM), and increased insulin release in transfected RIN-5F insulinoma cells and in HIT-T15 cells (EC_{50} = 3.5 nM) (Chu *et al.*, 2007). The effects on cAMP and insulin release were similar to those elicited by forskolin, and the incubation of a GPR119-selective siRNA rendered AR231453 inactive in HIT-T15 cells. Using isolated rat and mouse islets, AR231453 was shown to stimulate insulin secretion in a glucose-dependent manner with a magnitude similar to that of GLP-1. The administration of AR231453 to lean C57BL/6, KK^{Ay}, or $Lepr^{db/db}$ mice improved oral glucose tolerance, which was accompanied by an increase in plasma insulin levels in lean mice. The efficacy of AR231453 in lean mice (20 mg/kg) was comparable to that of the sulfonylurea glyburide (30 mg/kg, p.o.). However, in contrast to



AR231453 PSN632408

Figure 16.2 Structures of prototypical GPR119 agonists used as research tools.

glyburide, which stimulates insulin release in a glucose-independent fashion, administration of high doses of AR231453 (100 mg/kg) to fasted mice did not induce hypoglycemia or increase insulin release. AR231453 was inactive in GPR119-deficient mice, suggesting that the observed *in vivo* effects were indeed mediated by GPR119 (Chu *et al.*, 2007).

The effect of AR231453 on glycemic excursion was more pronounced when glucose was administered via an oral rather than an intraperitoneal route (Chu *et al.*, 2007). Additionally, AR231543 enhanced cAMP accumulation and GLP-1 release in GLUTag cells (Chu *et al.*, 2008). These data, along with the observations in the *gpr119*$^{-/-}$ mice, suggest that GPR119 agonists stimulate incretin release *in vivo*. Indeed, administration of AR231453 (10 mg/kg, p.o.) to lean mice stimulated a glucose-dependent increase in active GLP-1 levels. In addition, coadministration of AR231453 with the GLP-1 protecting DPP-IV inhibitor, sitagliptin, enhanced both plasma GLP-1 levels and oral glucose tolerance significantly more than with either agent alone (Chu *et al.*, 2008); no such enhancement was seen in GPR119-null mice. The role of GLP-1 release in the GPR119-mediated improvement of glucose tolerance was further demonstrated by Chu *et al.* (2008), showing that the efficacy of AR231453 was reduced in the presence of the GLP-1 antagonist exendin-(9–39) (Göke *et al.*, 1993; Kolligs *et al.*, 1995). In addition to increased GLP-1 release, administration of AR231453 to mice (10 mg/kg) was shown to increase plasma GIP levels prior to an oral glucose load and to augment the glucose-induced elevation of plasma GIP levels. The effect of AR231453 on plasma GIP levels was absent in *gpr119*$^{-/-}$ mice. Furthermore, using MIN6 pancreatic β-cells expressing GPR119, AR231453 was demonstrated to have a potential in protecting pancreatic β-cells through increased cAMP levels (Jones, 2006). Separately, researchers at Prosidion Ltd. (a subsidiary of OSI Pharmaceuticals, Inc.) have reported beneficial effects on disease progression with their GPR119 agonists following chronic oral administration in prediabetic *db/db* mice (Fyfe and Widdowson, 2007).

In addition to effects on glucose tolerance, synthetic GPR119 agonists from Prosidion have also been shown to modulate food intake and body weight (Fyfe *et al.*, 2007b, 2008b; Overton *et al.*, 2006). Overton *et al.* (2006) demonstrated that the GPR119 agonist PSN632408 (Fig. 16.2) produced a concentration-dependent increase in cAMP levels (EC_{50} = 1.9 μM) similar to the endogenous ligand OEA (EC_{50} = 2.9 μM) in an HEK-293 cell line stably expressing hGPR119. In acute rat studies, PSN632408 (100 mg/kg, i.p. and p.o.) reduced 24 h cumulative food intake, and upon subchronic administration (100 mg/kg, p.o.) to both diet-induced obese (DIO) and high-fat-fed rats, the compound reduced food intake, body weight gain, and white adipose tissue deposition. Although Overton *et al.* (2006) indicated that PSN632408 was a selective GPR119 agonist, subsequent work from Ning *et al.* (2008) has demonstrated that PSN632408 and OEA significantly differ from each other in their ability to mobilize calcium in MIN6c4 cells.

Since GPR119 agonists have been shown to increase GLP-1 levels and because administration of GLP-1 itself is known to reduce food intake, the hypophagic effects seen with PSN632408 were proposed by Overton *et al.* (2006) to be mediated, at least in part, by activation of GPR119. Data from GPR119-knockout mice confirming the specificity of the PSN632408-induced hypophagia and weight loss were not reported in this study by Overton *et al.* (2006), and the hypophagic effects of small molecular weight GPR119 agonists at doses that improve glucose tolerance have not been reported in peer-reviewed literature by other groups.

Taken together, GPR119 agonists have a potential to regulate glucose homeostasis by stimulating glucose-dependent insulin release directly by enhancing pancreatic β-cell function, and indirectly by increasing levels of the incretin hormones GLP-1 and GIP. In addition, preliminary data suggest that orally active GPR119 agonists could prevent weight gain, and thereby help in controlling obesity and related disorders. Several pharmaceutical research organizations have ongoing medicinal chemistry programs in this field and four companies have disclosed that they are currently investigating GPR119 agonists in human clinical trials.

V. GPR119 Agonists: Medicinal Chemistry

The emerging interest in investigating the therapeutic potential of orally active GPR119 agonists is exemplified by the multitude of patent applications that have appeared in recent years (see Jones and Leonard, 2009; Jones *et al.*, 2009; Shah, 2009). Based on the available structure–activity relationship (SAR) data, most GPR119 agonists can be viewed as containing two important pharmacophores: an aryl or heteroaryl moiety substituted with a hydrogen-bond accepting group, and a piperidine moiety N-capped with a carbamate or an isosteric heterocycle. These two structural motifs are usually connected through a central spacer containing a heterocyclic ring or an acyclic chain. The following sections provide an overview of the multiple classes of GPR119 agonists, along with the available biological data, reported by various pharmaceutical organizations.

A. Arena Pharmaceuticals

The GPR119 medicinal chemistry efforts at Arena initiated upon identification of **1** (Fig. 16.3) as a high-throughput screening (HTS) hit (Semple *et al.*, 2008). Although this compound demonstrated activity as an inverse agonist in a membrane cyclase assay ($IC_{50} = 84$ nM), its nitro-pyrimidine core provided the platform for conducting a parallel synthesis. Replacement of the pyrazolo unit with a phenyl ring containing an H-bond-accepting group at the

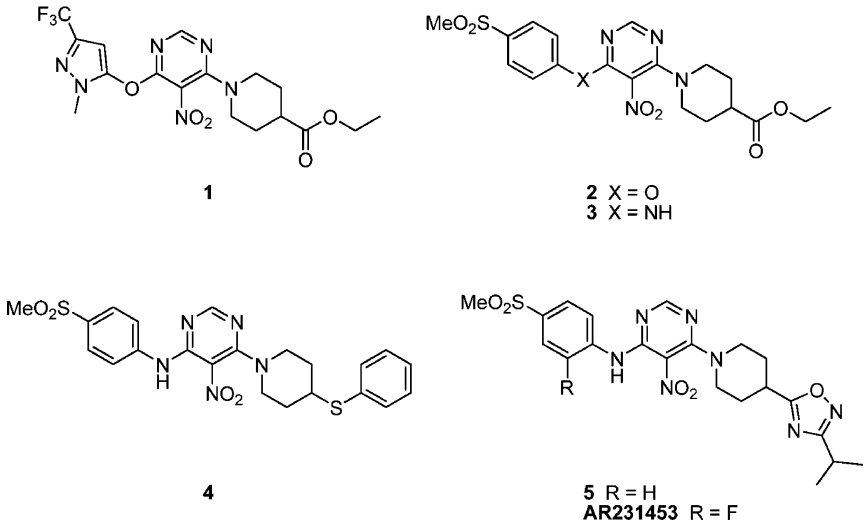


Figure 16.3 First generation GPR119 agonists from Arena Pharmaceuticals.

4-position, in particular, a methylsulfone substituent, provided full agonists; the agonist activity was maintained with both the ether and an aniline linker (e.g., **2**: $EC_{50} = 1.9$ μM, **3**: $EC_{50} = 1.2$ μM; Fig. 16.3). On the other side of the molecule, exchanging the labile ester functionality with a thiophenyl moiety resulted in GPR119 agonists with improved potency (e.g., **4**: $EC_{50} = 50$ nM). Further optimization was conducted by replacing the membrane cyclase assay with a more reproducible melanophore dispersion assay (Potenza *et al.*, 1992; Semple *et al.*, 2008). An isopropyl-substituted oxadiazole was identified as an optimal substituent on the piperidine ring (e.g., **5**: $EC_{50} = 5.8$ nM) and incorporation of a 2-F substituent on the aniline moiety provided a further boost in potency. This resulted in the discovery of Arena's prototypical compound AR231453 ($EC_{50} = 0.68$ nM) discussed in previous sections. Although AR231453 demonstrated robust *in vivo* activity in the mouse oGTT, it did not show significant effect on glucose excursion in a rat oGTT. This was believed to be due to the poor exposure of the compound in rats (10 mg/kg, p.o.: $t_{max} = 1$ h, $C_{max} = 0.25$ μM, AUC = 263 h ng/mL, and $t_{1/2} = 1.1$ h) compared to mice (10 mg/kg, p.o.: $t_{max} = 0.5$ h, $C_{max} = 9.84$ μM, AUC = 19803 h ng/mL, and $t_{1/2} = 3.4$ h).

The next generation GPR119 agonists from Arena were derived from a 4-(piperidin-4-yloxy)pyrimidine structural class (Jones *et al.*, 2004, 2005a,b, 2007). Replacement of the undesirable aromatic nitro group on the central ring with a variety of small substituents was shown to be tolerated (Fig. 16.4). For example, the C5 unsubstituted analog **8** and the C5 cyano analog **9** reduced the glucose excursion by 39% (10 mg/kg, p.o.) in rat oGTTs

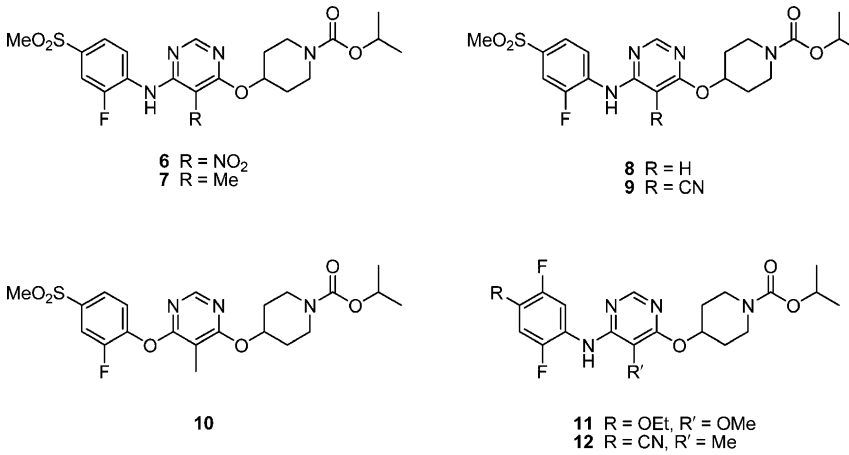
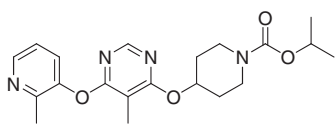


Figure 16.4 Representative 4-(piperidin-4-yloxy)pyrimidine-based GPR119 agonists from Arena.

(Jones *et al.*, 2005a,b). Similar to the first generation analogs, the left-side structural motif in the 4-(piperidin-4-yloxy)pyrimidine series consisted of an aryl moiety attached to the central ring either via a nitrogen or an oxygen linker (e.g., **10**). Although the methylsulfone moiety seems to be a preferred *para*-substituent on the aryl ring, other groups have also been claimed at this position in Arena's GPR119 patent applications. By way of illustration, the ethoxy analog **11** (Jones *et al.*, 2007) exhibited a 34% inhibition of glucose excursion in rats (10 mg/kg, p.o.), and similarly, the cyano compound **12** (Jones *et al.*, 2005b) reduced the glucose excursion by 38% (30 mg/kg, p.o.).

Further SAR efforts were directed toward improving the solubility and this was achieved by replacing the left-side phenyl ring with a pyridyl ring (Fig. 16.5). The methylpyridyl analog **13** possessed an improved aqueous solubility of 40 mg/mL and demonstrated promising *in vitro* and *in vivo* activity; the available data on three pyridyl analogs are summarized in Fig. 16.5 (Jones and Lehmann, 2007, 2008; Jones *et al.*, 2008). Furthermore, the pyridyl analogs demonstrated good pharmacokinetic properties, presumably due to the improved solubility. For example, compounds **14**–**16** exhibited a linear rising-dose pharmacokinetic profile in fasted male SD rats—AUC (3, 30, 300 mg/kg) = **14**: 3.59, 79.82, 285.99 μg h/mL; **15**: 4.73, 55.9, 515.32 μg h/mL; **16**:14.91, 65.91, 418.53 μg h/mL (Jones and Lehmann, 2008; Jones *et al.*, 2008).

Several modifications around the central core have been described in Arena's patent applications. Replacement of the pyrimidine ring with a pyridine provided compound **17** (Fig. 16.6), which caused 43% inhibition of glucose AUC in the rat oGTT at a dose of 30 mg/kg (Jones *et al.*, 2005b). Analogs containing a bicyclic central core based on isomeric pyrazolopyrimidine,



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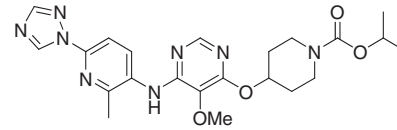

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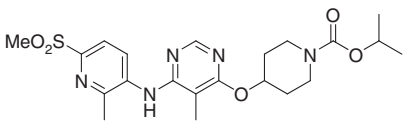

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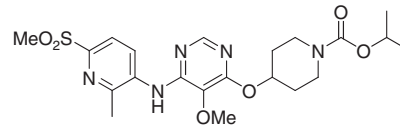

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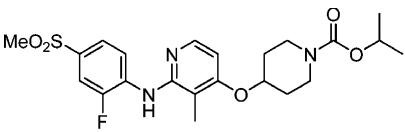
Compound	EC_{50} (nM); membrane cyclase assay				Rat oGTT, % I of glucose excursion
	Human (h)	Dog (d)	Mouse (m)	Rat (r)	
13	2	1	41	44	70, 24, 22 % (30, 3, 0.3 mg/kg)
14	2	4	57	81	60% (dose not specified)
15	2	8	43	42	38% (10 mg/kg)

Figure 16.5 SAR of methylpyridine analogs discovered at Arena.



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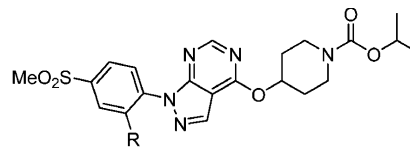


18 R = F
19 R = H

Figure 16.6 Selected Arena-discovered GPR119 agonists derived from central core modifications.

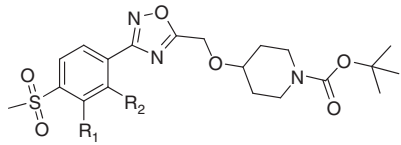
imidazopyrimidine, triazolopyrimidine, isoxazolopyrimidine, quinoline, and pyridopyrimidine ring systems have also been investigated extensively (Jones *et al.*, 2005c). Of particular interest is the fused-pyrazolo analog **18** (Fig. 16.6), which has been highlighted in a single-compound Arena process patent (Gharbaoui *et al.*, 2006a). Although the biological data for this compound have not been disclosed, the corresponding *des*-fluoro analog **19** was reported to exhibit an EC_{50} of 12.7 nM in the melanophore assay (Jones *et al.*, 2005c); incorporation of an *ortho*-fluoro substituent on the phenyl ring in other Arena series has typically resulted in improved potency. Data on the dose-escalation

pharmacokinetics (10–1000 mg/kg, p.o.) generated in SD rats have also been presented for **18** (Jones and Lehmann, 2008; Jones *et al.*, 2008).

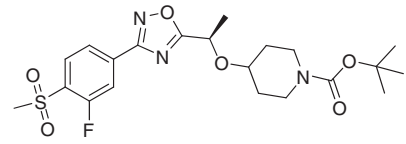
In December 2004, Arena announced a collaboration agreement with Ortho-McNeil Pharmaceutical, Inc., under which two Arena-discovered GPR119 agonists were selected for preclinical development (Arena Pharmaceuticals, Inc., Press Release, December 23, 2004; http://arna.client.shareholder.com/releasedetail.cfm?ReleaseID=320778; last accessed March 12, 2010). The first compound, APD668 (also known as JNJ28630355), displayed high GPR119 potency across various species ($hEC_{50} = 0.47$ nM, $mEC_{50} = 0.98$ nM, $rEC_{50} = 2.51$ nM; melanophore dispersion assay) and demonstrated good *in vivo* activity (3–30 mg/kg, p.o.) in rat and mouse oGTT studies (Semple, 2008). Chronic administration in prediabetic ZDF rats (10, 30 mg/kg, qd, 8 weeks) demonstrated APD668 to inhibit increases in blood glucose levels without causing hypoglycemia. The compound dose-responsively inhibited glucose excursion in an oGTT at week 9, suggesting no tachyphylaxis liability. Compared to a known DPP-IV inhibitor, APD668 was found to be more potent at a dose of 30 mg/kg. In addition to delaying the onset of hyperglycemia, APD668 delayed elevation of HbA1c and also decreased the levels of triglycerides and free fatty acids. Furthermore, APD668 demonstrated a reduction in food intake (30 mg/kg) causing a slight decrease in body weight. The structure of APD668 (JNJ28630355) was recently disclosed as compound **10** (Gharbaoui *et al.*, 2006b; Jones 2010). In February 2006, Ortho-McNeil initiated a phase 1 clinical trial of APD668 for the treatment of T2D (Arena Pharmaceuticals, Inc., Press Release, February 07, 2006; http://arna.client.shareholder.com/releasedetail.cfm?ReleaseID=320321; last accessed March 12, 2010). The initial clinical studies evaluated healthy volunteers and patients with T2D, and encouraging results were announced from this study. However, APD668 was put on hold to advance a potentially more potent GPR119 agonist discovered at Arena (Arena Pharmaceuticals, Inc., Press Release, January 07, 2008; http://arna.client.shareholder.com/releasedetail.cfm?ReleaseID=320208; last accessed March 12, 2010). In December 2008, Ortho-McNeil–Janssen Pharmaceuticals, Inc., initiated phase 1 clinical trials of the second Arena-discovered GPR119 agonist, APD597 (also known as JNJ38431055), for the treatment of T2D (Arena Pharmaceuticals, Inc., Press Release, December 15, 2008; http://arna.client.shareholder.com/releasedetail.cfm?ReleaseID=354391; last accessed March 12, 2010). The structure of APD597 has not been disclosed.

B. Prosidion Ltd.

The GPR119 agonist program at Prosidion evolved from their earlier lead PSN632408 (Fig. 16.2; $EC_{50} = 5.6$ μM, $E_{max} = 110\%$). Replacement of the left-side pyridine ring with the more commonly employed methanesulfonyl phenyl motif (Fig. 16.7), while retaining the oxadiazole core, was



20 R_1=H, R_2=H
21 R_1=H, R_2=F
22 R_1=F, R_2=H



PSN119-2

Figure 16.7 Oxadiazole-based GPR119 agonists from Prosidion.

shown to be tolerated (e.g., **20**: EC_{50} = 3.8 μM, E_{max} = 243%) (Fyfe *et al.*, 2008b). As observed with Arena analogs, introducing a fluoro substituent *meta* to the sulfone resulted in improved potency (e.g., **21**: EC_{50} = 1.8 μM, E_{max} = 200%). However, moving the fluoro group adjacent to the sulfone moiety provided analogs with superior EC_{50} and E_{max} values (e.g., **22**: EC_{50} = 0.9 μM, E_{max} = 375%). A narrow SAR was observed in this region, as exchanging the fluoro group with other substituents (–Cl, –Me, –OMe, $–NH_2$, $–NMe_2$) resulted in diminished activity. On the other hand, incorporating an (*R*)-methyl group to the methyleneoxy linker provided a more potent analog, PSN119-2 (EC_{50} = 0.4 μM, E_{max} = 358%) (Fyfe *et al.*, 2008b). The stereochemistry of the methyl group was shown to be important since the corresponding (*S*)-isomer was considerably less potent and efficacious (EC_{50} = 5.3 μM, E_{max} = 172%); both the enantiomers of the corresponding ethyl counterparts were also inferior in potency and efficacy. PSN119-2 was shown to enhance insulin secretion from the β-cell-derived hamster HIT-T15 cell line (EC_{50} = 18 nM) and GLP-1 release from the L-cell-derived mouse GLUTag cell line (EC_{50} = 8 nM). *In vivo*, PSN119-2 improved oral glucose tolerance without causing hypoglycemia when administered to male SD rats (10, 30 mg/kg, p.o.). The compound was also shown to reduce 24 h food intake in rats compared to the vehicle (10, 30 mg/kg, p.o.), although the effect was not as pronounced as that seen with the anorectic agent sibutramine (5 mg/kg, p.o.).

GPR119 agonists containing a central acyclic alkoxylene or alkylene spacer instead of the oxadiazole core have also been explored extensively by scientists at Prosidion (Fig. 16.8). Compound **23** was the initial hit (Fyfe *et al.*, 2007b), which demonstrated good potency (EC_{50} = 0.5 μM) but poor efficacy (E_{max} = 33%). As seen with the earlier series, an improvement in potency and efficacy was obtained upon replacing the pyridine ring with the 3-fluoro-4-methanesulfonylphenyl moiety (e.g., PSN119-1M: EC_{50} = 0.2 μM, E_{max} = 392%). Considerable biological data have been presented (Fyfe *et al.*, 2007b, 2007c, 2007d) on the sulfoxide analog, PSN119-1 (EC_{50} = 0.5 μM, E_{max} = 407%). In an oGTT in high-fat-diet-fed rats (20 mg/kg, p.o.), PSN119-1 demonstrated a positive glucose lowering effect, and unlike

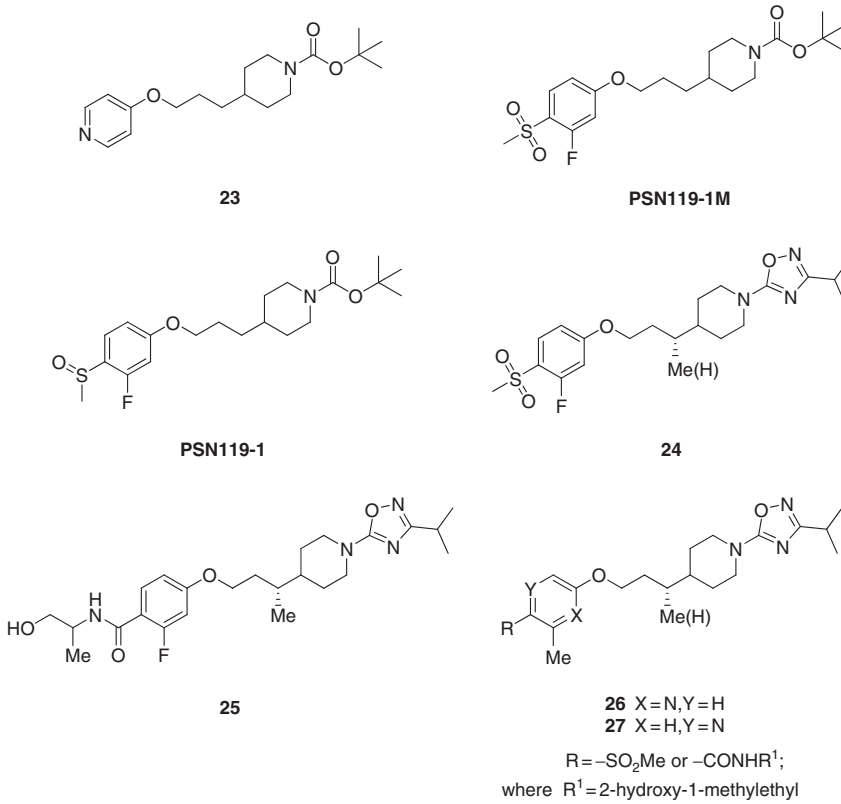
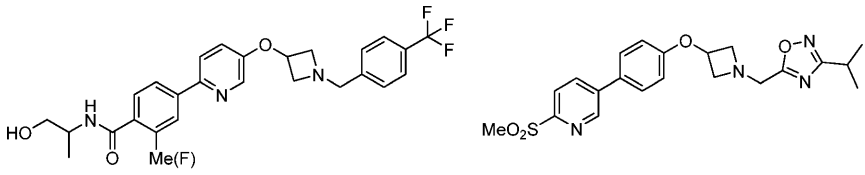


Figure 16.8 Linear core-based GPR119 agonists from Prosidion.

repaglinide (1 mg/kg), it did not cause hypoglycemia. The antihyperglycemic effects were further confirmed in an oGTT in diabetic ZDF rats. Chronic studies in young *db/db* mice over a 21-day treatment period (100 mg/kg, qd, p.o.) showed the compound to be capable of slowing the progression of diabetes as highlighted by its ability to improve glucose tolerance in an oGTT at the end of the 21-day treatment. Furthermore, PSN119-1 enhanced GLP-1 release in murine GLUTag cells (EC_{50} = 153 ± 70 nM) and also inhibited gastric emptying upon oral administration in SD rats (30 mg/kg). In a rat study evaluating its hypophagic actions, PSN119-1 demonstrated a statistically significant reduction in 24 h food intake (50 mg/kg, p.o.), comparable to the antiobesity agent sibutramine (5 mg/kg, p.o.). *In vivo*, PSN119-1 was reported to be extensively metabolized to the corresponding sulfone, PSN119-1M (Fig. 16.8).

More recently, the Prosidion group described GPR119 agonists in which the potentially labile *tert*-butylcarbamate functionality was replaced



28 29

Figure 16.9 Azetidine-based GPR119 agonists from Prosidion.

with bioisosteric heteroaryl groups, in particular with an oxadiazole similar to Arena's AR231453 (e.g., **24**–**27**; Fig. 16.8) (Bertram *et al.* 2008; Fyfe *et al.*, 2008c,d,e,f). Most examples retained the linear ether chain, which was optionally substituted with a chiral methyl group, preferably in *R*-configuration. Replacement of the left-hand side phenyl group with a pyridine ring and exchange of the typically used methylsulfone moiety with other substituents, in particular with hydroxyalkyl amide side chains, were also described; substitution at the adjacent position with fluorine or a methyl group was tolerated for these analogs. Several azetidine-based GPR119 agonists (Fig. 16.9) have also been disclosed by Prosidion (Fyfe *et al.*, 2009a,b). These analogs featured an appropriately substituted biaryl moiety connected to the azetidine through an oxygen atom. The azetidine nitrogen was generally substituted with a benzylic moiety (e.g., **28**) or with a five-membered heterocycle (e.g., **29**).

Although the data on specific compounds have not been disclosed in the recent Prosidion patent applications, preferred analogs within these inventions were claimed to exhibit an EC_{50} of less than 1 μM (HIT-T15 cAMP and insulin secretion assays) and to statistically reduce glucose excursion in rat oGTTs ($\leq$10 mg/kg, p.o.). Selected analogs were also reported to demonstrate a statistically significant hypophagic effect at a dose of $\leq$100 mg/kg.

Optimization of the above described chemical series resulted in identification of the clinical candidate PSN821 (OSI Pharmaceuticals Press Release, September 3, 2008; http://osip.client.shareholder.com/releasedetail.cfm?ReleaseID=370909; last accessed March 12, 2010), the structure of which has not been disclosed. In preclinical studies (Fyfe *et al.*, 2008g), PSN821 was shown to stimulate insulin secretion from HIT-T15 cells (EC_{50} = 153 ± 16 nM) and GLP-1 secretion from GLUTag cells (EC_{50} = 130 ± 42 nM). In male diabetic ZDF rats, oral administration of PSN821 (at single doses of 3, 10, and 30 mg/kg) significantly and dose-dependently reduced glucose excursions in oGTTs. At the 3 mg/kg dose, these effects were comparable to the DPP-IV inhibitor P32/98 (50 mg/kg); more significant and pronounced activity was observed at the 10 and 30 mg/kg doses. Furthermore, the compound remained effective in reducing glucose excursions in an oGTT after 10 days of treatment indicating

that tachyphylaxis had not occurred. Daily oral dosing of PSN821 (30 mg/kg) for 8 weeks in prediabetic ZDF rats significantly lowered nonfasting blood glucose concentrations and HbA1c levels. In addition, oral administration of PSN821 (30 mg/kg, qd) for 4 weeks in weight-stable DIO Wistar female rats significantly reduced body weight at levels comparable to those elicited by the antiobesity agent sibutramine (5 mg/kg). Thus, in preclinical studies, PSN821 demonstrated the potential to improve glycemic control and to achieve weight loss via oral administration. In phase 1 trials, PSN821 was well tolerated in both healthy volunteers (up to 3000 mg doses) and in patients with T2D (up to 1000 mg doses), where evidence of positive glucose lowering was observed in response to a standard nutrient challenge (OSI Pharmaceuticals Press Release, May 11, 2009; http://osip.client.shareholder.com/releasedetail.cfm?ReleaseID=383187; last accessed March 12, 2010).

The coadministration of a GPR119 agonist with a DPP-IV inhibitor has been shown to enhance plasma GLP-1 levels and to improve oral glucose tolerance, significantly more than with either agent alone. The discovery team at Prosidion has explored a unique approach of combining DPP-IV inhibition and GPR119 agonism in a single molecule (Barba *et al.*, 2009). Introduction of the cyanopyrrolidine pharmacophore of known DPP-IV inhibitors on the aryl motif of their GPR119 agonists provided compounds, which displayed dual activity as agonists of GPR119 and inhibitors of DPP-IV (e.g., **30**); the difluoropyrrolidine analog **31** is another example of a dual DPP-IV inhibitor/GPR119 agonist (Fig. 16.10). Limited biological data are available from this SAR effort. PSN-IV/119-1 (structure not disclosed) was recently reported to exhibit a DPP-IV IC_{50} of 0.2 μmol/L and GPR119 EC_{50} of 2.24 μmol/L (Swain *et al.*, 2009). Oral administration of PSN-IV/119-1 at a dose of 30 mg/kg in diabetic ZDF rats led to a greater reduction in glucose AUC compared to the DPP-IV inhibitor sitagliptin (58% vs. 22%); at a lower dose of 10 mg/kg, the activity was comparable to sitagliptin (20 mg/kg).

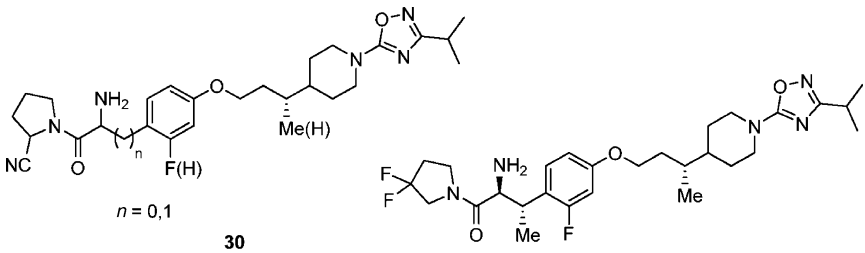


Figure 16.10 Representative compounds from Prosidion having dual activity as GPR119 agonists and DPP-IV inhibitors.

C. Metabolex

GPR119 agonists from Metabolex are based on a five-membered central heterocyclic core that is linked directly to the piperidine motif at its C4 position and to the aryl motif through an oxymethylene spacer (Chen *et al.*, 2008, 2009; Ma *et al.*, 2009; Song *et al.*, 2010; Wilson *et al.*, 2009). Among the various five-membered heterocycles reported (e.g., thiazole, oxazole, oxadiazole, pyrazole, triazole, etc.), the thiazole core was retained in a majority of the examples. Substituents on the piperidine motif included carbamates and bioisosteric heterocycles, and among these, an appropriately substituted pyrimidine moiety was highlighted as a preferred nitrogen capping group. The aryl motif consisted of a phenyl or pyridine ring having a hydrogen accepting group at the *para* position; in addition to the typically utilized methylsulfone functionality, a tetrazole moiety appeared in several examples. Figure 16.11 shows representative GPR119 agonists that showed

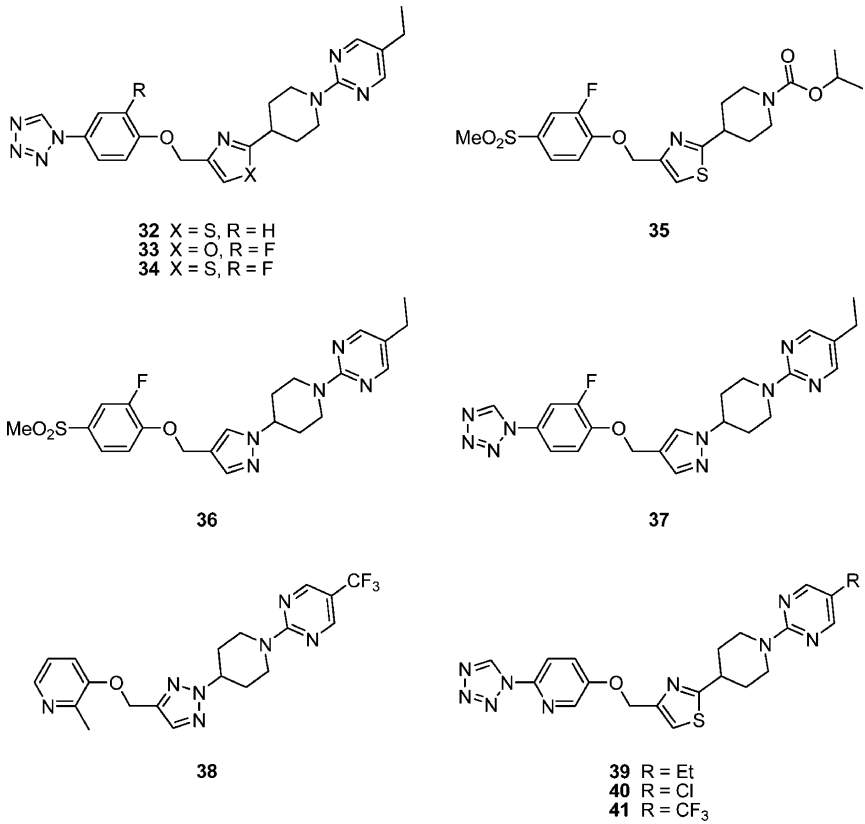


Figure 16.11 Selected GPR119 agonists based on five-membered central heterocyclic cores from Metabolex.

activity at 10 μM in the fluorescence resonance energy transfer (FRET) assay in which a decrease in the FRET signal corresponded to an increase in intracellular cAMP levels.

Metabolex advanced their orally available GPR119 agonist MBX2982 (**32**; Fig. 16.11) into clinical trials of T2D (McWherter, 2010; Roberts *et al.*, 2009, 2010; Wilson *et al.*, 2009); rights to this compound were recently acquired by Sanofi-aventis. In preclinical studies, MBX2982 was shown to increase cAMP levels in CHO cells expressing human GPR119 (EC50=3.9 nM) and to stimulate GSIS from isolated islets. The compound also demonstrated enhanced insulin secretion during hyperglycemic clamps in rats, and acutely lowered glucose excursion and increased plasma GLP-1 and GIP levels during oGTTs in mice and rats; these *in vivo* effects were enhanced with coadministration of the DPP-IV inhibitor sitagliptin. Furthermore, MBX2982 significantly slowed gastric emptying in mice and delayed diabetes onset upon chronic administration to high-fat-fed female ZDF rats without any evidence of tachyphylaxis. Recently, Metabolex announced positive results from phase 1 trials of MBX2982. In healthy volunteers, an ascending dose of the drug (10–1000 mg) was shown to be well tolerated without any dose-related adverse events (Metabolex, Inc., Press Release, November 12, 2008; http://www.metabolex.com/news/nov122008.html; last accessed March 12, 2010). MBX2982 exhibited a half-life suitable for once-daily dosing and also demonstrated dose-dependent reductions in glucose and increases in GLP-1 levels following a mixed meal. In prediabetic subjects (individuals with impaired fasting glucose or impaired glucose tolerance), repeated daily dosing for 4 consecutive days with 100 or 300 mg improved glucose excursion after a mixed meal and decreased the exposure to glucose during a graded glucose infusion (the latter response was attributable to elevated insulin secretion). In a 5-day placebo-controlled multiple ascending dose study (25, 100, 300, and 600 mg) in prediabetic subjects, decreased glucose excursions were observed after a mixed meal or glucose tolerance test with all doses (Metabolex, Inc., Press Release, October 13, 2009; http://www.metabolex.com/news/oct132009.html; last accessed March 12, 2010). MBX2982 has been advanced to phase 2 trials evaluating its efficacy, safety, tolerability, and pharmacokinetics following daily administration for 4 weeks in patients with T2D (http://www.clinicaltrials.gov/ct2/show/NCT01035879?term=MBX2982&rank=1; last accessed March 12, 2010).

D. Bristol–Myers Squibb

The first series of GPR119 agonists reported by Bristol–Myers Squibb featured a [6,5], [6,6], or [6,7] bicyclic central core (Fevig and Wacker, 2008a,b). Representative examples containing pyrimidine-fused pyrrazole, triazole, and morpholine ring systems are shown in Fig. 16.12 (**42**–**44**). An *in vitro* luciferase assay using transfected HEK293 cells was employed to determine the

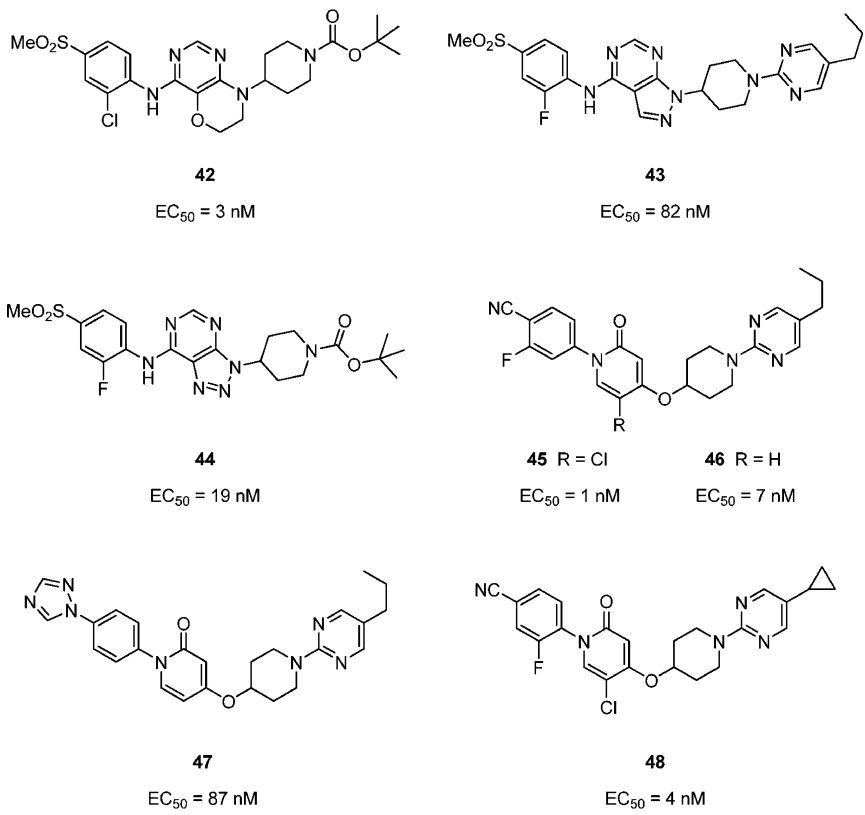


Figure 16.12 SAR of selected GPR119 agonists from Bristol–Myers Squibb based on fused-pyrimidine and pyridone scaffolds.

potency, and EC_{50} values ranging from 19 to 3546 nM were reported for the fused bicyclic derivatives. The second BMS series (Wacker *et al.*, 2009a,b, 2010) featured a pyridone central core that was N-substituted with the aryl motif and linked to the piperidine motif at the 4-position through an oxygen linker (**45**–**48**; Fig. 16.12); pyridazone analogs have also been claimed as GPR119 modulators (Wacker *et al.*, 2010). The general substitution pattern on the aryl and the piperidine groups was consistent with competitors' GPR119 agonists. EC_{50} values ranging from 1 nM to 7 μM in a human tetracycline-inducible cAMP assay were reported for the pyridone analogs.

E. GlaxoSmithKline

Replacement of Arena's pyrazolopyrimidine ring system (e.g., **18**, **19**; Fig. 16.6) with a dihydropyrrolopyrimidine scaffold was shown to be successful by researchers at GlaxoSmithKline (Ammala and Briscoe, 2008;

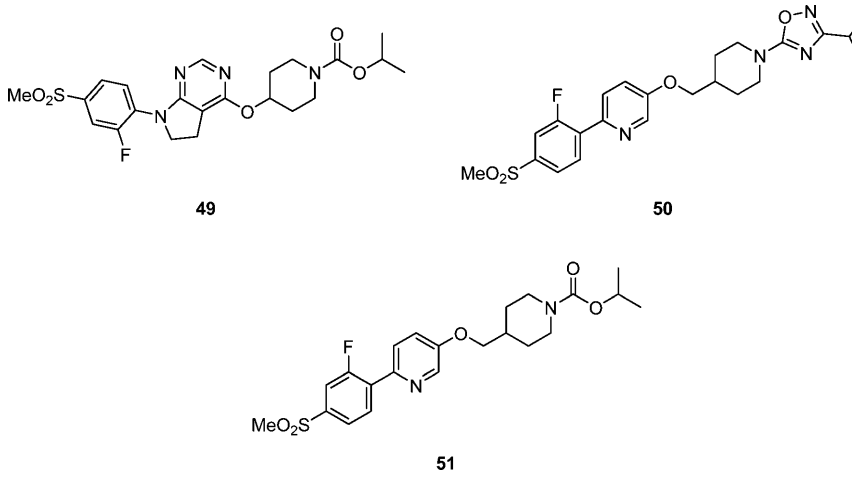


Figure 16.13 Representative GPR119 agonists from GlaxoSmithKline.

Katamreddy *et al.*, 2008). The prototypical compound **49** (Fig. 16.13) demonstrated an EC_{50} of 40 nM in a CHO6CRE reporter assay. This compound was also shown to stimulate GDIS from rat islet cells and to enhance the release of GLP-1 from GLUTag cells (EC_{50} = 22 nM). In a rat hyperglycemic clamp model, **49** augmented GSIS (1–10 mg/kg) which was consistent with the 1.4-fold increase in peak insulin levels and 43% inhibition of glucose AUC observed during an intravenous glucose tolerance test. In an oGTT, **49** reduced the glucose AUC by 28% (30 mg/kg) and 38% (10 mg/kg), respectively, in mice and rats. Dose-dependent increases in circulating total GLP-1 and GIP levels were also observed with this compound. The improved glucose tolerance and enhanced incretin hormone levels were shown to be GPR119-dependent as the compound was ineffective in GPR119-knockout mice. Furthermore, fasting glucose and insulin were not significantly affected by this GPR119 agonist compared to the vehicle. Compound **49** also enhanced whole body insulin sensitivity as demonstrated by euglycemic hyperinsulinemic clamp studies in normal rats. In addition to the pyrrolopyrimidine scaffold, a series of GPR119 agonists based on monocyclic six-membered aryl and heteroaryl cores have also been reported by GlaxoSmithKline (Carpenter *et al.*, 2010; Fang, *et al.*, 2008). The pyridyl-ethers **50** and **51** (Fig. 16.13) are representative examples that demonstrated improved glucose tolerance and elevated GLP-1 and GIP levels in mice (30 mg/kg, p.o.). Other GPR119 agonists in this series contained a benzene, pyrazine, or pyradazine central core. The des-fluoro analog of **50** was chosen as the clinical candidate GSK1292263 (hGPR119 pEC50 = 6.9, rat GPR119 pEC50 = 6.7). GSK1292263 augmented insulin secretion and decreased glucose AUC in rodent glucose

tolerance tests; an increased incretin secretion (GLP-1 and GIP) was also observed (Carpenter, 2010; Nunez, *et al.*, 2010). GSK1292263 retained its oGTT activity after 8 weeks of dosing and decreased HbA1c in ZDF rats after 7 weeks. In rodent models, this compound did not inhibit gastric or gall bladder emptying, and had no effect on body weight. In phase 1 studies, GSK1292263 was well tolerated (10 mg to 400 mg single rising dose) and displayed a dose-dependent decrease in glucose AUC (0-3 h) during an oGTT with an increase in circulating gut hormone levels. Coadministration with sitagliptin increased plasma active GLP-1 concentrations and lowered total GLP-1, GIP and PYY levels; no effects on gastric emptying were observed with GSK1292263.

F. Biovitrum

Analogs having a close resemblance to the pyridine ethers **50** and **51** have been claimed by Biovitrum AB for the treatment of GPR119-related disorders (Fig. 16.14). The central heterocyclic ring consisted of a pyridine (Brandt *et al.*, 2008a), pyradazine (Brandt *et al.*, 2008b), pyrimidine (Brandt *et al.*, 2008c), or pyrazine (Johansson *et al.*, 2009) nucleus, which was connected to the piperidine ring via an optionally substituted amino methylene (e.g., **52**, **53**) or an oxymethylene linker (e.g., **54**, **55**). Compounds **52**, **54,** and **55** were reported to have EC_{50} values of 22, 46, and 14 nM, respectively, in a human GPR119 cAMP HTRF (homogenous time-resolved fluorescence) assay. More recent analogs were characterized by a

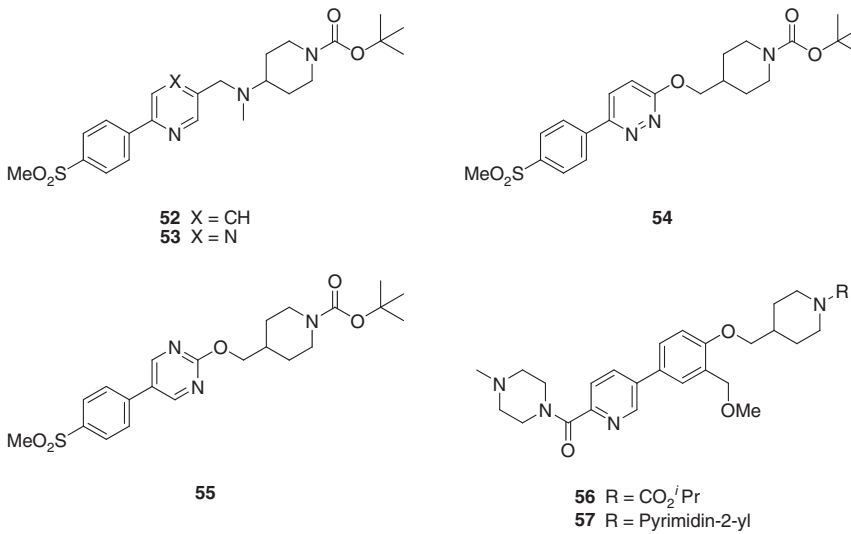


Figure 16.14 Representative GPR119 agonists from Biovitrum.

methoxymethyl-substituted phenyl core (Bremberg *et al.*, 2009); an appropriately substituted amide moiety, including the piperazine amide in **56** and **57**, was highlighted in several examples.

G. Merck

The first series of GPR119 agonists from Merck was derived from a bipiperidine scaffold in which one of the piperidine rings was N-linked to an aryl moiety and the other was nitrogen-capped with a carbamate or a bioisosteric heterocycle (e.g., **58**; Fig. 16.15; Wood *et al.*, 2008a,b). The more recent analogs from Merck (Wood *et al.*, 2009) were based on a linear core similar to Prosidion, and featured a cyclopropyl containing ether chain that connected the aryl and piperidine motifs (e.g., **59**, **60**; Fig. 16.15). The aryl moiety was typically a phenyl, pyridine, or pyrimidine group substituted with an H-bond accepting group such as a sulfone, cyano, or five-membered heterocycle. A pyrimidine ring optionally substituted with an alkyl or halogen was shown to be a preferred piperidine nitrogen capping group. Although the data for specific Merck analogs are not available, several compounds have been claimed to exhibit an $EC_{50} < 10$ nM in the cAMP HTRF assay.

Multiple structural classes of GPR119 agonists have been investigated by Schering–Plough (Fig. 16.15). The earlier spirocyclic azetidine and azetidinone derivatives (e.g., **61**, **62**) were claimed as GPR119 agonists and also as T-type calcium channel blockers and NPC1L1 antagonists (Harris *et al.*, 2008). Compound **61** was described as a modest GPR119 agonist (cAMP $IC_{50} = 1922$ nM); replacement of the amide functionality with a urea resulted in a potent T-type calcium channel blocker, **62** (IW hCav3.2 $IC_{50} = 23$ nM). More potent GPR119 agonists (EC_{50} range: 40 nM to 14 μM) were derived from novel fused pyrimidinone ring systems, which included 7-substituted 5,6,7,8-tetrahydropyrido[3,4-*d*]pyrimidin-4(3H)-one and 6-substituted 5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3H)-one analogs (Boyle *et al.*, 2008a, 2008b). An optionally substituted benzhydryl or an alkyl- or cycloalkyl-substituted benzyl moiety was highlighted as a pyridone–nitrogen substituent in both the series. On the tetrahydropyridine side, nitrogen capping groups included carbamates for the 3,4-*d* series (e.g., **63**), and benzylic groups for the 4,3-*d* series (e.g., **64**). Fused quinazolinones represented by compound **65** have also been claimed as agonists of GPR119 (Harris *et al.*, 2009). Another recent filing from Schering–Plough (Xia *et al.*, 2009) included GPR119 agonists which were similar in structure to Arena's pyrimidine ethers but featured novel bridged-piperidine ring systems (e.g., **66**). Although biological data on specific analogs have not been reported, undisclosed GPR119 agonists within several Schering–Plough patents have been claimed to be efficacious in mouse oGTT studies.

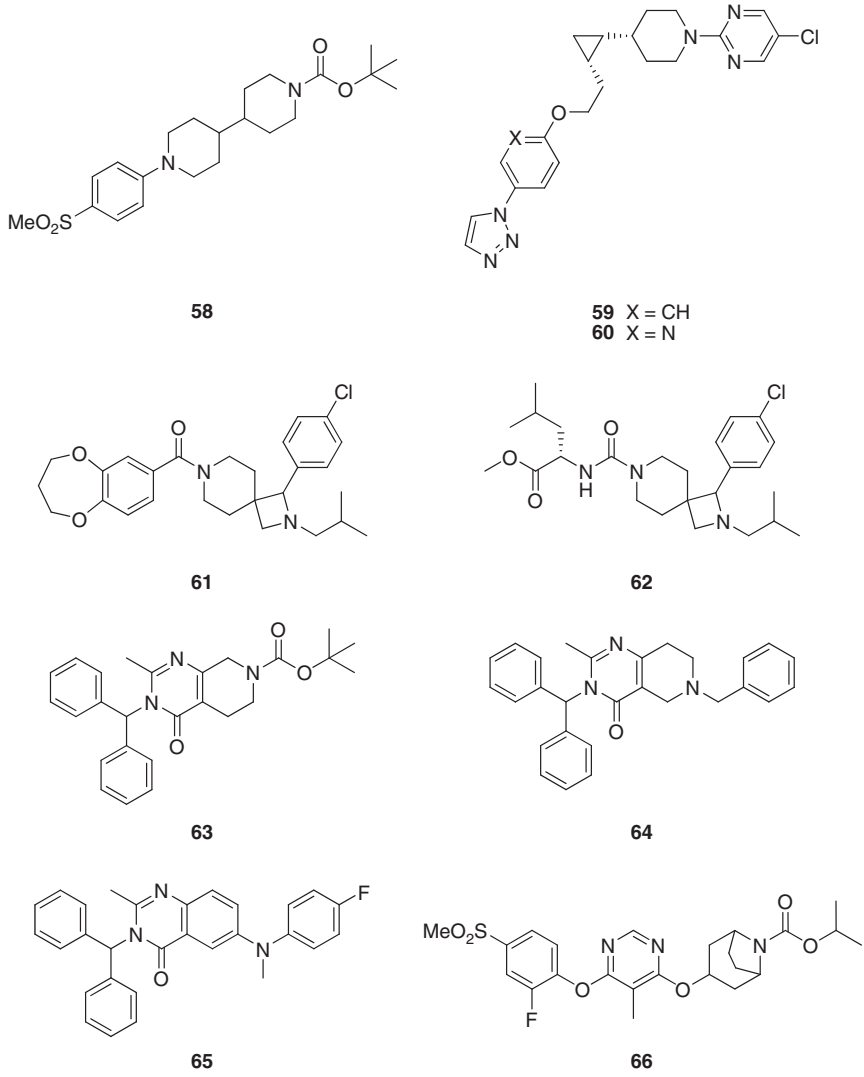


Figure 16.15 The structures of selected GPR119 agonists discovered at Merck and Schering–Plough (now Merck).

H. Genomics Institute of the Novartis Research Foundation (GNF)

Genomics Institute of the Novartis Research Foundation (GNF) has disclosed an extensive set of GPR119 agonists containing a heterocyclic sulfonamide as a novel left-side structural motif (Fig. 16.16) (Alper *et al.*, 2008, 2009a; Azimioara *et al.*, 2009). A cAMP assay using Flp-In-CHO-hGPR119 cells

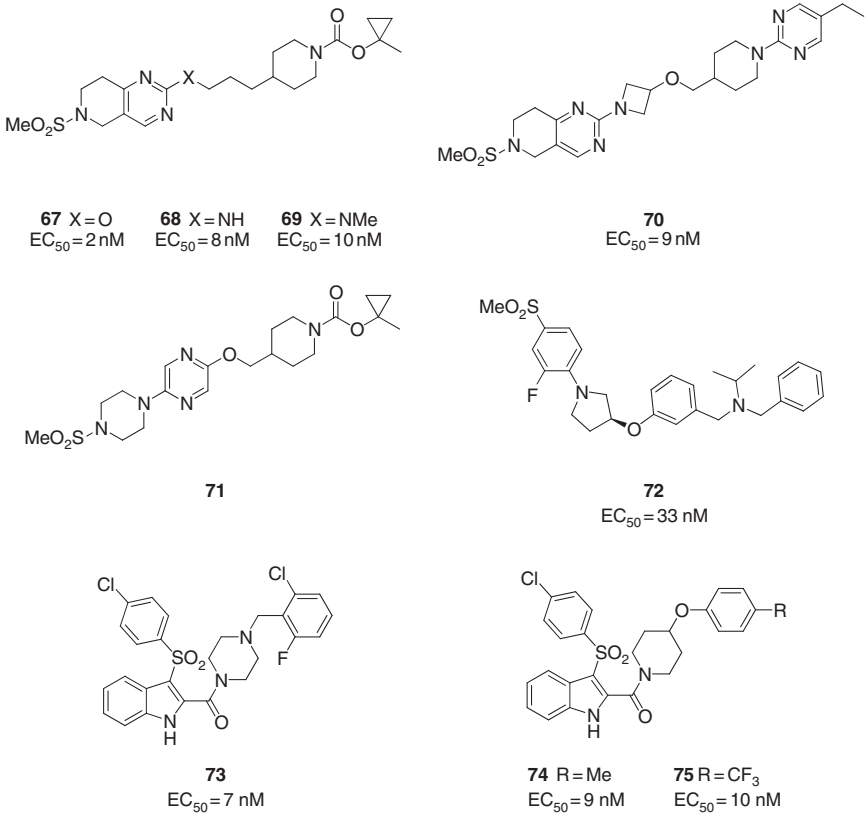


Figure 16.16 Novel GPR119 agonists from Genomics Institute of the Novartis Research Foundation (GNF).

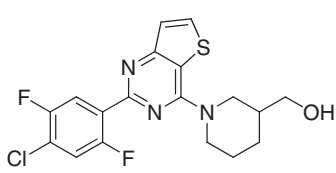
was employed to evaluate these compounds and the tetrahydropyrido[4,3-*d*] pyrimidine analog **67** was one of the most potent analogs reported with an EC_{50} value of 2 nM (Azimioara *et al.*, 2009). In addition to the tetrahydropyrido[4,3-*d*]pyrimidine unit, which has been highlighted in several examples, tetrahydroisoquinoline (Alper *et al.*, 2008) and tetrahydro-1,6-naphthyridine (Azimioara *et al.*, 2009) ring systems were also utilized successfully. Exchanging the oxygen atom in the central spacer of **67** for an amino or methylamino linker was shown to be tolerated (e.g., **68**: EC_{50} = 8 nM, **69**: EC_{50} = 10 nM); however, changing the position of the heteroatom or introducing an additional heteroatom in the spacer had a negative effect on the potency (Azimioara *et al.*, 2009). On the right hand side, replacement of the carbamate functionality with the typically employed oxadiazole or pyrimidine moieties provided several analogs with EC_{50} values below 10 nM. Numerous other modifications around the central core and the sulfonamide motif, as exemplified by the azetidine **70** (Azimioara *et al.*, 2009)

and the piperazinyl-pyrazine **71** (Alper *et al.*, 2009a), have also been described. More recently, two patent applications from GNF highlighted structurally distinct analogs that did not contain the typical structural framework observed in most GPR119 agonists (Alper *et al.*, 2009b,c). These compounds were also shown to be potent GPR119 agonists (e.g., **72**–**75**; Fig. 16.16).

I. Astellas

Compounds effective in stimulating insulin secretion and inhibiting the increase of blood sugar levels have been reported by Astellas. These were derived from a bicyclic scaffold in which a pyrimidine ring was fused to an aromatic (e.g., thiophene, thiazole, and pyridine) or a nonaromatic (e.g., dihydrothiophene, dihydrofuran, and cycloalkyls) heterocycle. Compound **76** (Fig. 16.17) is representative of the first class in which the thieno[3,2-*d*] pyrimidine core was retained in several examples (Yonetoku, *et al.*, 2006a). In the second series, a cyclic sulfone fused to the pyrimidine moiety (e.g., **77**; Fig. 16.17) was highlighted among other nonaromatic heterocycles (Yonetoku, *et al.*, 2006b). Substitution on the pyrimidine ring typically included a halogenated phenyl group at the 2-position and a nitrogen heterocycle at the 4-position; in the nonaromatic fused series, cycloalkyl groups at the 2-position of the pyrimidine ring were also described. In mouse oGTT studies, compounds **76** and **77** demonstrated 34% and 36% reduction in blood glucose levels, respectively.

Detailed pharmacological data on two undisclosed GPR119 agonists from Astellas have been presented. The first generation analog, AS1535907 (Yoshida, *et al.*, 2009), increased intracellular cAMP levels in GPR119 transfected HEK293 cells (EC_{50} = 1.5 μM) and enhanced insulin secretion in the mouse NIT-1 pancreatic β-cell line and rat perfused pancreas. *In vivo*, this compound improved glucose tolerance in an oGTT in normal and *db/db* mice following oral administration at a dose of 10 mg/kg. After 2 weeks of treatment, AS1535907 was shown to markedly increase pancreatic insulin content, insulin mRNA, and the number of insulin-positive cells in the pancreatic islets of *db/db* mice (30 mg/kg, b.i.d.). Further SAR



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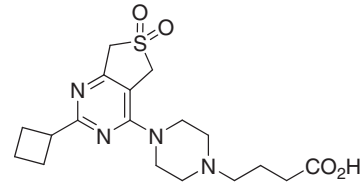

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Figure 16.17 Fused-pyrimidine-based GPR119 agonists from Astellas.

optimization resulted in the second generation compound, AS1907417 ($hEC_{50} = 1.1$ μM), which improved upon the metabolism and efficacy liabilities associated with AS1535907 (Tanaka, *et al.*, 2009). This compound significantly enhanced GDIS in MIN-6 pancreatic β-cell lines and also increased plasma and pancreatic insulin levels in diabetic *db/db* mice. In normal mice, this GPR119 agonist improved glucose tolerance in an oGTT (3 mg/kg, p.o.) but did not affect plasma glucose or insulin levels in fasted mice. Furthermore, in rodent models of diabetes, AS1907417 (10 mg/kg, p.o.) also reduced the levels of HbA1c, triglycerides, and free fatty acids.

VI. Conclusions

The combined stimulation of insulin and incretin release observed with GPR119 agonists provides a unique opportunity to regulate glucose homeostasis in patients with T2D. The glucose-dependent mechanism of these agents differentiates them from the sulfonylureas and insulin, which are associated with a high risk of hypoglycemia. Orally available GPR119 agonists also present an advantage over the currently available GLP-1 mimetics which suffer from the necessity of parenteral administration. Furthermore, preliminary data from animal studies have suggested that GPR119 agonists may have a potential to exert β-cell protective effects and to delay progression of diabetes. The use of a GLP-1 secretagogue (e.g., a GPR119 agonist) in combination with DPP-IV inhibition is also an important avenue of investigation. Such a strategy may not only provide improved glycemic control, but also induce weight loss, a feature observed with GLP-1 mimetics but not with DPP-IV inhibitors. Encouraging results have been reported from phase 1 clinical trials assessing the safety, tolerability, and proof of pharmacology of orally available GPR119 agonists. While there are some data demonstrating the potential for the GPR119 receptor desensitization *in vitro*, tachyphylaxis to the improved glycemic control with repeat dosing of GPR119 agonists in rodent models or clinical trials has not been reported. Several companies are advancing GPR119 agonists into phase 2 clinical trials for the treatment of T2D. The results of these trials are eagerly awaited to determine the long-term safety and durability of this therapeutic approach.

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