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Review Article

Targeting β-cell cyclic 3′5′adenosine monophosphate for the development of novel drugs for treating type 2 diabetes mellitus. A review

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Abstract

Cyclic 3′5′AMP is an important physiological amplifier of glucose-induced insulin secretion by the pancreatic islet β-cell, where it is formed by the activity of adenylyl cyclase, especially in response to the incretin hormones GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependent insulinotropic peptide). These hormones are secreted from the small intestine during and following a meal, and are important in producing a full insulin secretory response to nutrient stimuli. Cyclic AMP influences many steps involved in glucose-induced insulin secretion and may be important in regulating pancreatic islet β-cell differentiation, growth and survival. Cyclic AMP (cAMP) itself is rapidly degraded in the pancreatic islet β-cell by cyclic nucleotide phosphodiesterase (PDE) enzymes. This review discusses the possibility of targeting cAMP mechanisms in the treatment of type 2 diabetes mellitus, in which insulin release in response to glucose is impaired. This could be achieved by the use of GLP-1 or GIP to elevate cAMP in the pancreatic islet β-cell. However, these peptides are normally rapidly degraded by dipeptidyl peptidase IV (DPP IV). Thus longer-acting analogues of GLP-1 and GIP, resistant to enzymic degradation, and orally active inhibitors of DPP IV have also been developed, and these agents were found to improve metabolic control in experimentally diabetic animals and in patients with type 2 diabetes. The use of selective inhibitors of type 3 phosphodiesterase (PDE3B), which is probably the important pancreatic islet β-cell PDE isoform, would require their targeting to the islet β-cell, because inhibition of PDE3B in adipocytes and hepatocytes would induce insulin resistance.

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Introduction

The prevalence of diabetes mellitus, especially of type 2 diabetes, is increasing and the World Health Organisation predicts a world total of > 300 M by 2030. The high prevalence of diabetes combined with the associated increased mortality and morbidity, primarily as a result of macrovascular disease and microvascular long-term complications, make it a major health problem. Overwhelming evidence, especially from the DCCT study (The Diabetes Control and Complications Trial Research Group 1993) for type 1 diabetes and the UK Prospective Diabetes Study (Turner 1998) for type 2 diabetes suggested that good metabolic control would markedly reduce mortality and morbidity.

It is now broadly accepted that type 2 diabetes results from both peripheral insulin insensitivity and impaired insulin secretion (Kahn 2003). There is progressive islet β-cell failure in patients with type 2 diabetes and a reduction in the β-cell mass (Porte & Kahn 2001). Thus the islet β-cell remains an important target for the development of drugs for treating type 2 diabetes. Drugs are required that both enhance insulin secretion in response to normal physiological meal-related nutrient stimuli and that prevent the progressive loss of islet β-cell mass. Augmentation of meal-related insulin secretion by drugs should be glucose-dependent. This would ensure that increased insulin secretion occurs when required, the corollary being that insulin secretion would remain at a basal rate between meals, avoiding hyperinsulinaemia and thus hypoglycaemia, as seen with sulphonylureas, and the potential cardiovascular risks associated with chronic hyperinsulinaemia and insulin resistance (Juhan-Vague & Alessi 1997; Bastard et al 2000). Physiological, meal-related insulin secretion occurs in response to absorbed nutrients, primarily glucose. Glucose stimulates insulin secretion following its transport into and metabolism in the

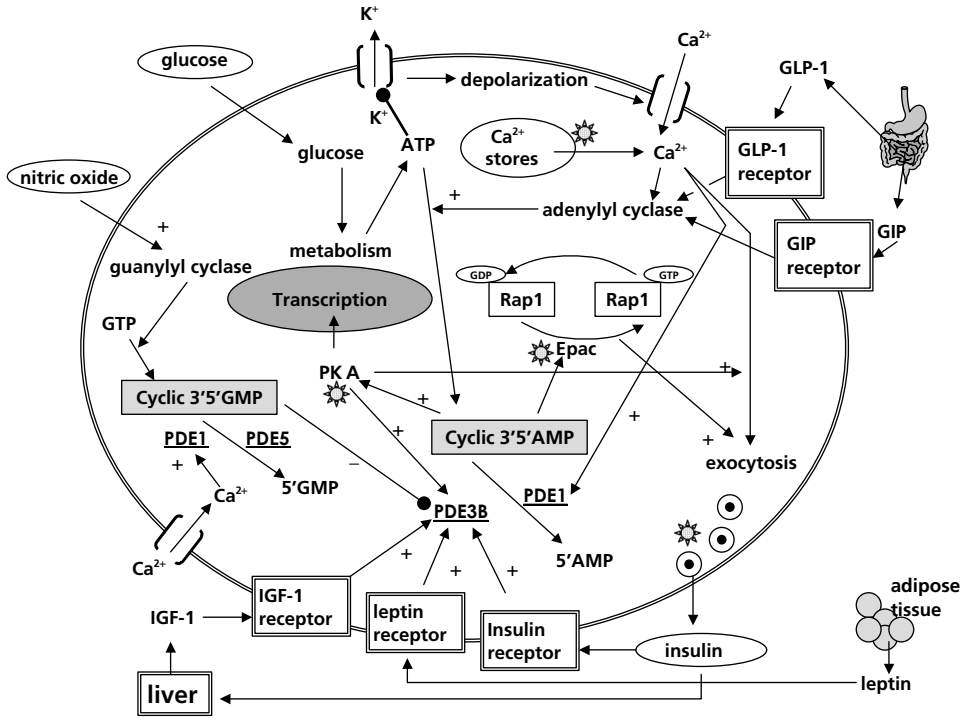

Figure 1 Schematic diagram of the pancreatic islet β-cell showing the sites of action (indicated by the star symbol) of cyclic 3′5′AMP in modulating glucose-induced insulin secretion and other β-cell activities. The pathways for the formation and destruction of cAMP and the hormonal factors influencing these pathways are shown also.

β-cell, generating ATP which closes K_{ATP} channels, resulting in depolarization and calcium influx (Figure 1). The effect of glucose on insulin secretion can be amplified by signalling pathways involving inositol trisphosphate and diacylglycerol derived from activation of phospholipase C (Howell et al 1994; Gilon & Henquin 2001) and by cyclic 3′5′AMP following activation of adenylyl cyclase (Howell et al 1994). Glucose itself has long been known to elevate the pancreatic islet β-cell cAMP content (Grill & Cerasi 1973). Although glucose-induced insulin secretion does not appear to require cAMP or the protein kinase A (PKA)-system (Grill & Cerasi 1973; Sharp 1979; Persaud et al 1990; Lester et al 1997), increases in cAMP augment the direct effect of glucose. This cyclic nucleotide is generally accepted as an important amplifier of glucose-induced insulin release (Holz & Habener 1992), particularly when its levels are increased by glucose itself (Harndähl et al 2002) and, importantly, by various gut hormones implicated as incretins, which are secreted from the small intestine in response to the presence of glucose and other nutrients in the gut. It has also been suggested that cAMP is a competence factor for normal islet β-cell responsiveness to glucose (Schuit 1996; Huypens et al 2000). Numerous hormones that stimulate insulin secretion increase islet β-cell cAMP, including glucagon (Huypens et al 2000), ACTH (Al-Majed et al 2004), and pituitary adenylate cyclase-activating polypeptide (PACAP; Filipsson et al (2001)). On the other hand, hormones inhibiting insulin secretion (galanin (Drews et al 1994), adrenaline (Peterhoff et al 2003)), reduce cAMP, although their effects on cAMP may not necessarily explain the inhibition of secretion. The major incretins are glucose-dependent insulinotropic polypeptide (GIP) secreted by intestinal K-cells and glucagon-like peptide 1 secreted by intestinal L-cells. These hormones augment glucose-induced insulin secretion through activating adenylyl cyclase, leading to an increase in islet β-cell cAMP. Their importance is illustrated clearly by the marked glucose intolerance and impairment of insulin secretion seen in mice lacking receptors for both hormones (Preitner et al 2004).

Secretion and actions of glucagon-like peptide-1 (GLP-1)

GLP-1 is derived from the processing of the preproglucagon gene in intestinal L-cells (Lund et al 1982). Various products of the post-translational processing of proglucagon exist including GLP-1(7–36)amide and GLP-1(7–37), which are equipotent stimulators of glucose-dependent insulin secretion (Holst et al 1987; Kreymann et al 1987; Mojsov et al 1987). For the purpose of this review, GLP-1 refers to the active form GLP-1(7–36)amide.

GLP-1 is secreted from intestinal L-cells in response to the absorption of glucose, other sugars, fatty acids and to a minor extent amino acids (MacIntosh et al 2001; Feinle et al 2002; Brubaker & Anini 2003). The GLP-1 secretory actions of metabolizable nutrients may be linked to cellular ATP generation, K_{ATP} channel blockade and elevation of intracellular Ca^{2+} (Gribble & Reimann 2002). There is also a neurally-mediated arm to GLP-1 secretion and a

stimulatory effect of GIP. These pathways may be especially important for release of GLP-1 from the distal region of the small intestine (Holst et al 1987; Filipsson et al 2000). The insulinotropic actions of GLP-1 are evident in islets (Komatsu et al 1989; Weir et al 1989) in normal and diabetic animals (Hendrick et al 1993; Scrocchi et al 1998; O'Harte et al 2000a, 2000b, 2001) and in healthy and diabetic volunteers (Gutniak et al 1992).

Other physiological actions of GLP-1 target the pancreatic islets, liver, adipose tissue and skeletal muscle. In pancreatic islets, GLP-1 stimulates insulin and somatostatin secretion (Holst et al 1987; Mojsov et al 1987; Eissele et al 1990; Creutzfeldt et al 1996) and inhibits glucagon secretion (Creutzfeldt et al 1996). GLP-1 stimulates insulin gene transcription (Drucker et al 1987; Fehmann & Habener 1992; Skoglund et al 2000), pancreatic islet cell proliferation (Buteau et al 1999; Perfetti et al 2000), and β-cell replication (Edvell & Lindstrom 1999). GLP-1 leads to the differentiation of pancreatic ductal AR42J cells into glucagon- and insulin-producing cells (Zhou et al 1999), through a PDX-1 dependent pathway (De La Tour et al 2001; Hui et al 2001). Lesser known actions of GLP-1 include promotion of glucose uptake and glycogen formation in liver and skeletal muscle (Valverde et al 1994; Villanueva-Penacarrillo et al 1994; O'Harte et al 1997). GLP-1 also stimulates lactate production, glucose uptake and glycogen storage in skeletal muscle (O'Harte et al 1997).

Secretion and actions of GIP

The 42 amino acid peptide GIP was isolated from a crude porcine CCK extract and shown to inhibit histamine-induced gastric acid secretion (Brown et al 1969). However, its major physiological role as a glucose-dependent stimulator of insulin secretion is now well recognized (Gault et al 2003a).

GIP is secreted from intestinal K-cells by glucose and other actively transported sugars and by fatty acids. Amino acids however are only a weak stimulus. Although GIP stimulus-secretion coupling pathways are poorly understood, there appears to be a close link with K-cell metabolism (Tseng et al 1994). Indeed it appears likely that signal transduction of intestinal K- and L-cells will be found to have many parallels to other glucose-sensitive secretory cells, most notably the pancreatic β-cell (Purrello & Rabuazzo 2000). Notably, insulin forms an effective feedback loop by inhibiting nutrient stimulated GIP secretion (Bryer-Ash et al 1994).

GIP is a potent glucose-dependent stimulator of insulin secretion in all in-vitro and in-vivo systems tested (Pederson et al 1998a, 1998b; Drucker 2003; Vilsboll & Holst 2004). Additional actions on the β-cell include stimulation of proinsulin gene transcription and translation (Fehmann & Göke 1995; Wang et al 1996), enhancement of the growth, differentiation and survival of pancreatic β-cells (Trumper et al 2001; Pospisilik et al 2003). Extra pancreatic actions of GIP appear to enhance its glucose-lowering ability by inhibiting hepatic glucose production (Elahi et al 1986) and promoting glucose uptake in isolated mouse diaphragm muscle (O'Harte et al 1997). Functional GIP receptors have also been identified on adipocytes (Yip et al 1998), where GIP has been shown to stimulate glucose transport (Eckel et al 1979), fatty acid synthesis (Oben et al 1991) and lipoprotein lipase activity (Knapper et al 1995).

Cyclic AMP in the pancreatic islet β-cell

GLP-1 and GIP exert their insulinotropic actions through activation of G-protein coupled receptors (GPCRs) (Thorens 1992; Usdin et al 1993). This results in activation of adenylyl cyclase and an increase in intracellular cAMP. Their effects in stimulating insulin synthesis and in the enhancement of the growth, differentiation and survival of pancreatic β-cells are probably also mediated by cAMP. However, there is evidence that other pathways, such as the transactivation of epidermal growth factor receptors, may be involved in stimulation of β-cell proliferation (Buteau et al 2003). Cyclic AMP has many effects within the β-cell, as described below (see also Figure 1).

Insulin secretion Several mechanisms may mediate the effect of cAMP in augmenting glucose-induced insulin secretion. These include increased opening of voltage-sensitive Ca^{2+} channels (Kanno et al 1998), calcium-induced Ca^{2+} release (Kang et al 2001), activation of ryanodine receptors in the endoplasmic reticulum (Islam et al 1998; Holz et al 1999), stimulation of β-cell lipolysis (Yaney et al 2001) and direct effects on exocytosis (Harndähl et al 2002). Most actions of cAMP in the β-cell appear to be mediated through PKA-catalysed phosphorylation events. There is evidence for specific targeting of PKA to particular subcellular locations within the β-cell by A-kinase anchoring proteins (AKAPs) and disruption of AKAPs inhibits, for example, the effects of cAMP in increasing intracellular Ca^{2+} and stimulating insulin secretion (Lester et al 1997). However, some effects of the cyclic nucleotide on exocytosis are partly PKA-independent (Renstrom et al 1997). PKA-independent effects on exocytosis may be mediated by the cAMP-binding proteins known either as cAMP-regulated guanine nucleotide exchange factors (GEFs) or exchange proteins activated by cAMP (Epacs) which target the small G-protein Rap1 (Kopperud et al 2003). The pancreatic islet β-cell expresses both Epac 1 and Epac 2 (Holz 2004). Treatment of pancreatic islets with antisense oligodeoxynucleotides against Epac reduced the effect of a permeant cAMP analogue in augmenting glucose-induced insulin secretion (Kashima et al 2001). A recently described, novel cAMP analogue, 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′-5′-cyclic monophosphate (8-pCPT-2′-O-Me-cAMP) activated Epac but not PKA. This analogue was shown to mobilize Ca^{2+} from intracellular Ca^{2+} stores via Epac-mediated Ca^{2+}-induced Ca^{2+} release in human pancreatic β-cells and INS-1 insulin-secreting cells (Kang et al 2003). The cAMP-mediated insulinotropic action of the incretin factors glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1) (Drucker et al 1987) are mediated in part through PKA, but PKA-independent actions have also been demonstrated and probably involve Epac, as evidenced by the effects of antisense oligodeoxynucleotides against Epac (Kashima et al 2001).

Other roles in the β-cell Glucose and the insulinotropic hormones have actions on the β-cell which extend beyond the acute stimulation of insulin secretion. These include increased insulin synthesis and the modulation of β-cell growth, differentiation and apoptosis. In addition to its role in amplifying glucose-induced insulin secretion, cAMP may mediate or modulate these other effects. Glucose-mediated increases in insulin synthesis involve the phosphorylation of the transcription factor pancreatic duodenal homeobox-1 (PDX-1) and its translocation to the nucleus (Elrick & Docherty 2001). While the effects of glucose itself on PDX-1 are not mediated by cAMP, there is strong evidence for the importance of cAMP in GLP-1-dependent stimulation of PDX-1 in the β-cell, as well as its translocation to the nucleus and its activation of the insulin gene promoter (Wang et al 2001). However, the role of cAMP in regulating insulin synthesis is unclear since the adenylyl cyclase activator forskolin or the cAMP analogue 8-bromo-cAMP suppressed insulin transcription in INS-1 cells in a PKA-independent manner (Ding et al 2003). Cyclic AMP may mediate effects of glucose in stimulating the expression of immediate early response genes such as *c-myc* (Jonas et al 2001) and *c-fos* (Susini et al 1998). In this context, cAMP can either activate or inhibit the p42/p44 mitogen-activated protein kinase pathway depending on the cell type and conditions (Calleja et al 1997). Glucose itself activates the p42/p44 MAPK pathway (Benes et al 1998, 1999) and this effect is amplified, although not mediated by cAMP. Indeed, the activation of this pathway by GIP is cAMP and PKA-dependent (Ehses et al 2002). Regulation of gene expression by cAMP may have relevance to effects on β-cell growth, differentiation and apoptosis. For example, Andersen et al (1996) showed that elevated cAMP levels protected rat islets against interleukin-1β-mediated induction of nitric oxide synthase and nitric oxide production. Excessive nitric oxide production appears to mediate subsequent apoptosis of β-cells. On the other hand, β-cell lines were made more susceptible to apoptosis following exposure to dibutyryl cAMP (Loweth et al 1996) or the cAMP elevating agent forskolin (Ahmad et al 2000b).

Formation of cAMP in the β-cell

The pancreatic islet β-cell was shown to express several of the nine adenylyl cyclases (AC), including AC1, AC2, AC3, AC4, AC5, AC6 and AC8 (Leech et al 1999; Guenifi et al 2000; Delmeire et al 2003), including the calcium-calmodulin activated AC1, AC3 and AC8.

Destruction of cyclic nucleotides in pancreatic islet β-cell

The hydrolysis of cAMP and cyclic GMP (cGMP) to their biologically inactive 5′ derivatives is catalysed by the family of enzymes known as the cyclic nucleotide phosphodiesterases, which provide the only known system for destroying these cyclic nucleotides. Currently there are 11 known gene families of CN-PDEs (PDE1–PDE11), comprising more than 50 enzymes with differences in their substrate selectivity (cAMP vs cGMP), kinetics, allosteric regulation, tissue distribution and susceptibility to pharmacological inhibition (reviewed by Perry & Higgs 1998; Soderling & Beavo 2000; Mehats et al 2002). Table 1 shows the substrate, the K_m values, particular properties and inhibitors of some of these enzymes, particularly those which have been identified in pancreatic islets. In view of the clear importance of cyclic nucleotides, especially cAMP, in the pancreatic islet β-cell, it is important to understand the nature and the roles of the phosphodiesterase (PDE) enzymes expressed in pancreatic islet β-cells.

PDE isoforms present in the islets

Most studies to elucidate the PDE isoforms present in the islet β-cell have been undertaken using pancreatic islets, which contain four endocrine cell types; these comprise the insulin secreting β-cells, the glucagon secreting A cells, the somatostatin secreting D cells and the pancreatic polypeptide (PP) cells. Non-endocrine cells, including blood vessels are also present. To look more selectively at the islet β-cell, insulin-secreting cell lines have been used extensively, although these have limitations as models for native β-cells. Evidence was obtained for the presence of PDE1 (calcium-calmodulin activated), PDE3 (cyclic 3′5′ GMP-inhibited) and PDE4 (cAMP-specific) in islets and β-cell lines.

There is good evidence for the presence of PDE3 in insulin secreting cells for the role of this isoform in regulating the cAMP pool relevant to insulin secretion. Thus membrane and cytosolic fractions of islet homogenates contained a low K_m (1.4–2.2 μM) cAMP PDE activity (Shafiee-Nick 1995) that was inhibited by 60–70% using 10 μM cGMP (Furman & Pyne 1990). The pellet fraction of homogenates of BRIN BD11 cells was also found to contain a cAMP PDE activity that was potently (IC50 0.7 μM) inhibited (up to 30–40%) by cGMP (Ahmad et al 2000b). Of the two isoforms of type 3 phosphodiesterase (PDE3), PDE3A and PDE3B, only PDE3B appears to be expressed in the β-cell. Western blotting of extracts of rat pancreatic islets and the β-cell line HIT-T15, using a polyclonal antibody against a GST-PDE3B, revealed a single protein band corresponding in size to the PDE3B protein found in extracts of rat epididymal adipose tissue (Zhao et al 1997). Immunostaining of rat islets showed that PDE3B was expressed only in cells that were co-stained with anti-insulin antibodies (Zhao et al 1997). Reverse transcription-polymerase chain reaction (RT-PCR) using BRIN-BD11 cell RNA and PDE3B-specific primers demonstrated a product showing > 97% sequence homology with rat adipose tissue PDE3B (Ahmad et al 2000a, b).

The selective PDE3 inhibitors SK&F 94836 and Org 9935 potently inhibited rat islet PDE activity, especially in membrane fractions (Shafiee-Nick et al 1995) with up to 85% of membrane-bound PDE being inhibited. Similar observations were made in human islets (Shafiee-Nick et al 1994) and in the BRIN BD11 insulin secreting cell line (Ahmad et al 2000a), although in homogenates of this cell line, unlike in islets, the drugs inhibited cAMP hydrolysis only in the membrane fractions. Rat and human islets were shown by Parker et al (1995) to contain a milrinone-sensitive PDE, accounting for up to 70% of

Table 1 Properties of phosphodiesterases that have been identified in the pancreatic islet β-cell

Enzyme	Reference	Substrate	K_m (μM)	Properties/comment	Inhibitors
PDE1A PDE1B PDE1C	Han et al (1999) Clapham & Widerspin (2001) Yan et al (1995)	 cGMP cAMP	 3 1–30	Activated by Ca/calmodulin. Evidence for PDE1C in insulin secreting cells	Zaprinast (1/5), vinpocetine (1A) SCH51866 (also 5,9,10A), 8-MM-IBMX (1C)
PDE3A PDE3B	Perry & Higgs (1998), Mehats et al (2002) Pyne et al (1987), Harrison et al (1986)	cAMP	0.5	Inhibited by cGMP Phosphorylation by PKA and insulin-sensitive kinase. Evidence for PDE3B but not PDE3A in insulin secreting cells	Milrinone, Org 9935, siguazodan, cilostamide, enoximone, OPC3911. No evidence that any of these is selective for PDE3B vs PDE3A
PDE4A PDE4B PDE4C PDE4D	Torphy (1998), Houslay (1998)	cAMP	0.2–4	Phosphorylation by PKA and p42/p44 MAPK (PDE4D3). There is no indication which subtype(s) present in insulin secreting cells.	Rolipram, RP73401, zardaverine, CP80633, CDP840, LAS31025, SB207499
PDE5	Corbin & Francis (1999)	cGMP	2	Phosphorylation by PKA/PKG	Zaprinast, sildenafil, dipyridamole, SCH51866 (also 1,9, 10A)
PDE6	Pittler et al (1990)	cGMP	60–100	Has been identified recently in pancreatic β-cells (Kaminski & Morgan 2004)	Zaprinast, dipyridamole
PDE8A, PDE8B	Fisher et al (1998)	cAMP	0.7	IBMX-insensitive. The only evidence for its presence in insulin secreting cells is the observation that approximately 10% of the cAMP PDE activity is insensitive to IBMX.	Dipyridamole
PDE10A	Soderling & Beavo (2000)	cAMP cGMP	0.05 3		SCH51866 (also 1,5,9) dipyridamole

total islet PDE activity. The PDE3 activity found in the soluble fraction of homogenates of rat islets might be PDE3A from blood vessels or other non-β-cell tissue, since PDE3A was shown exclusively in the cytosolic fractions of all tissues studied, whereas PDE3B was expressed in particulate fractions (Liu & Maurice 1998).

Other isoforms also contribute to islet β-cell PDE activity. Both islets (Sugden & Ashcroft 1981; Lipson & Oldham 1983; Capito et al 1986) and the β-cell lines BRIN BD11 (Ahmad et al 2000a) and β-TC3 (Han et al 1999) contain a Ca-calmodulin activated PDE. The PDE1/PDE5 inhibitor zaprinast produced a 14–30% inhibition in membrane and cytosolic fractions of rat islet homogenates (Shafiee-Nick et al 1995) and 25% inhibition of cAMP/cGMP PDE activity in BRIN BD11 cells (IC50∼1–5 μM) (Ahmad et al 2000a). Han et al (1999) showed that the presence of a PDE that was inhibited by zaprinast (IC50 4.5 μM) and 8-MM-IBMX (IC50 7.5 μM) but not by vinpocetine (IC50 > 100 μM for PDE1C vs 1.4 and 9.8 μM for PDE1A and PDE1B, respectively). These findings were compatible with the presence of PDE1C but not PDE1A or PDE1B and this was supported by RT-PCR (Han et al 1999).

A role for the cAMP-selective PDE4 in the hydrolysis of β-cell cAMP was supported by the inhibition of islet (Shafiee-Nick et al 1995; Parker et al 1995) or BRIN-BD11 cell (Ahmad et al 2000a) PDE by the selective PDE4 inhibitor rolipram. Han et al (1999) demonstrated the expression of PDE4A and PDE4D in βTC3 cells using RT-PCR.

The importance of PD3B in regulating insulin secretion

Our studies, using biochemical and pharmacological approaches, first suggested the role of PDE3 in modulating glucose-induced insulin secretion in islets of rat (Furman & Pyne 1990; Shafiee-Nick et al 1995) and man (Shafiee-Nick et al 1994). This has been generally confirmed by others in islets (Parker et al 1995; Zhao et al 1997; Harndähl et al 2002), and by ourselves and others in insulin secreting cell lines (Ahmad et al 2000a; Harndähl et al 2002). The importance of PDE3B in regulating β-cell cAMP in the context of insulin secretion was demonstrated by adenovirus-mediated over-expression of PDE3B in a β-cell line or in islets (Harndähl et al 2002) and by using transgenic animals over-expressing PDE3B in the β-cell (Harndähl et al 2004). Those studies clearly showed that glucose-induced, as well as GLP-1-induced insulin secretion was impaired by PDE3B over-expression in-vitro and in-vivo.

Although there is very strong evidence supporting the role of PDE3B in the β-cell, some studies have suggested the importance of other PDEs. Rolipram, a selective PDE4 inhibitor, augmented glucose-induced insulin secretion in β-cell lines (Han et al 1999; Ahmad et al 2000a), although not in islets (Shafiee-Nick et al 1995). This discrepancy

between islets and cell lines may be due to differences in the compartmentalization of PDE4 isoforms, allowing PDE4 to interact with relevant cAMP pools in transformed cell lines but not in native β-cells. A role for PDE1 in both the β-TC3 cell line and in islets was suggested by Han et al (1999) who showed that 8-MM-IBMX, purported to be a selective inhibitor of the Ca-calmodulin activated PDE1C isoform, augmented glucose-induced insulin secretion. The importance of PDE1 in regulating β-cell cAMP remains to be determined.

Exploitation of cAMP mechanisms in the development of drugs to treat type 2 diabetes

From the foregoing it is clear that increasing β-cell levels of cAMP will augment glucose-induced insulin secretion. Therapeutically, this could be achieved by activating β-cell adenylyl cyclase using analogues of GLP-1 or GIP, increasing the availability of endogenous GLP-1 and GIP by preventing their normal very rapid breakdown or by preventing the breakdown of cAMP in the β-cell using inhibitors of phosphodiesterase selective for the most important β-cell isoform i.e. PDE3B.

GLP-1 analogues

The metabolic stability and biological activity of many GLP-1 analogues have been investigated (Deacon et al 1998; Knudsen et al 2000; Xiao et al 2001; Green et al 2003a, b, 2004a). So far, limited clinical trials have been conducted using GLP-1 (Gutniak et al 1992; Meier et al 2003; Meneilly et al 2003), GLP-1 analogues (Rolin et al 2002; Chang et al 2003; Holz & Chepurny 2003) and exendin-4 (Egan et al 2002, 2003). However, clear improvements in glucose tolerance have been observed in experimental animals (Holst 1999).

N-terminal (His^7 modified) analogues of GLP-1

Substitution of His^7 with several other amino acids indicated that the imidizole ring of His^7 was crucial for GLP-1 action (Hareter et al 1997). It was shown that N-terminal extension of GLP-1, with insertion of an acetyl group at the α-amino group of His^7, did not significantly affect GLP-1 action. N-terminally extended peptides with imidizole-lactic acid, N-methyl, and α-methyl groups at His^7 (Table 2) are more resistant to dipeptidyl peptidase IV (DPP IV) than native GLP-1, but exhibit reduced affinity for the GLP-1 receptor and compromised ability to stimulate cAMP production (Hareter et al 1997). His^7-glucitol-GLP-1 was shown to be resistant to DPP IV whilst maintaining antihyperglycaemic activity in-vivo (Table 2) (O'Harte et al 2000a, b). Most recently, two additional analogues, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1, have been shown to be completely resistant to DPP IV and human plasma degradation, as well as exhibiting potent receptor binding, cAMP production and insulin secretory activity in-vitro (Table 2). In obese diabetic *(ob/ob)* mice, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 displayed potent insulinotropic actions, with N-pyroglutamyl-GLP-1 being particularly effective compared with the antihyperglycaemic effects of the native peptide (Green et al 2004b).

Position 2 (Ala^8 substituted) analogues of GLP-1

Substitution of Ala^8 for Gly conferred increased resistance to DPP IV and, despite a reduced affinity for the GLP-1 receptor, corrected the fasting hyperglycaemia and glucose intolerance of diabetic mice (Burcelin et al 1999) (Table 2). Similarly, substitution with Ser significantly increased the plasma stability of GLP-1 without impairing its insulinotropic activity in rats (Ritzel et al 1998) (Table 2). Substitution of Ala^8 for Thr, Gly, Ser and α-aminoisobutyric acid (Aib) significantly prolonged biological half-lives in-vivo compared with native GLP-1, but nevertheless bound to the GLP-1 receptor with high affinities (Deacon et al 1998) (Table 2). However, only native GLP-1 and (Aib^8)GLP-1 significantly improved insulin output over basal conditions (Deacon et al 1998). Similarly, analogues with D-Alanine (D-Ala), Ser and Gly substitutions at position 8 were substantially more resistant to DPP IV than native GLP-1, and had similar or enhanced biological half-life and potency (Siegel et al 1999a, b) (Table 2). Substitution of Ala^8 for Gly and aminohexanoic acid (Aha) (Table 2) produced analogues that stimulated insulin secretion and intracellular cAMP to a similar degree to native GLP-1 (Doyle et al 2001). In-vivo they lowered circulating blood glucose and increased blood insulin concentrations in Zucker rats. More recently, Green et al (2003b) characterized two novel Ala^8-substituted analogues of GLP-1, namely (Abu^8)GLP-1 and (Val^8)GLP-1. These were shown to be completely resistant to the actions of DPP IV or human plasma (Table 2). Receptor binding studies demonstrated that (Abu^8)GLP-1 and (Val^8)GLP-1 bound the GLP-1 receptor with high affinity, but that this was reduced compared with native GLP-1 (Table 2). Although active stimulators of intracellular cAMP production, (Abu^8)GLP-1 and (Val^8)GLP-1 were 1.5- and 3.5-fold less potent, respectively, than native GLP-1 (Green et al 2003b). Despite these losses in receptor affinity and cAMP production, this did not compromise insulinotropic activity either in-vitro or in-vivo (Table 2). This may be partially explained by the diverse mechanisms of action of GLP-1 on the pancreatic β-cell. Glucose tolerance tests in obese diabetic *(ob/ob)* mice showed that (Abu^8)GLP-1 had similar in-vivo glucose-lowering ability to native GLP-1, while (Val^8)GLP-1 was up to 37% more potent. This glucose-lowering activity was coupled with enhanced insulin releasing activity following peptide administration. (Abu^8)GLP-1 and (Val^8)GLP-1 appeared to be equipotent insulin secretagogues in-vivo (Green et al 2003b). Amino acid substitution of GLP-1 at Ala^8 appears to be a particularly viable strategy for generating worthwhile stable therapeutic candidate peptides for the treatment of type 2 diabetes.

Position 3 (Glu^9 substituted) analogues of GLP-1

Substitution of Glu^9 by either Ala or Asp dramatically altered GLP-1 receptor affinity and biological activity (Xiao et al 2001). Green et al (2003a) investigated how modifications at Glu^9 affected degradation of GLP-1 by

Table 2 Properties of various GLP-1 analogues

Position modified	Analogue	DPP IV resistance	In-vitro properties			In-vivo activities	
			Decreased	Unchanged	Increased	Glucose lowering	Insulin secretion
Position 7 GLP-1	Native GLP-1	N		√		+	+
	N-acetyl-GLP-1	++	√			−	+
	N-glucitol-GLP-1	++		√		N	N
	D-His^7-GLP-1	++	√			U	U
	N-Imi-GLP-1	++	√			U	U
	N-Me-GLP-1	++	√			U	U
	N-α-Me-GLP-1	+	√			U	U
	N-pGlu-GLP-1	++	√			+	N
Position 8 GLP-1	D-Ala^8-GLP-1	++	√			N	+
	Abu^8-GLP-1	++	√			N	N
	Aha^8-GLP-1	++	√			N	N
	Aib^8-GLP-1	++			√	N	N
	Gly^8-GLP-1	+	√			+	N
	Ser^8-GLP-1	+	√			+	+
	Thr^8-GLP-1	++	√			U	U
	Val^8-GLP-1	++	√			+	N
Position 9 GLP-1	Ala^9-GLP-1	U	√			−	U
	Asp^9-GLP-1	U	√			N	U
	Lys^9-GLP-1	++	√*			− *	− *
	Phe^9-GLP-1	+	√			−	−
	Pro^9-GLP-1	++	√			N	N
	Tyr^9-GLP-1	+	√			−	−
Various fatty acid modifications	LysPal^{26}-GLP-1	++	√			−	−
	Abu^8, LysPal^{26}-GLP-1	++	√			−	−
	Val^8- LysPal^{26}-GLP-1	++	√			−	−
	NN2211	++		√		+	+
	CJC-1131	++		√		+	U
	Ly315902	U		U		U	N
	Exendin-4(1–39)	++			√	+	+

U = unknown effect; N = neutral effect (i.e. same as native GLP-1), + improvement, ++ marked improvement; − deterioration in activity; * = antagonist like activity observed. Aha = aminohexanoic acid, Aib = aminoisobutyric acid. Abu = 2-aminobutryic acid, Sar = sarcosine, Sar, Imi = imidizole-lactic acid group; Me = methyl group; Fmoc = N-(9-fluorenyl)methoxycarbonyl group.

DPP IV. Notably substitution of Glu^9 with Pro^9 yielded a peptide which retained all of the normal biological actions and was substantially more stable than native GLP-1 (Table 2). Other analogues, (Phe^9)GLP-1 and (Tyr^9)GLP-1, were less resistant than (Pro^9)GLP-1 to the action of DPP IV but were more resistant than native GLP-1 (Green et al 2003a). Of these novel analogues, only (Pro^9)GLP-1 was highly potent in lowering plasma glucose and raising insulin levels in-vivo in a commonly used animal model of type 2 diabetes (Green et al 2003b). Furthermore, replacing Glu^9 with Lys^9 (Table 2) produced a GLP-1 analogue which was profoundly resistant to DPP IV degradation and which possessed cellular and metabolic actions similar to those of the established antagonists, GLP-1(9–36)amide and exendin (9–39) (Green et al 2004c).

Acylated and other analogues of GLP-1

To overcome renal clearance and extend bioactivity beyond just a few hours, GLP-1 analogues have been generated with attachment of acylated groups at selected residues (Ruiz-Grande et al 1990; Gault et al 2002a; Holz & Chepurny 2003). Lys^{26}-(N-ϵ-(γ-Glu(N-α-hexadecanoyl)))-GLP-1, otherwise known as NN2211, has an extended half-life of 8 h in man and a promising metabolic profile (Table 2) (Rolin et al 2002). Other analogues modified in this way possess prolonged half-lives. Lys^{34}-(octanoyl)-GLP-1 (Ly315902) has a half-life of 3–6 h in dogs (Chou et al 1997) and Lys^{37}(2-(2-(2-maleimidopropionamido (ethoxy)ethoxylacetamide)))-GLP-1 (CJC-1131, Table 2) has a half-life of 18 h in rats (Kim et al 2003). Green et al (2004a) indicated that acylated GLP-1 peptides could be problematic in terms of bioactivity and bioavailability in acute experiments (Table 2). Recently CJC-1131, which is a GLP-1 analogue with albumin attached to extend plasma half-life, has been reported to have beneficial actions (Kim et al 2003). Other recent clinical trials have examined exenatide (AC-2993), which is based on the exendin naturally-occurring peptide from the Gila monster which acts as an agonist on the GLP-1 receptor (Nielsen et al 2004).

GIP analogues

Although much early attention was devoted to the study of GLP-1 analogues, recent studies indicate that stable forms of GIP may be particularly promising as potential antidiabetic agents. Thus, a number of studies have been published examining the in-vitro activities of a range of GIP fragments and analogues (O'Harte et al 1999; Kühn-Wache et al 2000; Hinke et al 2001, 2004; Gault et al 2002a, b, c, d, 2003a, b, c; Manhart et al 2003). A family of selective designer human GIP (1–42) analogues (Figure 2) modified at positions Tyr^1 (O'Harte et al 1999, 2000a, 2002; Gault et al 2002b), Ala^2 (Hinke et al 2002; Gault et al 2003a, b), and Glu^3 (Gault et al 2002b, 2003c) have generated particularly promising results in animal models of type 2 diabetes.

N-terminal (Tyr^1 modified) analogues of GIP

Several novel Tyr^1-modified analogues of GIP have been developed recently, including N-acetyl-, N-Fmoc-, N-glucitol-, N-palmitate-, and N-pyroglutamyl-GIP (O'Harte et al 1998, 1999, 2000a, 2000b, 2002; Gault et al 2002a). These analogues modified at the α-amino region of Tyr^1 exhibited complete resistance to purified DPP IV with in-vitro half-lives greater than 12 h compared with 2.3 h for native GIP (Table 3). All Tyr^1-modified analogues stimulated increases of 2–10-fold in cAMP production and approximate 1.4-fold increases in insulin secretion in-vitro. Subtle differences in individual analogues were noted. N-Fmoc- and N-palmitate-GIP (Table 3) appeared to be 14–20% less potent in-vitro than N-acetyl-, N-glucitol- and N-pyroglutamyl-GIP. In *ob*/*ob* mice, Tyr^1-modified analogues were noticeably superior at stimulating insulin release and lowering blood glucose compared with native GIP. Consistent with in-vitro data, these results indicated that N-acetyl-, N-glucitol- and N-pyroglutamyl-GIP were slightly more potent than either N-Fmoc- or N-palmitate-GIP (O'Harte et al 2002; Gault et al 2002b). Of the Tyr^1-modified analogues tested, N-acetyl-GIP (O'Harte et al 2002) appeared to be the most impressive. Importantly, the severe insulin resistance and β-cell defect of *ob*/*ob* mice (including poor response to native GIP) was largely overcome by N-acetyl-GIP, making the use of such an analogue for type 2 diabetes therapy a feasible objective. Thus any defect in the insulinotropic response to GIP in diabetes appears to be overcome by these stable and chemically modified GIP agonists.

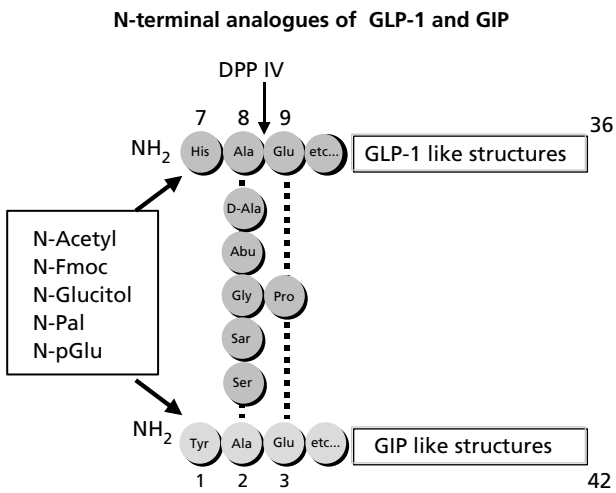

Figure 2 N-terminal analogues of GLP-1 and GIP showing the positions of the substituents referred to in Tables 2 and 3.

Position 2 (Ala^2 substituted) analogues of GIP

Ala^2-substituted GIP analogues, including (Abu^2)GIP, (Gly^2)GIP, (Sar^2)GIP, (Ser^2)GIP and (D-Ala^2)GIP have been synthesized and tested for DPP IV stability and biological activity (Hinke et al 2002; Gault et al 2003a, b) (Table 3). (Abu^2)GIP and (Sar^2)GIP did not exhibit resistance to DPP IV and had disappointing in-vitro biological activities. However, both analogues displayed antihyperglycaemic and insulinotropic activity comparable with native GIP when administered to *ob*/*ob* mice (Table 3) (Gault et al 2003b). In contrast, (Gly^2)GIP and (Ser^2)GIP (Gault et al 2003a) were more resistant to DPP IV than native GIP, and displayed enhanced ability to elevate cAMP and stimulate insulin secretion in-vitro (Table 3). These actions resulted in (Gly^2)GIP and (Ser^2)GIP having significantly improved insulinotropic and antihyperglycaemic activities in *ob*/*ob* mice compared with native GIP. Similarly, Hinke et al (2002) investigated the substitution of L-alanine in position 2 of GIP with D-alanine (Table 3). This enzyme-resistant analogue exhibited moderately reduced biological activity in-vitro, but significantly improved the glycaemic excursion in *fa*/*fa* VDF Zucker rats. (D-Ala^2)GIP demonstrated similar activity to (Gly^2)GIP or (Ser^2)GIP (Table 3) (Gault et al 2003a). Despite notable improvements in biological activity compared with native GIP, the efficacy of Ala^2-substituted analogues was not as impressive as Tyr^1-modified GIP analogues (Table 2).

Position 3 (Glu^3 substituted) analogues of GIP

Substitution of Glu^3 with Pro produced a novel GIP receptor antagonist, (Pro^3)GIP, which was completely resistant to DPP IV mediated degradation (Table 3) (Gault et al 2002c). (Pro^3)GIP inhibited GIP-stimulated cAMP production and insulin secretion with high sensitivity and specificity in-vitro. Furthermore, (Pro^3)GIP effectively countered the insulin-releasing and antihyperglycaemic actions of the native GIP in *ob*/*ob* mice (Gault et al 2002c, 2003c). These actions were similar to the GIP(3–42) antagonist but more specific than exendin(9–39) (Gault et al 2003c). The possible therapeutic usefulness of this selective and potent GIP-receptor antagonist has been emphasized by studies which suggested that GIP plays a key role in lipid metabolism and in the development of both genetically-inherited and diet-induced obesity (Miyawaki et al 2002).

DPP IV inhibitors

Significant improvements of metabolism and glycaemic control in diabetes have been reported following treatment

Table 3 Properties of various GIP analogues

Position modified	Analogue	DPP IV resistance	In-vitro properties			In-vivo activities	
			Decreased	Unchanged	Increased	Glucose lowering	Insulin sercetion
Position 1 GIP	Native GIP	N		√		N	N
	N-Acetyl-GIP	++			√	+	+
	N-Fmoc-GIP	++			√	+	+
	N-Glucitol-GIP	++		√		+	+
	N-Pal-GIP	++			√	+	+
	N-pGlu-GIP	++			√	+	+
Position 2 GIP	D-Ala^2-GIP	++		√		+	+
	Abu^2-GIP	N		√		−	−
	Gly^2-GIP	+		√		+	+
	Sar^2-GIP	++	√			−	−
	Ser^2-GIP	+		√		+	+
Position 3 GIP	Pro^3-GIP	++	√*			− *	− *

N = neutral effect (i.e. same as native GLP-1), + improvement, ++ marked improvement; − deterioration in activity; * = Antagonist like activity observed. Abu = 2-aminobutryic acid, Fmoc = N-(9-fluorenyl)methoxycarbonyl group, Pal = palmitate, Sar = sarcosine.

with a number of DPP IV inhibitors (Pederson et al 1998b; Balkan et al 1999; Pauly et al 1999; Ahren et al 2000; Deacon et al 2001, 2002). Administering NVP-LAF237 with pioglitazone appeared to normalize glucose concentrations in adult obese Zucker rats (Burkey et al 2002). Another DPP IV inhibitor, FE 999011, delayed the onset of type 2 diabetes in Zucker diabetic rats (Sudre et al 2002). Oral administration of the DPP IV inhibitor, P32/98, to VDF (*fa*/*fa*) Zucker rats caused sustained improvements in glucose tolerance, insulin sensitivity and β-cell glucose responsiveness (Pospisilik et al 2002a). Treating these animals for a prolonged period with P32/98 also improved hepatic and peripheral insulin sensitivity (Pospisilik et al 2002b). In streptozotocin-induced diabetic rats, β-cell survival and islet neogenesis was enhanced by P32/98 (Pospisilik et al 2003). Additionally, type 2 diabetic subjects showed improved metabolic control following administration of NVP DPP728 two to three times daily over four weeks (Ahren et al 2002).

Despite beneficial actions of DPP IV inhibitors on glycaemic control, the physiological actions of DPP IV inhibition on many other regulatory peptide substrates (at least 35) could be adversely affected (Mentlein 1999). Until a fully comprehensive evaluation of the consequences of widespread DPP IV inhibition has been carried out in clinical trials, the potential of DPP IV inhibitors in diabetes therapy will remain uncertain. However, some encouragement can be taken from the lack of obvious abnormalities in DPP IV knock-out animals (Cheng et al 1999). A major advantage of using DPP IV inhibitors is the possibility of oral administration; however, uncertainty exists regarding their efficacy (Holst 2003).

PDE inhibitors

Selective inhibition of PDE3 in the islet β-cell will augment meal-related insulin secretion, because of the amplification of the effect of GIP and GLP-1. If PDE3 inhibitors are to be useful for the treatment of type 2 diabetes mellitus, they would need to be selective for islet β-cell PDE3. However, the important islet PDE3 isoform is PDE3B, which also appears to be the isoenzyme in the liver and adipose tissue (Reinhardt et al 1995), where its activation mediates some of the effects of insulin. Indeed, our in-vivo work showed that potent PDE3 inhibitors markedly augmented glucose-induced elevations of plasma insulin concentrations, but did not modify plasma glucose concentrations (El-Metwally et al 1997). The lack of effect on plasma glucose may have been due to concomitant inhibition of hepatic and adipose tissue PDE3. Milrinone, another PDE3 inhibitor, improved glucose tolerance in the normal mouse but was hyperglycaemic in the *ob*/*ob* mouse, despite elevations in plasma insulin (Parker et al 1997). That group showed that milrinone antagonized insulin-mediated inhibition of lipolysis in isolated adipocytes and insulin-mediated inhibition of glucose production in isolated hepatocytes. Interestingly, Graham et al (2001) showed that caffeine, a non-selective PDE inhibitor, augmented glucose-induced elevations in serum insulin concentrations, while tending to elevate blood glucose during oral glucose tolerance tests in young, healthy male volunteers, again suggesting insulin resistance. These observations may explain why changes in blood glucose were not reported in the major clinical trial of milrinone for heart failure (Packer et al 1991). Although highly speculative, hyperinsulinaemia associated with insulin resistance might have contributed to the higher mortality in milrinone-treated patients in this trial, as hyperinsulinaemia/insulin resistance is associated with increased production of PAI-1 (Bastard et al 2000), thereby impairing fibrinolysis.

Another intriguing possibility lies in the potential of PDE inhibitors to prevent β-cell loss in both type 1 and type 2 diabetes. The non-selective PDE inhibitor pentoxifylline and the PDE4-selective agent rolipram were shown to reduce

insulitis and prevent diabetes in NOD (non-obese diabetic) mice (Liang et al 1998). While this effect may be explained by inhibition of cytokine production by pro-inflammatory cells such as macrophages, there may also be a contribution from an inhibition of nitric oxide production by islet cells in response to cytokines. Thus pentoxifylline and the selective PDE3 inhibitor cilostamide blocked nitric oxide production by mouse islet cells and a β-cell line (NIT-1) in response to stimulation by lipopolysaccharide and inflammatory cytokines (Besay & Prud'homme 2001) and prevented the expression of inducible nitric oxide synthase both in-vitro and in-vivo in the NOD mouse. This supports earlier findings that IBMX reduced interleukin-1β-induced nitric oxide synthase expression and nitric oxide production in rat islets, an effect mimicked by dibutyryl cAMP (Andersen et al 1996). However, much more work is needed to clarify the role of cAMP in preventing β-cell death, especially in view of the promotion of apoptosis by exposure to dibutyryl cAMP (Loweth et al 1996) or forskolin (Ahmad et al 2000b) in β-cell lines. Indeed, we have reported that serum withdrawal-induced apoptosis of BRIN BD 11 cells is associated with impaired IGF-1-dependent regulation of PDE3B (Ahmad et al 2000b), which is predicted to induce increases in intracellular cAMP, perhaps leading to dysfunctional regulation of BAD/Bcl2 and thereby promoting apoptosis.

Conclusions

The development of GLP-1 and GIP analogues and the DPP IV inhibitors has opened up entirely new and exciting approaches to treating type 2 diabetes mellitus. Some of these agents have now reached Phase 3 clinical trials. The potential for the use of PDE3 inhibitors is limited because of the lack of selectivity of current drugs for the PDE3B isoform. Recently a benzyl vinologous amide substituted aryldihydropyridazinone was shown to potently inhibit PDE3, with a 6.8-fold selectivity for PDE3B compared with PDE3A, suggesting the possibility of achieving PDE3B-selectivity (Edmondson et al 2003). Even if selective PDE3B inhibitors were developed they would need targeting to the pancreatic β-cell, to avoid insulin resistance mediated by inactivating the enzyme in adipocytes and hepatocytes. As there is evidence for a role for cAMP in regulating GLP-1 secretion, it may also be possible to target PDE in the intestinal L-cell as a way of increasing the secretion of the hormone. We have recently found PDE3B to be expressed in intestinal mucosal cells (Pyne et al, unpublished observations) thus allowing the intriguing possibility that PDE3B may be involved in regulating GLP-1 secretion. Targeting intestinal mucosal L-cell PDE3B may raise fewer problems than attempting the selective targeting of the pancreatic islet β-cell enzyme.

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