Alternative Signaling Pathways of the Glucose-dependent Insulinotropic Polypeptide (GIP) Receptor

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ABSTRACT

Glucose-dependent insulinotropic polypeptide (GIP) regulates pancreatic β-cell function by binding to its cognate Family B, G protein-coupled receptor and elevating intracellular cAMP and Ca^{2+}. The main rationale for the studies described in this Thesis was that multiple interacting intracellular signal transduction pathways were proposed to mediate GIP's pleiotropic actions on the pancreatic β-cell. Moreover, the ability of GIP to potentiate insulin secretion is blunted in some type 2 diabetics, implying potential defects at the receptor signaling level. Therefore, the major aim was to characterize further the intricate network of GIP receptor signaling pathways underlying β-cell processes using β-cell models. Using GIP receptor-transfected CHO-K1 cells and βTC-3 and INS-1 tumour cell lines, it was possible to correlate GIP receptor activation with the regulation of lipid signaling (arachidonic acid release), K^{+}_{ATP} channel-independent events, MAP kinase signaling (Raf→Mek1/2→ERK1/2→p90RSK and p38 MAPK), and CREB signaling. These events were demonstrated to be functionally relevant for insulin secretion, cell growth and survival (MAPK signaling), and insulin gene transcription (CREB signaling) respectively.

Through insulin secretion studies and pharmacological approaches, GIP was shown to regulate the secretion of insulin via activation of PLA_2 and K^{+}_{ATP} channel-independent mechanisms in βTC-3 cells. In an attempt to elucidate novel signals coupled to the GIP receptor, we examined the expression of 75 protein kinases and 25 protein phosphatases in CHO-K1, βTC-3, and INS-1 cells. This has allowed the partial mapping of intracellular signal transduction pathways for these cell models. From this, GIP receptor coupled signaling events were studied using phospho-specific antibodies, transfection techniques, pharmacological inhibitors, and gene reporter assays. Studies in CHO-K1 cells expressing the GIP receptor and in INS-1 β-cells have implicated cAMP/PKA signaling in the regulation of the mitogenic ERK1/2 module, Raf→Mek1/2→ERK1/2→ p90RSK. Results further suggest that the GIP receptor is coupled to β-cell survival via cAMP mediated inhibition of p38 MAPK and caspase-3 activity. Finally, coupling of the GIP receptor to rat insulin promoter activity was shown to occur via cAMP/PKA and a CREB family transcription factor. These events were found to be independent of phospho-regulation of S133 CREB/S117 CREM/S63 ATF-1, and suggest that the tightly regulated phosphorylation of these transcription factors by GIP may be involved in novel signals regulating β-cell function.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AA	Arachidonic acid
ACTH	Adrenocorticotropic hormone
AFC	7-amino-4-(trifluoromethyl) coumarin
AKAP	A-kinase anchoring protein
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
ATF-1/2	Activating transcription factor-1/2
βARKct	β-adrenergic receptor kinase C-terminus
BHLH	Basic helix loop helix
BMX	Bone marrow X kinase
BSA	Bovine serum albumin
BTK	Bruton tyrosine kinase
BZIP	Basic leucine zipper
CaMK	Calmodulin-dependent kinase
CAMP	Cyclic adenosine monophosphate
cAMP-GEF	cAMP guanine nucleotide exchange factor
CCK	Cholecystokinin
CD45	Leukocyte common antigen CD45 transmembrane phosphatase
Cdk	Cyclin-dependent kinase
CHO	Chinese hamster ovary
CICR	Calcium induced calcium release
CK	Casein kinase
Cot/Tpl-2	Cancer Osaka thyroid oncogene/Tumor progression locus-2
CPKA	Catalytic subunit of PKA
CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modulator
CSK	C-terminus Src kinase
CT	Carboxy terminal
DAG	Diacylglycerol
DAPK	Death-associated protein kinase
DMEM	Dulbecco's modified eagle media
DP IV	Dipeptidylpeptidase IV
EGF	Epidermal growth factor
ERK1/2	Extracellular regulated kinase-1/2
FAK	Focal adhesion kinase
FFA	Free fatty acid
FKHR	Forkhead transcription factor
Fyn	Fyn oncogene related to SRC
GCK	Germinal center kinase

GFP	Green fluorescent protein
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GIP	Glucose-dependent insulinotropic polypeptide
GIPR-/	GIP receptor knockout
Gln	Glutamine
GLP-1/2	Glucagon-like peptide-1/2
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
GRK2	G-protein receptor kinase-2
GRP	Gastrin releasing peptide
GSK-3	Glycogen synthase kinase-3
HELSS	Haloenolactone suicide substrate
HPA	Hypothalamus-pituitary-adrenal
Hpk	Hematopoetic progenitor kinase
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor-1
IKK	Inhibitor NF-κB kinase
IP_3	Inositol-1,4,5-triphosphate
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
$iPLA_2\beta$	ATP-stimulatable, Ca^{2+}-independent cytosolic PLA_2 isoform β
JAK	Janus kinase
JNK	Jun N-terminal kinase
K^+_{ATP}	ATP sensitive K^+ channel
KAP	Cyclin-dependent kinase associated phosphatase
KIC	α-ketoisocaproic acid
KRBH	Modified Kreb's ringer with Hepes and BSA
Ksr	Kinase suppressor of Ras 1
LAR	LCA antigen-related (LAR) transmembrane tyrosine phosphatase
Lck	Lymphocyte specific protein-tyrosine kinase
Leu	Leucine
LHRH	Leutinizing hormone releasing hormone
Lyn	Oncogene Lyn
MAP	Mitogen-activated protein
MAPK	MAP kinase
MAPKAP-2	MAPK activated protein-2
MKK/Mek	MAPK kinase
MKKK/Mekk	MAPK kinase kinase
MKP-1/2/3	MAP kinase phosphatase-1/2/3
Mnk	MAPK interacting kinase
Mos	v-Mos Moloney murine sarcoma viral oncogene homolog 1
MSK-1	Mitogen and stress-activated kinase-1

Mst1	Mammalian sterile 20-like 1
NAADP	Nicotinic acid adenine dinucleotide phosphate
Nek	NIMA (never in mitosis) related kinase 2
NSCC	Non-specific cation channel
p90RSK	90 kDa Ribosomal S6 kinase
PAC1	MAP kinase phosphatase PAC1
PACAP	Pituitary adenylate cyclase activating protein
PAK	p21 Activated kinase
PC1/3	Proconvertase-1/3
PDGF	Platelet-derived growth factor
PDK	3-Phosphoinositide-dependent kinase
PDX-1	Pancreatic duodenal homeobox transcription factor
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Cyclic GMP-dependent protein kinase
PKR	DsRNA-dependent protein kinase
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PP1/C-alpha	Protein phosphatase 1 catalytic subunit alpha isoform
PP1/C-beta	Protein phosphatase 1 catalytic subunit beta isoform
PP1/C-gamma	Protein phosphatase 1 catalytic subunit gamma isoform
PP2A/A-alpha/beta	Protein phosphatase 2A, A regulatory subunit alpha and beta isoforms
PP2A/C alpha	Protein phosphatase 2A catalytic subunit alpha isoform
PP2A/C beta	Protein phosphatase 2A catalytic subunit beta isoform
PP2B/A alpha	Protein phosphatase 2B catalytic subunit alpha isoform
PP2C	Protein phosphatase 2C
PP2C beta	Protein phosphatase 2C catalytic subunit beta isoform
PP2C/delta	Protein phosphatase 2C catalytic subunit delta isoform
PP2X/C	Protein phosphatase 2X catalytic subunit
PP5, PPT	Protein phosphatase 5 catalytic subunit
PPV/C	Protein phosphatase 6 catalytic
PPX/A'2	Protein phosphatase 2X catalytic subunit
PTEN	Phosphatidylinositol 3'-phosphate and protein phosphatase
PTP	Protein tyrosine phosphatase
PTP-PEST	Protein tyrosine phosphatase with PEST sequences
Pyk	Protein tyrosine kinase
Raf1	Oncogene Raf1
Rb	Retinoblastoma
RIA	Radioimmunoassay
RIP	Rat insulin promoter
RNAi	RNA interference

ROK	RhoA kinase
Rp-cAMPS	Adenosine 3,5-cyclic phosphorothioate-Rp
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
SIRP	Signal regulatory protein substrate of PTP1D phosphatase
SOCS	Suppressor of cytokine signaling
Src	Oncogene Src
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
Syk	Spleen tyrosine kinase
TSH	Thyroid stimulating hormone
UTR	Untranslated region
VDCC	Voltage-dependent calcium channel
VIP	Vasoactive intestinal polypeptide
Yes	Yamaguchi sarcoma viral oncogene homolog 1
ZAP70	Zeta-chain (TCR) associated protein kinase
ZIPK	ZIP kinase

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"If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts he shall end in certainties." Sir Francis Bacon.

Oddly enough I began my PhD with this quotation in mind, having used it in a self-assessment essay that same year. It is not only appropriate to the study of science, but also to most other facets of life. Aside from the 'doubts' that generate questions in science, a PhD is also about the 'doubts' of a person, that ultimately allow for the growth of an individual, facilitated in an intellectual forum. There are numerous individuals that have contributed to my growth and who have inspired me throughout my PhD and I am indebted to all of them.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

The maintenance of glucose homeostasis is achieved via an intricate network of regulatory pathways involving the anabolic hormone insulin, and its catabolic counterpart, glucagon. In addition to these pancreatic islet hormones, there exists an axis regulating postprandial insulin secretion between the small intestine and the pancreatic Islets of Langerhans. This enteroinsular axis is composed of hormonal, neural, and direct substrate effectors, which regulate insulin secretion from the pancreatic β-cell. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1) are the main incretin peptides comprising the hormonal arm of this axis, both being secreted from intestinal endocrine cells in response to carbohydrates, fats, and amino acids. GIP was initially characterized by its ability to inhibit gastric acid secretion, and in 1973 was discovered to have insulinotropic effects on the pancreatic β-cell. GIP is now known to be a major regulator of pancreatic β-cell function, by regulating insulin secretion, insulin gene transcription, and β-cell growth and survival. These effects are mediated by GIP binding to its cognate G-protein coupled receptor (GPCR), resulting in the elevation of intracellular cAMP and Ca^{2+}. Given the blunted effects of GIP in some type 2 diabetics, and the downregulation of the GIP receptor in an animal model of this disease, studies aimed at elucidating coupling of GIP receptor signal transduction to functional β-cell processes are warranted.

1.2 THE DISCOVERY OF GIP

1.2.1 The incretin concept and the discovery of GIP

It had been known for a long time that a large bolus of oral glucose did not result in the production of the glucosuria seen during similar doses of glucose given intravenously (i.v.) (Bernard 1877). Based on this observation and the discovery of secretin in 1902, Moore, Edie, and Abram suggested in 1906 that the duodenum must supply a "chemical excitant for the internal secretion of the pancreas" (Moore et al. 1906). Twenty years later La Barre and colleagues demonstrated that i.v. injection of crude secretin produced hypoglycaemia in dogs via stimulation of the endocrine pancreas (Zunz and La Barre 1929; La Barre and Still 1930). They

coined the term "incretin", based on the fact that the secretin preparation contained two active principles: an incretin stimulating the internal secretion of the pancreas, and an excretin, stimulating the exocrine pancreas (La Barre and Still 1930). This term was forgotten until the 1960s, after which the development of the insulin radioimmunoassay (RIA) allowed for the demonstration that a much greater insulin response to oral glucose was observed when compared to i.v. glucose (Elrick et al. 1964; McIntyre et al. 1964). By simple subtraction, Perley and Kipnis estimated that 50 % of the insulin secreted after oral glucose is due to gastrointestinal factors (Perley and Kipnis 1967).

The term, enteroinsular axis, was subsequently coined by Unger and Eisentraut (Unger and Eisentraut 1969) to describe the connection between the small intestine and the pancreatic islets of Langerhans. This axis encompasses nutrient, neuronal, and hormonal signals from the gut that act on the islets to secrete insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), and pancreatic polypeptide (PP cells). The term incretin, was further defined by Creutzfeld to include the hormonal component of the enteroinsular axis that 1) must be released by nutrients, particularly carbohydrates, and 2) must stimulate insulin secretion in the presence of elevated glucose levels at physiological concentrations (Creutzfeldt 1979; Creutzfeldt and Ebert 1985).

Despite fulfilling these requirements today, GIP was first isolated as a potent inhibitor of gastric acid and pepsin secretion from impure preparations of cholecystokinin-pancreozymin (CCK-PZ) by Brown, Pederson and colleagues (Brown et al. 1969, 1970; Brown and Dryburgh 1971). Thus, the acronym "GIP" was originally defined as gastric inhibitory polypeptide, owing to its enterogastrone effects. However, similar preparations of CCK were demonstrated to have insulinotropic activity (Rabinovitch and Dupre 1972; Dupre et al. 1973). This led to the eventual purification of porcine GIP and confirmation of the Dupre hypothesis, suggesting insulinotropic GIP actions. Intravenous infusion of GIP concomitant with glucose was shown to result in significantly increased insulin secretion when compared to glucose infusion alone (Dupre et al. 1973). This glucose-dependence was further supported by studies *in vivo* in dogs (Pederson et al. 1975), humans (Elahi et al. 1979) and in the perfused rat pancreas (Pederson and Brown 1976). It was recognized that this inherent feature of GIP would provide a threshold against inappropriate insulin secretion. Therefore, the peptide hormone was re-designated 'glucose-dependent insulinotropic polypeptide' to reflect these novel physiological actions (Brown and Pederson 1976).

Today, GIP and glucagon-like peptide-1 (GLP-1) are considered to be the two main incretins that make up the hormonal component of the enteroinsular axis. Early studies claiming

an incretin role for gastrin and secretin have been refuted due to the lack of gastrin secretion in response to oral glucose and the inability of secretin to promote insulin secretion at physiological concentrations. The role of CCK as an incretin has also been controversial due to its lack of effect in humans and relatively insignificant effect on postprandial insulin secretion. Today it is believed that GIP and GLP-1 are responsible for 50-70 % of postprandially secreted insulin (Fehmann et al. 1995).

1.2.2 The GIP gene and its evolution

GIP is a member of the pituitary adenylate cyclase activating polypeptide (PACAP)/glucagon/secretin superfamily of peptides which to date includes 6 genes encoding 9 bioactive peptides (reviewed in Sherwood et al. 2000). The complete porcine 42 amino acid sequence of GIP was initially reported in 1971 (Brown and Dryburgh 1971) and corrected in 1981 (Jörnvall et al. 1981). The GIP gene has now been sequenced in humans (Moody et al. 1984), pigs (Jörnvall et al. 1981), cows (Carlquist et al. 1984), rat (Higashimoto et al. 1992), and mouse (Schieldrop et al. 1996), where there exists a > 90 % identity at the amino acid level implying an important physiological role for the hormone. Comparison of the exon gene arrangement of the PACAP/glucagon superfamily reveals that these genes were derived from a common ancestral gene >1000 million yrs. ago via gene duplication events (Bell 1986; Sherwood et al. 2000).

The human GIP gene has been localized to chromosome 17q, spanning approximately 10 kbp (Inagaki et al. 1989). The GIP gene is encoded by 6 exons, with the GIP hormone encoded on exons 3 and 4. Exon 1 encodes the 5' untranslated region (UTR), exon 2 the signal peptide common in most superfamily members (this is exon 1 in secretin), exon 5 mostly the distal cryptic peptide, and exon 6 the 3' UTR (Inagaki et al. 1989). The rat and human genes give rise to 144 and 153 amino acid precursors respectively (Inagaki et al. 1989; Higashimoto and Liddle 1993), which are processed by proteolytic cleavage of the signal peptide and the amino and carboxy-terminal cryptic peptides.

With the characterization of the human GIP gene (Inagaki et al. 1989), the promoter region has also been partially examined (Someya et al. 1993). The promoter contains SP1, AP-1 and AP-2 consensus sites, and is additionally regulated by two cAMP responsive elements (-159 to –152). More recent analysis of the rat GIP promoter has also led to the identification of a

similar cAMP response element (-161 to –154), and AP-1 and AP-2 sites (Higashimoto and Liddle 1993; Boylan et al. 1997). The distal promoter has also been found to contain a functional GATA element, thought to be responsible for cell-specific expression of GIP (Boylan et al. 1997).

The regulation of GIP gene expression has been relatively unexplored. Rat GIP mRNA levels were increased in the small intestine and submandibular salivary gland by glucose administration (Tseng et al. 1994, 1995; Higashimoto et al. 1995). In addition, glucose was also found to have direct effects on GIP mRNA expression (3-fold increas in response to an elevation of glucose from 5 to 25 mM) in the intestinal tumour cell line STC_{6-14} (Schieldrop et al. 1996). In support of its effects on secretion, fat was also able to increase GIP mRNA levels, and sustain increases longer than glucose (Higashimoto et al. 1995). Dietary fasting has also resulted in both increases and decreases in GIP mRNA levels (Sharma et al. 1992; Higashimoto et al. 1995).

GIP shares the greatest identity with GLP-1 compared to all other superfamily peptide members. In humans, there is 41 % identity of amino acids between these two peptides (and their receptors), implying they have been evolutionarily separated for a long time. However, the origin of GIP has only been traced to mammals, whereas GLP-1 is present in birds, reptiles, and even in jawless fish. Thus, until fish have been carefully analyzed for the expression of GIP, the exact separation of these two genes will be difficult to assess (reviewed in Sherwood et al. 2000).

1.2.3 GIP distribution and secretion

The distribution of GIP was originally found to be confined to K-cells of the proximal intestine in humans (Polak et al. 1973; Buchan et al. 1978), but extends down to the ileum in rat and dog (Buchan et al. 1982). However, there is also evidence of immunoreactive GIP and GIP mRNA localized via *in situ* hybridization to the ductal cells of the submandibular salivary gland in rats (Tseng et al. 1993; Tseng et al. 1995). Further elucidation of the role of GIP in the salivary gland and support of this finding in humans has remained elusive. Most recently, GIP mRNA has been detected by RT-PCR in the human and mouse stomach (Yeung et al. 1999). Distinct GIP cells were further characterized in the mouse stomach by in situ hybridization and immunohistochemical analysis (Yeung et al. 1999). Surprisingly, in the same study, a human GIP promoter driven transgene also resulted in reporter expression in the mouse pancreas.

GIP is secreted in response to intraluminal nutrients from K-cells located mostly in the duodenum and jejunum. Secretion of GIP has been detected in response to intraduodenal glucose (Cataland et al. 1974; Pederson et al. 1975), fat (Falko et al. 1975), and protein (Wolfe et al. 2000) or amino acids (Thomas et al. 1978). Following a mixed meal, GIP concentrations have been reported to increase to 35-235 pM from 12-92 pM during fasting (5-6 fold on average) (Alam and Buchanan 1993). These broad ranges have been attributed to poor cross reactivity of antisera raised against porcine GIP for human or rat forms (Alam and Buchanan 1993).

The sparse distribution of K-cells has made it difficult to examine the nature of stimulus-induced GIP secretion. With the demonstration that GIP-containing cells can be purified by subcloning (Kieffer et al. 1995A), the molecular basis of nutrient-induced GIP secretion can be further investigated. In accordance with its role as an incretin, oral glucose is able to increase IR-GIP in humans (Cataland et al. 1974), dogs (Pederson et al. 1975), and rats (Pederson et al. 1982), whereas i.v. glucose cannot. The requirement for sodium-dependent active transport of monosaccharides supports the hypothesis that carbohydrates act on the K-cell directly (Morgan et al. 1979). Recent evidence has begun to highlight similarities between K-cells and pancreatic β-cells. Both cells express glucokinase and the inward rectifying K^+_{ATP} channel subunit (Cheung et al. 2000; Ramshur et al. 2002). However, studies on STC-1 (K) cells expressing insulin under control of the GIP promoter suggest that insulin secretion (and presumably also GIP secretion) is independent of glycolysis and the K^+_{ATP} channel (Ramshur et al. 2002).

The ingestion of fat in humans is thought to be an even more potent secretagogue of GIP than glucose (Brown et al. 1975; Morgan 1996). In fact, fat results in a more prolonged elevation of the incretin, although in the absence of elevated glucose, there are no insulinotropic actions of GIP (Cleator and Gourlay 1975; Pederson et al. 1975; Falko et al. 1975; Morgan 1996). The ability of protein or protein-digestion products to stimulate GIP release physiologically has been less clear. Thomas and colleagues reported that an amino acid mixture served as a potent stimulus for GIP and insulin secretion in humans (Thomas et al. 1978). However, other studies failed to show increases in GIP secretion following ingestion of protein rich meals of cod or steak (Cleator and Gourlay 1975; Sarsan et al. 1980). Recent studies, however, contended that peptone can stimulate GIP secretion in the rat (Wolfe et al. 2000).

A neural component to incretin secretion has been suggested, as bilateral subdiaphragmatic vagotomy in conjunction with gut transection completely abolished fat induced GLP-1 release (Rocca and Brubaker 1999). However, these vagal inputs may be more important in mediating GLP-1 secretion, since studies indicating that parasympathetic and

sympathetic nerves can stimulate, inhibit, or have no effects on GIP release have been published (McIntosh 1991).

1.2.4 Metabolism of GIP

The notion that GIP may be degraded to an inactive polypeptide fragment was first suggested after the identification of GIP_{3-42} in 1981 (Brown et al. 1981; Jörnvall et al. 1981). Since these observations, GIP and GLP-1 have both been identified as substrates of the enzyme dipeptidyl peptidase IV (DP IV; also known as CD26), which also displays activity towards other glucagon/secretin peptide family members (Mentlein et al. 1993; Deacon et al. 1995; Kieffer et al. 1995B). The selectivity of DP IV for the incretins is due to its specificity for N-terminal penultimate Pro>Ala>Ser, where both GIP and GLP-1 have alanine residues. Both polypeptides are N-terminally cleaved very rapidly after being secreted (half-life of 1-2 min) resulting in the complete loss of insulinotropic activity of GIP (Brown et al. 1981; Kieffer et al. 1995B). Thus, it becomes evident that studies examining circulating GIP levels must differentiate between biologically active (GIP_{1-42}) and inactive (GIP_{3-42}) peptide forms before conclusions can be drawn regarding its role in disease states. This begs a re-evaluation of early studies monitoring GIP levels, and may explain some of the discrepant findings among type 2 diabetic studies. While assays allowing the monitoring of active GLP-1 have been available for some years, measuring active GIP levels has only recently been possible in humans, and should further clarify the physiological role of GIP (Hoffman et al. 2001).

The importance of DP IV in regulating the inactivation of incretin hormones, and hence glucose homeostasis, has become clear from animal studies using DP IV inhibitors (Kieffer et al. 1995B; Pauly et al. 1999; Deacon et al. 2001) and the generation of DP IV knockout mice (Marguet et al. 2000). Administration of DP IV inhibitors can improve glucose tolerance acutely in both nondiabetic and diabetic animal models (Pederson et al. 1998; Deacon et al. 2001). Further, long term treatment of diabetic animal models with DP IV inhibitors ameliorates the diabetic phenotype (Pospisilik et al. 2002; Reimer et al. 2002; Sudre et al. 2002). Thus, the therapeutic paradigm has arisen, that by increasing the circulating levels of active GIP and GLP-1 postprandially, one can improve glucose homeostasis. The use of DP IV inhibitors and DP IV resistant incretin analogues in the treatment of type 2 diabetes mellitus has therefore been an area of growing interest. Furthermore, DP IV inhibitor studies have also begun to support the ever-

expanding role of incretin physiology in the regulation of insulin sensitive tissues, and beta cell growth and survival.

1.3 BIOLOGICAL ACTIONS OF GIP

1.3.1 GIP actions on the gastrointestinal tract

The concept of GIP as an enterogastrone was initially supported by the notion that this hormone is secreted from the small intestine and inhibited gastric acid secretion in the denervated stomach (Heidenain pouch) (Brown et al. 1969, 1970; Pederson and Brown 1972). However, subsequent studies challenged this notion, as GIP was only a weak inhibitor of gastric acid secretion in the innervated stomach in man (Maxwell et al. 1980) and dog (Soon-Shiong et al. 1979). Further insight was provided by McIntosh (1981), when it was demonstrated that GIP was able to stimulate somatostatin release in the perfused rat stomach, with vagal stimulation or acetylcholine inhibiting this release. This finding may explain the diminished capacity of GIP to act on the innervated stomach, and implies that paracrine effects of somatostatin may be crucial in mediating GIP actions. Despite data opposing the action of GIP in the stomach, the recent demonstration that GIP mRNA is expressed in the stomach of the rat and human (Yeung et al. 1999) further supports a role for GIP in regulating gastric physiology. Thus, a view held to date is that the enterogastrone effects of GIP may be species-specific and the hormone may act synergistically with other enterogastrones to facilitate inhibition of gastric acid physiologically.

There are also indications that GIP may be able to regulate gastrointestinal motility. In the dog stomach, GIP infusion can inhibit both pentagastrin and acetylcholine stimulated motor activity (Pederson and Brown 1972; Brown et al. 1975), while also causing relaxation of the cat stomach (Jansson et al. 1978). Further, GIP has also been observed to lower esophageal sphincter pressure in cats (Sinar et al. 1978), and reduce intestinal water and electrolyte transport in humans (Helman and Barbezat 1977). There is also evidence for effects of GIP on mesenteric blood flow (Fara and Salazar 1978). While the ability of GLP-1 to inhibit gastric emptying is well documented (Schirra et al. 1996; Willms et al. 1996; Nauck et al. 1997), further studies are required to confirm this role for GIP.

Due to the proximal location of the K-cell versus the L-cell (GLP-1 secreting cell) in the small intestine, and the apparent discrepancy in the kinetics of release (GLP-1 is secreted before nutrients enter the distal gut), a hypothesis has been put forward implicating GIP in the regulation of GLP-1 release. GIP was found to stimulate intestinal GLP-1 secretion *in vivo* in

rats (Roberge and Brubaker 1993), and *in vitro* in the vascularly perfused ileum (Dumoulin et al. 1995; Herrmann-Rinke et al. 1995). Further *in vitro* studies have confirmed these findings, as GIP is able to stimulate GLP-1 release from rat intestinal cells (Brubaker 1991; Huang and Brubaker 1995; Saifia et al. 1998) and canine L-cells (Damholt et al. 1998). This led to the notion of a proximal-distal loop whereby nutrients in the duodenum stimulate the release of GIP, which circulates to the ileal L-cells and promotes GLP-1 secretion. However, this concept has not been supported in human studies (Nauck et al. 1993A; Raufman 1996; Schirra et al. 1997). A more recent role for the neuropeptide GRP (gastrin-releasing peptide) in this loop, has suggested that GIP may act via the nervous system (vagal or mesenteric) to regulate GLP-1 secretion independent of direct effects on the L-cell (Roberge et al. 1996).

1.3.2 GIP actions on pancreatic Islets

Direct evidence for the insulinotropic action of GIP in humans was first provided by Dupre et al. (1973). With subsequent studies providing evidence for its secretion in response to nutrients, GIP was defined as an incretin and is currently thought to be the only true incretin other than GLP-1 (Creutzfeldt 1979). Dupre and colleagues went on to show that GIP had no insulinotropic properties in the euglycemic state (Dupre et al. 1973), thus initiating interest in the concept of glucose-dependence. A glucose threshold of approximately 5.5 mM has been observed in the perfused rat pancreas (Pederson and Brown 1976). To date, GIP has been shown to be insulinotropic *in vivo* in humans (Dupre et al. 1973; Elahi et al. 1979), dog (Pederson et al. 1975) and rodents (Lynn et al. 2001), and *in vitro* in the perfused pancreas (Pederson and Brown 1976), isolated islets (Siegel and Creutzfeldt 1985; Shima et al. 1988), and β-cell lines (Kieffer et al. 1993; Lu et al. 1993A; Montrose-Rafizadeh et al. 1994). Further, the establishment of a knockout mouse for the GIP receptor (GIPR -/-) (Miyawaki et al. 1999), has clearly supported the insulinotropic role of GIP despite the compensatory actions of GLP-1 (Pamir et al. 2003). The physiological role of GIP during fasting has received little attention, while its role in regulating postprandial insulin secretion is unequivocal. In fact, GIP has been reported to account for up to 70 % of the postprandial insulin response (Nauck et al. 1986; Tseng et al. 1996).

In addition to the ability of GIP to stimulate β-cell insulin secretion, GIP has also been documented to regulate β-cell gene expression. It is well established that GIP regulates insulin

gene (Lu et al. 1993A; Fehmann and Göke 1995) and protein expression (Wang et al. 1996). Further physiological evidence of this was recently established in our laboratory in studies on the GIPR -/- mice. Islet insulin mRNA and protein levels are both significantly decreased in GIPR null mice despite compensatory GLP-1 actions (Pamir et al. 2003). Further effects of GIP on β-cell genes were presented in a study where long-term incubation of RIN 1046-38 cells (a rat β-cell line) with GIP for 24 h augmented mRNA expression of the glucose transporter GLUT-1, and hexokinase I (Wang et al. 1996). It was proposed that GIP can act via several mechanisms, including increasing mRNA levels of insulin and crucial glycolytic enzymes, to enhance glucose stimulated insulin secretion.

There is also preliminary evidence for GIP effects on other islet cell types. GIP has been shown to stimulate glucagon secretion under low (< 5.5 mM) glucose conditions in the perfused rat pancreas (Pederson and Brown 1978), however, such responses were not observed in humans (Nauck et al. 1993A; Meier et al. 2001). A role for GIP in the regulation of somatostatin secretion (from δ-cells) has also been suggested (Schmidt et al. 1990).

Part of the present thesis is concerned with the novel role of GIP as a growth and survival factor for β-cells. During the course of this thesis, supportive work was published, implicating GIP in the regulation of cell growth (Trumper et al. 2001) and survival (Trumper et al. 2002) in a β-cell line (INS-1). Examination of GIPR-/- mice also provides support for this hypothesis, as these mice display dysregulation of islet size (Pamir et al. 2003). Furthermore, the use of DP IV inhibitors in streptozotocin-induced diabetic rats has implicated a role for GIP in the β-cell protective effects conferred by DP IV inhibitor treatment (Pospisilik et al. 2003). Although the definitive studies on the GIPR -/- mouse (vs. wild type) have not been completed, GIP appears to play a similar role to GLP-1 in its ability to regulate β-cell mass (Buteau et al. 1999; Xu et al. 1999; Stoffers et al. 2000).

1.3.3 GIP actions on adipose tissue

Due to the potent effects of fat ingestion on GIP secretion, which are not insulinotropic in the absence of elevated glucose (Pederson et al. 1975), an hypothesis that GIP has effects on lipid metabolism and adipose tissue seemed reasonable. It was first shown that GIP dose-dependently increased lipoprotein lipase activity in cultured preadipocytes (Eckel et al. 1979). Subsequent work also supported a role for GIP in disposal of circulating triglycerides (Wasada et

al. 1981; Beck 1989; Ebert et al. 1991). Most studies point towards an overall anabolic effect of the hormone on adipose tissue (Ebert and Creutzfeldt 1987; Hauner et al. 1988; Beck 1989). However, recent data from cultured mouse adipocytes (3T3-L1 cells) suggest that GIP may be lipolytic in the fasting state when circulating insulin levels are minimal (McIntosh et al. 1999). In this manner, GIP was hypothesized to contribute to the priming of β-cells in the fasting state via elevated FFA (free fatty acids). In fact, the generation of GIP receptor knockout mice (GIPR -/-) has suggested a critical role for GIP in the regulation of adipose tissue mass (Miyawaki et al. 1999, 2002). These animals were resistant to high-fat diet induced obesity, and cross breeding of GIPR -/- mice with genetically obese mice (*ob/ob*) resulted in an amelioration of the latter condition. In humans, however, there is no clear evidence for a role for GIP at the level of adipose tissue (Jorde et al. 1984).

1.3.4 GIP actions on other glucose sensitive and insensitive tissues

GIP has been shown to inhibit glucagon-stimulated hepatic glucose production in rat (Hartmann et al. 1986) and human (Elahi et al. 1986), however, owing to the absence of any detectable GIP receptors in the liver, this is thought to be via indirect means (Usdin et al. 1993). There is also evidence for the involvement of GIP in regulating glucose uptake in skeletal muscle (O'Harte et al. 1998). Similar to the anabolic actions of insulin, GIP was found to stimulate glucose uptake, oxidation, glycogenesis, and lactate formation in mouse abdominal muscle (O'Harte et al. 1998). Interestingly, as with the liver, there has been no evidence for the GIP receptor at the skeletal muscle level (Kaplan and Vigna 1994).

Further biological actions of GIP have been shown to occur at the level of blood vessel endothelial cells (affecting mesenteric, splanchnic, and portal blood flow) (Fara and Salazar 1978; Kogire et al. 1988, 1992), the anterior pituitary (Ottlecz et al. 1985), osteoblasts (Bollag et al. 2000, 2001), and the adrenal cortex (Mazzocchi et al. 1999; Nussdorfer et al. 2000).

1.3.5 Structure function determination of biological actions of GIP

Analysis of the bioactive domain of the GIP_{1-42} polypeptide has been ongoing with the aim of developing shorter length bioactive peptides. GIP_{1-30} has been shown to have similar

insulinotropic activity to the full length polypeptide in the perfused rat pancreas, but with reduced somatostatinotropic activity (Pederson et al. 1990; Morrow et al. 1996). In fact, GIP_{19-30} was found to stimulate insulin secretion weakly in the perfused pancreas preparation (Morrow et al. 1996). Contrary to these studies, the N-terminal region of GIP has also been shown to be crucial for its bioactivity, since GIP is rapidly cleaved and inactivated to GIP_{3-42} by DP IV *in vivo* (Kieffer et al. 1995B). Recent studies have indeed demonstrated that the N-terminal domain, GIP_{1-14}, also possesses insulinotropic activity (Hinke et al. 2001). Thus, there appears to be four domains in GIP_{1-42}. The N-terminal 14 amino acids are sufficient to induce insulin secretion, as are the 12 amino acids 19-30. The 12 C-terminal amino acids (30-42), however, are important in the somatostatinotropic activity of GIP exhibited in the rat stomach (Hinke et al. 2001). Finally, the high-affinity binding domain $GIP_{6-30NH2}$ has been identified as a GIP receptor antagonist (Tseng et al. 1996; Gelling et al. 1997). Given that two domains appear to show insulinotropic bioactivity, Hinke et al. (2001) has proposed the notion of multiple contact residues contributing to high-affinity receptor binding.

1.4 THE GIP RECEPTOR AND SIGNAL TRANSDUCTION

1.4.1 Discovery of the GIP receptor and tissue expression

Prior to their cloning 1993 (Usdin et al. 1993), GIP receptors were detected in transplantable hamster insulinoma cells (Maletti et al. 1984), the insulin secreting β-cell line In 111 (Amiranoff et al. 1984), in human insulinomas (Maletti et al. 1987), and in the mouse beta cell line βTC-3 (Kieffer et al. 1993) via binding of iodinated GIP (^{125}I-GIP). These studies described binding sites with an IC_{50} in the nM range (0.2-7 nM). Futhermore, receptor cross-linking experiments allowed for separation and detection of a 64 kDa receptor (Couvineau et al. 1984; Amiranoff et al. 1986).

The GIP receptor cDNA was first isolated from a rat tumour cell line (RINm5F), and found to encode a putative seven transmembrane GPCR of 455 amino acids (Usdin et al. 1993). This was later confirmed by sequencing of the rat pancreatic islet GIP recepter (Wheeler et al. 1995). To date, the hamster (Yasuda et al. 1994) and human (Yamada et al. 1995) GIP receptors have also been cloned, showing > 80% sequence similarity. Based on structural and amino acid similarities, the GIP receptor has now been classified as a GPCR Family B member, as part of a

subgroup including receptors for glucagon, GLP-1/-2, growth hormone releasing hormone (GHRH), PACAP, vasoactive intestinal polypeptide (VIP) and secretin (Gether 2000). These receptors are characterized by their large extracellular domain, display little sequence identity to other GPCRs and, as with their ligands, are thought to share a common ancestral gene (Ulrich II et al. 1998). The GIP receptor shares the greatest amino acid sequence identity with glucagon (44 %) and GLP-1 (40 %) receptors (Usdin et al. 1993). The greatest variability between these receptors is in the intracellular carboxy-terminal tail region, while the extracellular N-terminus is relatively well conserved (35 % and 39 % respectively). Based on sequence analysis, the GIP receptor has a predicted molecular weight of approximately 50 kDa, however, previously mentioned crosslinking experiments (Couvineau et al. 1984; Amiranoff et al. 1986) and western blot analysis (Lewis et al. 2000) have revealed a 60-70 kDa protein. This supports the notion that the protein undergoes post-translational protein modifications (glycosylation) prior to surface expression (Amiranoff et al. 1986).

Initial characterization of GIP receptor mRNA by Northern blot, RT-PCR, and *in situ* hybridization analyses revealed widespread tissue distribution within adipose tissue, the adrenal cortex, brain, endothelium of major blood vessels, heart, intestine, lung, pancreas, and stomach (Usdin et al. 1993). Chapter 1.3 highlighted roles for GIP at most of these tissues, however, a role for GIP at the level of the brain, heart, and lung remains to be elucidated. Interestingly, GIP receptor mRNA and GIP binding sites have been localized to areas of the telencephalon (motor, somatosensory, and auditory), the midbrain (inferior colliculus), the forebrain (anterior olfactory nucleus, lateral septal nucleus, subiculum), and the pituitary (Usdin et al. 1993; Kaplan and Vigna 1994). However, in spite of receptor localization in the brain, no evidence exists for GIP mRNA transcripts (Higashimoto et al. 1992; Tseng et al. 1993) or immunoreactive GIP (Buchan et al. 1982). Thus, the relevance of the GIP receptor in the brain remains enigmatic.

Further examination of GIP receptor expression in islet cells was carried out by Moens and colleagues (1996) using FACS sorted rat α- and β-cells. The GIP receptor mRNA was confirmed to be expressed in β-cells, however, significant levels were also detected in the non β-cell sorted fraction (> 80 % α-cells, < 10 % β-cells). This finding was supported by studies on glucagonoma cells and functional receptor signaling data (Moens et al. 1996).

The most recently reported novel location of the GIP receptor is on rat osteoblast-derived cells (Bollag et al. 2000). GIP receptors (but not GLP-1 receptors) were detected at the mRNA and protein level, in rat osteoblasts and osteocytes, and were shown to be functionally capable of increasing type 1 collagen and alkaline phosphatase activity. Furthermore, 6-week GIP

treatment in ovariectomized rats was able to increase bone density to levels similar to ovariectomized controls (Bollag et al. 2001). The authors have proposed that GIP is part of an entero-osseous axis, whereby GIP coordinates nutrient utilization for bone formation in an analogous manner to insulin (Bollag et al. 2000).

1.4.2 GIP receptor signal transduction mechanisms: cAMP and Ca^{2+} signals

As a GPCR member of the Family B subgroup, the GIP receptor stimulates adenylate cylase and the production of cAMP. This has been confirmed in pancreatic tumour cell lines (Amiranoff et al. 1984; Maletti et al. 1987; Lu et al. 1993B; Hinke et al. 2000), a gastric cancer cell line (Gespach et al. 1984), isolated islets (Siegel and Creutzfeldt 1985; Lynn et al. 2001), FACS sorted α- and β-cells (Moens et al. 1996), heterologous expression models (Wheeler et al. 1995; Gelling et al. 1997), endothelial cells (Zhong et al. 2000), and osteoblast-like cell lines (Bollag et al. 2000). Reported affinities for cAMP production have ranged from EC_{50} values of approximately 200 pM (Moens et al. 1996) to 30 nM (Amiranoff et al. 1984).

Early evidence coupling the GIP receptor to increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) also supported the insulinotropic nature of the ligand (Wahl et al. 1992; Lu et al. 1993B; Usdin et al. 1993; Wheeler et al. 1995; Ding and Gromada 1997), due to the importance of $[Ca^{2+}]_i$ in insulin exocytosis. GIP has been shown to increase influx of extracellular Ca^{2+} into mouse islets (Wahl et al. 1992), and to increase $[Ca^{2+}]_i$ in HIT-T15 (Lu et al. 1993B), RINm5F (Usdin et al. 1993), and COS cells (Wheeler et al. 1995). Increases in $[Ca^{2+}]_i$ were concluded to be due to extracellular Ca^{2+} influx, as EGTA or nimodipine (a voltage-dependent L-type channel antagonist) were able to ablate these effects (Lu et al. 1993B). There was, however, no effect of GIP on IP_3 (inositol-1, 4, 5-triphosphate) production in HIT-T15 cells (Lu et al. 1993B), suggesting an alternative to phospholipase C (PLC)-induced mobilization of intracellular Ca^{2+} stores. Interestingly, GLP-1 also had no effect on inositol turnover in either HIT-T15 cells (Lu et al. 1993B) or others (fibroblasts and COS cells) (Widmann et al. 1994). However, there is also some evidence that GLP-1 can elevate IP_3 in COS cells (Wheeler et al. 1993) and β-cells (Zawalich et al. 1993; Zawalich and Zawalich 1996; MacDonald et al. 2002). Thus, the mechanism of regulation of intracellular Ca^{2+} stores by GIP remains uncertain and can only be speculated upon based on more recent GLP-1 studies.

In addition to β-cells, the importance of GIP induced mobilization of intracellular Ca^{2+} has also become evident in other cell systems. GIP has been shown to increase $[Ca^{2+}]_i$ in α-cells (Ding et al. 1997), endothelial cells (Zhong et al. 2000), and osteoblast-like cell lines (Bollag et al. 2000). However, the mechanism involved in coupling of the GIP receptor to these Ca^{2+} fluxes has also not been well elucidated. To date, possibilities include cAMP/protein kinase A (PKA) effects on Ca^{2+} channels, cAMP stimulated PKA-independent effects on ryanodine receptors involved in calcium induced calcium release (CICR), nicotinic acid adenine dinucleotide phosphate (NAADP) effects (Masgrau et al. 2003), or arachidonic acid effects on Ca^{2+} flux (Ehses et al. 2001).

The physiological relevance of these cAMP/PKA and $[Ca^{2+}]_i$ signals was later investigated in individual mouse β-cells (Ding and Gromada 1997). Pharmacological inhibition of PKA supported its role in regulating GIP-stimulated insulin secretion, however no direct link was made between cAMP/PKA signaling and $[Ca^{2+}]_i$ increases. From these studies it was concluded that GIP potentiated insulin secretion via cAMP/PKA signaling interacting distal to the increase in $[Ca^{2+}]_i$. Previous experiments had demonstrated that cAMP could elicit exocytosis in both a Ca^{2+}-dependent and Ca^{2+}-independent manner (Ämmälä et al. 1993). Thus, the current paradigm is that both cAMP and PKA can have independent effects on insulin exocytosis, the former may affect late stages of the exocytotic process and the latter can affect mobilization of the readily releasable pool of insulin granules (Gromada et al. 1998). This "sensitization" then results in a larger secretory response to Ca^{2+} (Gromada et al. 1998).

Most recently, a cAMP-dependent, PKA-independent mechanism of action underlying incretin stimulated insulin secretion has been established (Kashima et al. 2001), involving a critical role for a cAMP-GEFII-Rim2 complex. Cyclic AMP-GEFII (GEF = guanine nucleotide exchange factor) or Epac2 was shown to be responsible for 50 % of the potentiation mediated by incretins, whereas PKA was mostly responsible for the remainder. How these two independent signals feed into Ca^{2+} regulation has also recently been documented. The GLP-1 receptor can mediate CICR in INS-1 and human β-cells via Epac selective signals (Kang et al. 2003). The relevance of these cAMP-dependent, PKA-independent signals in regulating other important β-cell gene regulatory events remains to be established.

1.4.3 GIP receptor signal transduction mechanisms: protein kinase signals.

In addition to regulating cAMP and Ca^{2+} signals, there is also preliminary evidence for the involvement of protein kinases in GIP-mediated functions. As such, phosphatidylinositol 3-kinase (PI3K) has been implicated in GIP receptor mediated signal transduction (Straub and Sharp 1996; Kubota et al. 1997; Trumper et al. 2001). Straub and Sharp first reported that the PI3K inhibitor wortmannin could severely blunt the effects of GIP (as well as VIP and PACAP) on insulin secretion from HIT-T15 cells (Straub and Sharp 1996). Demonstration of a direct role for PI3K in mediating effects on insulin secretion remains to be studied. Further support for GIP-mediated PI3K actions was provided by Kubota and colleagues (Kubota et al. 1997), who demonstrated that GIP activation of mitogen-activated protein (MAP) kinase (also known as extracellular regulated kinases 1 and 2; ERK1/2) was also influenced by wortmannin. Most recently, direct evidence for PI3K activation by GIP was provided by Trumper in INS-1 cells (Trumper et al. 2001).

Despite these initial studies, there was very little information gained about GIP receptor signaling transduction and its impact on protein kinase cascades throughout the 1990s relative to GLP-1 and other GPCRs. Given the expansion of the signal transduction field throughout this period, there was much to be elucidated and the potential for identifying novel physiological actions based on these biochemical networks is an attractive possibility. The main focus of the present thesis was to identify novel signaling mechanisms of the GIP receptor and functionally relate them to tissue specific GIP actions.

1.4.4 Structure function relationships of the GIP receptor

Studies using GIP/GLP-1 receptor chimeras have revealed that the high affinity binding domain of the GIP receptor lies within the extracellular amino-terminus, with the first transmembrane domain being crucial for GIP-specific receptor activation (Gelling et al. 1997). Characterization of the carboxy-terminal (CT) tail of the GIP receptor demonstrated that a minimum receptor length of 405 amino acids is required for membrane expression (Wheeler et al. 1999). However, parallel studies by Tseng and Zhang reported membrane expression with a 399 amino acid chain length (Tseng and Zhang 1998). It also appears that the majority of the carboxy-terminal tail is not essential for coupling to adenylyl cyclase, a feature seen in other

GPCRs. However, specific amino acids within the proximal carboxy-terminal tail seem to facilitate this high affinity interaction (Wheeler et al. 1999).

Since the carboxy-terminal tail of GPCRs is responsible for coupling to effectors (G-proteins, adenylate cyclase), it is often phosphorylated to induce receptor desensitization (suppression of signaling) or internalization. Desensitization of the GIP receptor is thought to be due to phosphorylation upon prolonged exposure to ligand, a process shown to be mediated by G-protein receptor kinase-2 (GRK-2) (Tseng and Zhang 2000). Truncation of the final 30 amino acids of the CT tail, or mutation of serine residues 426/427 to alanine, both reduced GIP receptor internalization rate (Wheeler et al. 1999). Tseng and Zhang also demonstrated a role for cysteine 411 in GIP receptor desensitization (Tseng and Zhang 1998), however, no role was found for this amino acid in internalization (Wheeler et al. 1999).

1.5 GLP-1 AND THE GLP-1 RECEPTOR

1.5.1 Discovery of GLP-1 and secretion

The discovery of GLP-1 resulted from the cloning of the cDNA encoding the preproglucagons from the pancreata and intestine of angler fish (Lund et al. 1981, 1982). This led to recognition of a sequence that bore strong homology to GIP, and the proposal of a second incretin hormone encoded by the proglucagon gene (Lund et al. 1981). The three main sites of expression of the proglucagon gene include the α-cells of pancreatic islets, the L-cells of the distal ileum, colon, and rectum, and the nucleus tractus solitarius in the hindbrain (vagal nerve nucleus) (reviewed in Kieffer and Habener 1999). Today it is known that the major product of the proglucagon gene in the pancreas is glucagon, and in the intestine and brain post-translational processing produces GLP-1 (7-37 and 7-36amide) and GLP-2. The tissue-specific expression of these genes is accomplished via prohormone convertase (PC) mediated post-translational cleavage at specific basic amino acid residues. PC1/3 are thought to be responsible for the formation of GLPs in the intestine, while PC2 liberates glucagon in the α-cell (reviewed in Kieffer and Habener 1999).

Similar to the secretion kinetics of GIP, GLP-1 is released following nutrient ingestion in response to carbohydrates, fats, and proteins. However, release is thought to be biphasic, with hormonal and neural inputs mediating early release (15-30 min), and direct nutrient contact

mediating later secretion (30-60 min) (Kieffer and Habener 1999). The proposal of a proximal-distal loop regulating GLP-1 secretion was discussed earlier in terms of GIP actions. The presence of nutrients in the proximal intestine is thought to evoke GLP-1 secretion from distal L-cells in an endocrine and neural-dependent pathway (Kieffer and Habener 1999). Rapidly after its secretion into the circulation, GLP-1 is N-terminally degraded by DP IV, resulting in the liberation of GLP-$1_{9\text{-}36NH2}$ (reviewed in Kieffer and Habener 1999).

1.5.2 Biological actions of GLP-1

There has been a great deal of interest in the anti-diabetic actions of GLP-1, owing to its preserved insulinotropic action in type 2 diabetics. Largely for this reason, elucidation of the physiological actions of GLP-1 has received much greater attention than GIP. Despite parallel actions on the stomach and pancreatic β-cells, there is some divergence in the reported biological actions of the two polypeptides. This however, may simply be due to less emphasis on GIP-related research.

GLP-1 has been shown to inhibit gastric acid secretion (enterogastrone effects) (Schjoldager et al. 1989; O'Halloran et al. 1990) and gastric emptying (Schirra et al. 1996; Willms et al. 1996; Nauck et al. 1997). Similar to GIP, insulinotropic actions of GLP-1 on pancreatic β-cells are glucose-dependent (Kreymann et al. 1987; Holz et al. 1993), however, such glucose-dependence has not been well established with respect to proliferative/survival actions (Buteau et al. 1999, 2001; Li et al. 2003). Further actions on pancreatic islets include an inhibitory effect on glucagon secretion (Suzuki et al. 1989) and stimulation of somatostatin secretion (Heller and Aponte 1995). In contrast to GIP, there are reports that GLP-1 has effects on fasting glycemia (Baggio et al. 2000).

The actions of GLP-1 on other insulin-sensitive tissues (liver, skeletal muscle, and adipose tissue) are less well elucidated. The overall actions of GLP-1 on these tissues are anabolic, via stimulation of glycogenesis and lipogenesis. Most surprisingly, however, these actions are without well documented demonstration of the expression of the GLP-1 receptor in these tissues (reviewed in Kieffer and Habener 1999).

A more recently discovered biological action of GLP-1 is its role in regulating feeding behaviour via anorexigenic actions in the brain (Turton et al. 1996). GLP-1 receptors are present in the brain (as with GIPR mRNA), and administration of GLP-1 into the third intracerebral

ventricle of rats results in a profound decrease in food consumption (Turton et al. 1996). However, it is uncertain whether this occurs under physiological circumstances, since GLP-1 receptor knockout mice (GLP-1R -/-) exhibit normal feeding behaviour (Scrocchi et al. 1996). Studies in humans do, however, support a satiety effect of GLP-1 during infusion of the peptide (Flint et al. 1998; Toft-Nielsen et al. 1998).

Further actions of GLP-1 on lung tissue and the cardiovascular system have been reported. GLP-1 has been shown to cause surfactant secretion from pneumocytes (Benito et al. 1998). Centrally and peripherally administered GLP-1 receptor agonists are able to dose-dependently increased blood pressure and heart rate in rats (Yamamoto et al. 2002). The latter actions are not thought to be via direct actions of GLP-1 on the heart, but rather through central effects regulating sympathetic outflow and thus cardiovascular responses (Yamamoto et al. 2002).

There is also preliminary evidence for the involvement of GLP-1 in the hypothalamus-pituitary-adrenal (HPA) axis. GLP-1 can promote thyroid stimulating hormone (TSH), leutinizing hormone releasing hormone (LHRH), and adrenocorticotropic hormone (ACTH) release (Kieffer and Habener 1999; Nussdorfer et al. 2000).

As alluded to above, the most heavily pursued interest in GLP-1 physiology currently is its effects on β-cell fate. While many of these studies have used the long acting GLP-1 receptor agonist, exendin-4, data support the claim that the GLP-1 receptor can regulate β-cell proliferation (Buteau et al. 1999, 2001; Stoffers et al. 2000), differentiation/neogenesis (Xu et al. 1999; Hui et al. 2001; Abraham et al. 2002; Zhou et al. 2002), and survival (Li et al. 2003). Intriguingly, trophic actions of GLP-1 have also been extended to neuronal cells in culture (PC12 cells) (Perry et al. 2002A), and anti-apoptotic GLP-1 actions have been documented in hippocampal neurons (Perry et al. 2002B).

1.5.3 GLP-1 receptor and signal transduction

The GLP-1 receptor was first cloned by Thorens (Thorens 1992) and mRNA transcripts have been found in the brain, heart, hypothalamus, intestine, kidney, lung, pancreatic islets, and stomach. There is also evidence both for and against the expression of GLP-1 receptor mRNA in adipose tissue, liver, and skeletal muscle. However, investigators have continually shown *in vivo*

effects of GLP-1 on these tissues, arguing for indirect actions or an undiscovered novel GLP-1 receptor isoform responsible for these actions (reviewed in (Kieffer and Habener 1999)).

Shortly after its identification, the GLP-1 receptor was shown to stimulate adenylate cyclase in β-cell lines (Drucker et al. 1987; Lu et al. 1993B; Widmann et al. 1994), isolated islets and β-cells (Ahren et al. 1996; Moens et al. 1996) and various other tissues (Dhillon et al. 1993; Thorens et al. 1993; Wheeler et al. 1993). The EC_{50} for this response is similar to that reported for GIP (0.5 to 3 nM) (Fehmann et al. 1995). It follows that GLP-1 has also been reported to activate PKA using both pharmacological inhibition (Gromada et al. 1998) and direct activity assays (Fehmann et al. 1994; Gao et al. 2002). Furthermore, GLP-1 has been shown to increase $[Ca^{2+}]_i$ in numerous β-cell models (Wheeler et al. 1993; Holz et al. 1995; Ahren et al. 1996) and isolated β-cells (Ding et al. 1997).

With respect to β-cells, GLP-1 signaling has been investigated in terms of insulin secretion, insulin gene transcription, and growth/survival effects. Cyclic AMP/PKA affect both K^+_{ATP} channel closure (Holz et al. 1993; Gromada et al. 1997) and CICR via ryanodine-sensitive stores to promote insulin secretion (Wheeler et al. 1993; Holz et al. 1995, 1999; Ahren et al. 1996; Ding et al. 1997). GLP-1 can additionally exert effects on Ca^{2+} influx via effects on VDCC (voltage dependent calcium channels) and Ca^{2+}-activated nonselective cation channels (NSCC) (Leech and Habener 1997; Gromada et al. 1998; Leech and Habener 1998). The importance of PKA in these events has recently been called into question, however, with studies showing cAMP-GEF-dependent, PKA-independent effects on CICR and insulin secretion (Kang et al. 2001, 2003; Kashima et al. 2001; Tsuboi et al. 2003). Earlier literature implicated a role for PLC and IP_3 in mediating GLP-1 induced Ca^{2+} signaling, however, this pathway is now thought to play a minor role (MacDonald et al. 2002). In addition to regulating Ca^{2+} signaling, cAMP/PKA have been shown to potentiate insulin secretion beyond elevations in $[Ca^{2+}]_i$ as with GIP (Ämmälä et al. 1993; Yajima et al. 1999).

GLP-1 has also been shown to regulate insulin gene transcription in both a cAMP/PKA-dependent (Lawrence et al. 2002) and –independent manner (Skoglund et al. 2000; Chepurny et al. 2002). These effects are thought to be mediated via a CREB (cAMP response element (CRE) binding protein) family transcription factor acting on CRE elements of the rat insulin gene, however, the identity of this transcription factor remains elusive (Chepurny et al. 2002).

Finally, the roles of cAMP/PKA in the proliferative and survival effects of GLP-1 have not been addressed. The mitogenic actions of GLP-1 on the β-cell have been attributed to PI3K, PKCξ (protein kinase C), p38 MAP kinase signaling (Buteau et al. 1999, 2001), and

transactivation of the EGF (epidermal growth factor) receptor (Buteau et al. 2003). In addition, the pancreatic developmental transcription factor, PDX-1, has been implicated in the mitogenic actions of GLP-1, where GLP-1 has been shown to increase its DNA binding activity and its translocation to the nucleus in a cAMP/PKA-dependent manner (Buteau et al. 1999; Wang et al. 2001). As with GLP-1 receptor induced growth effects, survival effects were only discovered recently and have been attributed to caspase-3 inhibition in a cAMP-dependent manner (Li et al. 2003).

The GLP-1 receptor couples to multiple G-proteins in CHO-K1 cells, including $G_{\alpha s}$,$G_{\alpha q/11}$, and $G_{\alpha i1,2}$ (Montrose-Rafizadeh et al. 1999). Thus, in addition to the classical $G_{\alpha s}$ mediated cAMP/PKA pathway, there exists the possibility of other small G-protein coupled signals. These data, however, have not been confirmed in β-cells, and could be due to overexpression of the GLP-1 receptor in this heterologous system.

Activation of the ERK1/2 (Frodin et al. 1995; Buteau et al. 2001; Gomez et al. 2002) and p38 MAP kinases (Montrose-Rafizadeh et al. 1999; Buteau et al. 2001) has also been coupled to GLP-1 receptor activation in β-cell lines and islets. A recent study showed that GLP-1 activation of ERK1/2 involves PKA and Mek1/2, in a Ca^{2+}-dependent manner. However, input was found to be independent of Ras, Rap, and Raf isoforms (A-Raf, B-Raf, C-Raf) (Gomez et al. 2002). Activation of p38 MAP kinase by the GLP-1 receptor is dependent on MKK3/6 in CHO cells, and thought to be downstream of PI3K in INS-1 cells (Buteau et al. 1999).

1.6 PATHOPHYSIOLOGY OF THE INCRETINS

The role of the incretins in regulating glucose homeostasis is well documented and begs the question as to whether they contribute to the pathophysiology of diabetes or other diseases. No clear consensus has been reached regarding the roles of GIP or GLP-1 in the pathophysiology of diabetes mellitus or obesity, however, novel insights are beginning to emerge. In type 2 diabetics, IR-GIP levels have been reported as increased (Ross et al. 1977; Elahi et al. 1984; Jones et al. 1989), normal (Levitt et al. 1980; Service et al. 1984), or blunted (Groop 1989) following nutrient ingestion. In obese subjects, reports include elevated fasting IR-GIP levels (Salera et al. 1982; Mazzaferri et al. 1985), and either exaggerated (Creutzfeldt et al. 1978; Elahi et al. 1979; Jones et al. 1989; Fukase et al. 1993), normal (Lauritsen et al. 1980; Jorde et al. 1983; Amland et al. 1984), or blunted (Groop 1989) responses to oral nutrient ingestion. These

inconsistencies are likely due to inappropriate RIAs that quantify C-terminal peptide levels (both active GIP_{1-42} and inactive GIP_{3-42}). Recent evidence using N-terminal specific antibodies to GIP and GLP-1 suggest no defect in meal-regulated active GIP secretion, with an attenuation in late phase active GLP-1 secretion in type 2 diabetics (Vilsboll et al. 2001). However, despite this, the evidence that abnormalities in circulating GIP and GLP-1 concentrations are associated with type 2 diabetes is not strong.

Perley and Kipnis first demonstrated a reduced or absent incretin effect in type 2 diabetics (Perley and Kipnis 1967), and Nauck later reported that this may be due to the ablation of GIP responses in these patients (Nauck et al. 1993B). In fact, numerous studies have shown that type 2 diabetics are fully responsive to exogenous GLP-1, highlighting a role for this incretin as a therapeutic agent (Nauck et al. 1993B; Elahi et al. 1994). The evidence for blunted GIP actions has been extended to first degree relatives of type 2 diabetics and a subgroup of type 1 diabetics (Meier et al. 2001; Greenbaum et al. 2002).

The underlying mechanism resulting in blunted GIP actions in diabetes mellitus has been an area of interest in our laboratory and others. Chan and coworkers (1984) demonstrated that insulin responses to GIP were enhanced in pancreata of obese Zucker rats and the glucose threshold for the insulinotropic action of GIP was well below fasting levels. In 1998, two missense mutations in the GIP receptor were identified, however, these mutations were not associated with type 2 diabetes in a Japanese study group (Kubota et al. 1996; Almind et al. 1998). More recently, our laboratory reported a reduction in islet GIP receptor mRNA and protein content, underlying the blunted GIP effects in the Vancouver fatty Zucker rat model of type 2 diabetes (Lynn et al. 2001). This is in agreement with an earlier proposal that the underlying pathogenesis of type 2 diabetes mellitus might involve defective GIP receptor expression (Holst et al. 1997). Lynn et al. (2003) went on to show that expression of the GIP receptor is influenced by ambient glucose and fatty acid levels at the level of the β-cell. Fatty acids were found to upregulate GIP receptor mRNA under normoglycemic conditions, however, hyperglycemia resulted in a 70 % reduction in receptor mRNA which was no longer regulated by fat. Thus, nutrients are clearly able to regulate the GIP receptor and in this manner may contribute to the decreased responsiveness of GIP in type 2 diabetics.

In addition to diabetes pathophysiology, there is also a role for GIP in the etiology of food-dependent Cushing's syndrome. Abnormal overexpression of the GIP receptor in adrenal adenomas of these patients results in cortisol increases that are paralleled by postprandial GIP levels (Lacroix et al. 1992; De Herder et al. 1996; Chambre et al. 1998; N'diaye et al. 1998).

1.7 GLUCOSE METABOLISM AND INCRETIN STIMULATED INSULIN SECRETION

1.7.1 Glucose stimulation of β-cell insulin secretion

Blood glucose is tightly regulated by circulating insulin secreted from pancreatic β-cells. The β-cell responds to blood glucose fluctuations by sensing concentrations of glucose. Glucose equilibrates across the plasma membrane via a glucose transporter (GLUT) and is phosphorylated by glucokinase, the rate limiting enzyme of glycolysis. Pyruvate, the main product of glycolysis in the β-cell, enters the mitochondrial TCA cycle and provides the link between oxidative metabolism and insulin secretion. The ensuing increase in cellular ATP:ADP is due to electron transfer from the TCA cycle (in the form of NADH and $FADH_2$) to the respiratory chain. Thus, the paradigm is that membrane depolarization is facilitated through closure of ATP-sensitive K^+ channels (K^+_{ATP}), resulting in Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, and culminating in insulin granule exocytosis. However, this model is oversimplified and there now exist roles for K^+_{ATP}-independent glucose-stimulated insulin release, Ca^{2+}-independent insulin secretion, and additive signals regulating insulin granule exocytosis (glutamate, malonyl-CoA) (reviewed in Maechler and Wollheim 2001).

It has relatively recently been demonstrated that when the K^+_{ATP} channel is held open with diazoxide, and intracellular Ca^{2+} levels are elevated by cellular depolarization, that glucose is still capable of stimulating insulin secretion (Gembal et al. 1992; Sato et al. 1992). These results were supportive of earlier work demonstrating that glucose is still capable of stimulating insulin secretion even in the presence of sulfonylureas, drugs that close the K^+_{ATP} channel (Loubatieres-Mariani et al. 1973; Henquin 1980). This novel finding was therefore termed the K^+_{ATP} channel-independent pathway for glucose stimulated insulin secretion. Even in the event of complete ablation of Kir6.2 or SUR subunits (that make up the K^+_{ATP} channel), there is evidence for insulin secretion (Miki et al. 1998; Seghers et al. 2000). The term, "K^+_{ATP} channel-independent" has recently been reconsidered by Henquin, who has proposed the terms "triggering pathways", for K^+_{ATP} channel closing or depolarizing events, and "amplifying pathways" to denote insulin potentiation events that are independent of effects on the K^+_{ATP} channel, but dependent on Ca^{2+} increases (Henquin 2000).

1.7.2 Glucose-dependence of GIP actions on the β-cell

The notion of glucose-dependence of GIP action was introduced in section 1.2.1, with seminal experiments conducted by Dupre (1973) and a threshold level of 5.5 mM glucose later confirmed in the perfused rat pancreas (Pederson and Brown 1976). However, the mechanism underlying the glucose-dependency of GIP or other insulinotropic hormones is not well understood. Early experiments showed that glucose could be substituted with glycolytic intermediates, glyceraldehyde, α-ketoisocaproic acid, and leucine and glutamine, with GIP still able to potentiate insulin secretion in the perfused rat pancreas (Brown et al. 1981). Furthermore, mannoheptulose, a blocker of glycolysis, abolished GIP-stimulated insulin secretion suggesting that glucose metabolism is indeed a prerequisite for the insulinotropic actions of GIP (Mueller et al. 1982).

The interdependence between glucose and incretin actions is now thought to involve crosstalk between glucose metabolism (glycolysis and TCA flux) and cAMP-mediated GIP/GLP-1 receptor signaling. Holz and Habener (1992) proposed that this occurs at the level of the K^+_{ATP} channel, since both glucose and cAMP/PKA are able to affect channel activity. However, recent studies by Beguin et al. (1999) question the importance of GPCR cAMP/PKA effects on the K^+_{ATP} channel in the pancreatic β-cell. GIP was shown to increase phosphorylation of serine 372 of Kir6.2 in a PKA-dependent manner, which was paradoxically coupled to an increase in K^+_{ATP} channel activity. Cyclic AMP has also been shown to stimulate insulin secretion from rat islets independent of the K^+_{ATP} channel, but in a Ca^{2+} and glucose-dependent manner (Yajima et al. 1999). Therefore, it appears that GIP is a physiological mediator of K^+_{ATP} channel-independent insulin secretion. The glucose-dependence of this action implies that a glycolytic/TCA cycle metabolic intermediate is responsible for these amplifying actions of GIP actions on the β-cell.

Some interesting findings regarding the regulation of the K^+_{ATP} channel by PKA have begun to unravel the mystery of glucose-dependence of insulin secretion. Various groups have shown that when ADP levels are elevated, cPKA (the catalytic subunit of PKA) increases channel activity (contradictory evidence) (Béguin et al. 1999; Lin et al. 2000). In contrast, when ADP levels are reduced, cPKA inhibits K^+_{ATP} channel activity (Light et al. 2002). This may explain why there is no potentiation of insulin secretion by cAMP/PKA when glucose levels are low (high ADP) versus when glucose is elevated (low ADP; membrane depolarization and insulin secretion) (MacDonald et al. 2002), thereby lending evidence towards the glucose

dependence of incretin actions at the level of the K^+_{ATP} channel. However, this ADP/ATP dependence at the level of the K^+_{ATP} channel doesn't explain the glucose-dependence of K^+_{ATP} channel-independent actions of incretins.

1.7.3 Glucose-stimulated lipid signaling and β-cell insulin secretion

Early evidence suggested that insulin secretegogues such as glucose, acetylcholine, and certain amino acid accelerate islet phospholipid metabolism (Turk et al. 1987; Turk et al. 1993). During the investigation of the K^+_{ATP} channel model for glucose-stimulated insulin secretion, other second messengers were investigated for their potential to affect the fine tuning of this system. Lipid messengers such as diacylglycerol (DAG), nonesterified arachidonic acid, and arachidonate metabolites were the focus of early advances. Diacylglycerol is able to regulate PKC, and has been extensively studied as a second messenger in mediating muscarinic effects on islets. Isolated islets contain 30-36 % of total glycerolipid fatty acyl mass as arachidonic acid and glucose stimulation has been shown to increase amounts of nonesterified arachidonic acid from the micromolar range to concentrations of 50-200 μM. Arachidonic acid has also been linked to increases in intracellular [Ca^{2+}] and is therefore thought to participate in glucose modulation of intracellular [Ca^{2+}]. In this manner, arachidonic acid and its metabolites have been linked to glucose-stimulated insulin secretion (reviewed in (Turk et al. 1987; Turk et al. 1993; Simonsson and Ahren 2000)).

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the *sn-2* fatty acid substituents from glycerophospholipid substrates to yield a free fatty acid (arachidonic acid) and a 2-lysophopholipid (Balsinde and Dennis 1997). PLA_2 has been identified in both rat and human islets, as well as in various insulinoma cell lines (Chen et al. 1996; Ma et al. 1998,1999). These β-cell models express all three known classes of PLA_2: Ca^{2+}-dependent cytosolic PLA_2 (Ma et al. 1998; Persaud et al. 2002), ATP-stimulatable Ca^{2+}-independent cytosolic PLA_2 (Ma et al. 1997; Ramanadham et al. 1997) and secretory PLA_2 isoforms (Ramanadham et al. 1998). All three enzymes have been suggested to play a role in glucose-stimulated insulin secretion (Ma et al. 1997, 1998; Ramanadham et al. 1998, 1999). A role for ATP-stimulatable Ca^{2+}-independent cytosolic PLA_2 ($iPLA_2$, or the new designation $iPLA_2\beta$) has recently been linked to glucose-stimulated insulin secretion, arachidonic acid release, and increases in β-cell $[Ca^{2+}]_i$ (Ramanadham et al. 1993; Simonsson et al. 1998; Ramanadham et al. 1999; Simonsson and

Ahren 2000). Interestingly, overexpression of $iPLA_2\beta$ in INS –1 β-cells enhances glucose and cAMP-stimulated insulin secretion (Ma et al. 2001). Thus, it is tempting to speculate that the incretins may regulate insulin secretion via effects on $iPLA_2$ activity.

1.8 G-PROTEIN COUPLED RECEPTOR SIGNAL TRANSDUCTION

1.8.1 General overview

G-protein coupled receptors constitute > 1 % of the human genome and are the largest family of cell surface molecules involved in signal transmission. Receptors are activated by various ligands, including peptide and non-peptide neurotransmitters, hormones, growth factors, odorant molecules and light. These hepathelical receptors are the target of > 50 % of current therapeutic agents on the market (reviewed in Marinissen and Gutkind 2001). GPCRs are so-called because of their intimate interaction with heterotrimeric G-proteins, including Gα, Gβ, Gγ subunits, which undergo GDP/GTP exchange at the level of the Gα subunit. To date, 17 α-subunits, 5 β-subunits, and 12 γ-subunits have been identified. These subunits can then stimulate effector molecules such as adenylate and guanylate cyclases, phosphodiesterases, PLA_2, PLC, PI3Ks, activating or inhibiting the production of second messengers such as cAMP, cGMP, DAG, IP_3, and arachidonic acid. Direct actions of these G-proteins on channels can also promote ion flux (reviewed in Marinissen and Gutkind 2001 and Hur and Kim 2002). Many of these classical second messengers were originally thought to effect downstream signaling modules that were discrete from one another. However, this is not the case, and it is now evident that the transmission of input signals must be thought of in terms of 3-dimensional networks, consisting of junctions (signal integrators) and nodes (signal splitters) (reviewed in Jordan et al. 2000).

In addition to G-proteins and the classical effector molecules, protein kinases are a major downstream target of cell surface receptors responsible for intracellular signal transduction. Greater than 500 protein kinases are now known to make up almost 2 % of the human genome (Manning et al. 2002). Protein kinases are able to switch on or off cellular processes through phosphorylation of proteins at specific serine, threonine, or tyrosine residues. In this manner, such phosphorylation can control enzyme activity, interaction with other proteins or molecules, location, and propensity for cellular degradation. Protein kinases represent the majority of signaling molecules affected by GPCR input (reviewed by Johnson and Lapadat 2002).

The expansion of the cellular signal transduction field in the last ten years has made it evident that despite cell specificity, many parallel signal inputs exist, promoting independent physiological effects. It is unclear as to how intracellular specificity is achieved, and attention is shifting towards understanding the subcellular organization of these signaling modules in an attempt to explain this enigma. It is becoming clear that 'signal directing' molecules exist which are capable of targeting the broad specificity of many signaling proteins to cellular microenvironments. These scaffold proteins have been identified at the GPCR receptor level (β-arrestin), for cAMP/PKA (AKAPs, A-kinase anchoring proteins) and for MAP kinase modules (MP-1 for ERK1/2, JIP for JUN kinase, KSR). Thus, one must keep in mind cellular 3-dimensional organization when considering intracellular signaling events. When available data are combined from tissue distribution, subcellular localization of individual components and protein-protein complexes, a more complete picture of the cell-specific signaling networks will be developed (reviewed in Smith and Scott 2002).

1.8.2 GPCRs and MAP kinase signaling

MAP kinase (MAPK) molecules represent a highly conserved class of protein kinases present in unicellular organisms through to humans. These cytoplasmic serine/threonine kinases are cellular regulators of numerous processes including gene transcription, mitosis, movement, metabolism, differentiation, and metabolism. To date, five distinct mammalian MAP kinase signaling modules have been defined involving ERK1/2, p38 MAPK, Jun N-terminal kinase/stress-activated protein kinase (JNK, SAPK), ERK3, and ERK5 (BMK 1; big MAPK). The core of the MAPK pathway consists of three kinases that sequentially phospho-regulate one another (see Figure 1): the kinases directly upstream of the MAPK are members of the Mek family (or MKK), and these are activated by the Mek kinases (Mekk, or MKKK). To date, it seems by far the most complex family of proteins in this signaling module are the Mekks. It is thought that the purpose of having many different Mekks is that many inputs can influence these messengers, however, each is only associated with one specific Mek-MAPK cassette (reviewed in Garrington and Johnson 1999; Chang and Karin 2001; Chen et al. 2001; Pearson et al. 2001; Johnson and Lapadat 2002).

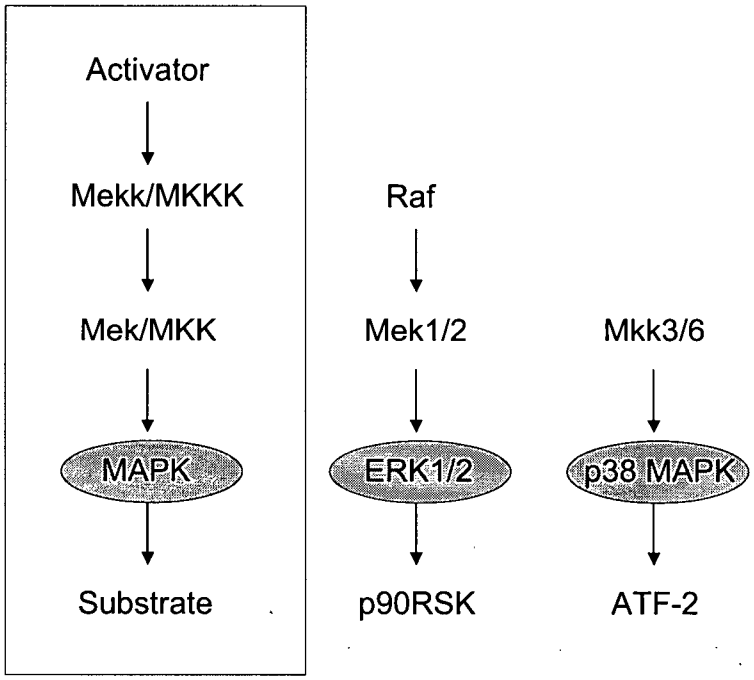


Figure 1: Prototypical MAPK module with examples of ERK1/2 and p38 MAPK cascades. See text for details. Abbreviations not stated in text include p90 ribosomal S6 kinase (p90RSK) and activating transcription factor-2 (ATF-2).

A prime example of the complexity of numerous signaling inputs all affecting a protein kinase module is the interaction of GPCRs with MAP kinase signaling (Figure 2). The first demonstration of a GPCR coupling to a MAPK module came from the observation that $G\alpha_i$ receptors activate MAPK via $G\beta\gamma$ subunits (Crespo et al. 1994). In addition to $G\beta\gamma$ subunits, coupling of GPCRs to the MAPK cascade has now been elucidated for $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ classes of G-proteins. Individual GPCRs are known to couple to multiple G-proteins, making these signal inputs even more complex. In general, $G\alpha_s$ signals via adenylate cyclase and cAMP while $G\alpha_i$ and $G\alpha_{q/11}$ are directly coupled to PLC, PKC and PI3K. However, due to almost unlimited cross-talk between receptors, G-proteins, and protein kinases, these boundries are starting to fall (reviewed in Liebmann 2001; Marinissen and Gutkind 2001). The best broad classification of how GPCRs stimulate MAP kinases includes the following three categories: 1. Signaling via classical G-protein effectors (e.g. PKA, PKC, $Ca^{2+}{}_i$); 2. Signaling via transactivation of classical receptor tyrosine kinases (EGF and platelet-derived growth factor (PDGF) receptors); 3. Signaling via β-arrestin mediated internalization of receptors (β-arrestin scaffolds that couple receptors to MAPK module kinases) (Luttrell 2002).

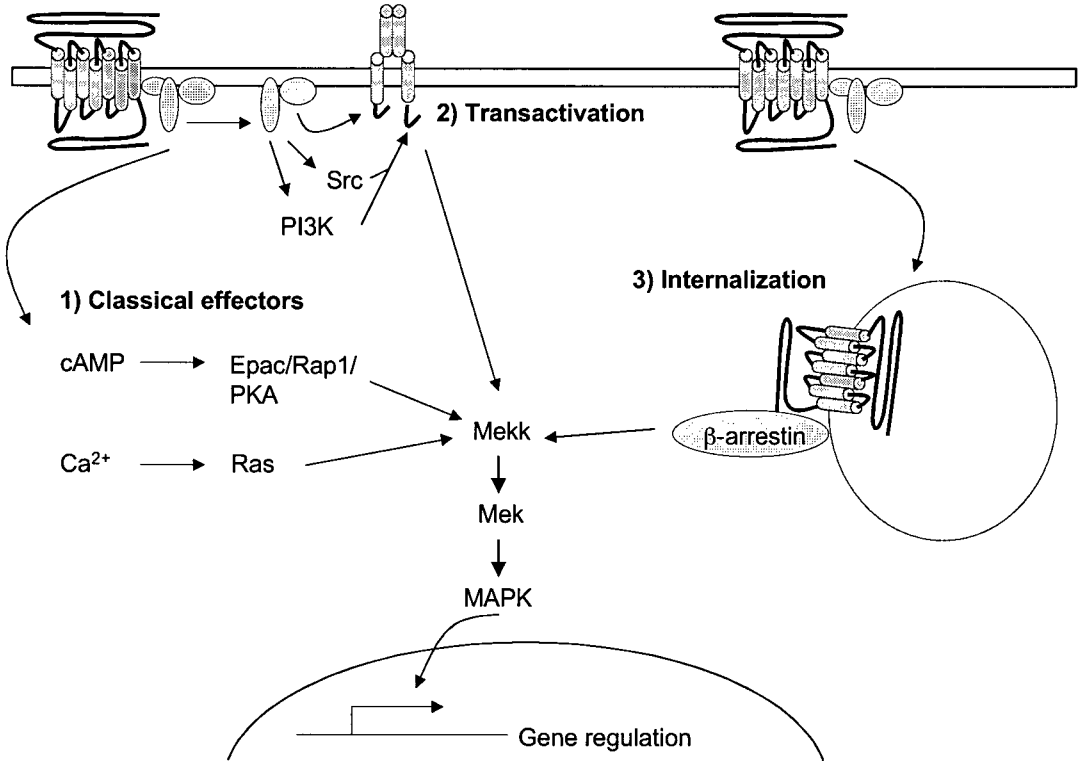


Figure 2: Coupling of GPCRs to MAPK signaling via classical effectors (1), transactivation of tyrosine receptor kinases (2), or internalization into endosomes (3). Seven transmembrane GPCR are depicted together with associated Gα, Gβ and Gγ subunits from left to right respectively. See text for details. Abbreviations not mentioned in text include Src, oncogene Src tyrosin kinase, and Ras, a small GTPase.

1.8.3 Gαs-coupled receptors and MAP kinase regulation

Signals conveyed through $G\alpha_s$-coupled GPCRs are extremely diverse. Although these receptors are classically thought to influence cellular events through adenylate cyclase/cAMP/PKA, it is now evident that additional proximal signals can mediate regulation of the ERK1/2 module. Depending on the cell type, such receptors have also been shown to activate MAPK via PKC, PI3K, and/or cross-talk with receptor tyrosine kinases (RTKs) (Liebmann 2001). Furthermore, the prototypical GPCR, the β_2-adrenergic receptor, has even been reported to undergo a switch, from $G\alpha_s$ to $G\alpha_i$, with subsequent activation of ERK1/2 by Gβγ subunits (Daaka et al. 1997; Maudsley et al. 2000). Finally, $G\alpha_s$ has been shown to directly

interact with, and activate, Src (Ma et al. 2000) and this protein-tyrosine kinase can regulate the ERK 1/2 module due to its ability to transactivate RTKs and influence the activity of the small G-protein Ras (via Grb2 and Sos) (Maudsley et al. 2000).

Family B $G\alpha_s$-coupled GPCRs, including glucagon, GLP-1, VIP, and PACAP, are all coupled to the production of cAMP and have been shown to activate ERK1/2 MAP kinases (Frodin et al. 1995; Barrie et al. 1997; Montrose-Rafizadeh et al. 1999; Yamaguchi et al. 2000; Buteau et al. 2001; Jiang et al. 2001). In fact, PACAP has even been linked to ERK1/2 activation via PKC in the neuronal PC12 cell line (Barrie et al. 1997), and there is evidence for PI3K-dependent ERK1/2 activation by CGRP and GLP-2 (Jasleen et al. 2000). Until recently, however, the role of cAMP in ERK1/2 activation was controversial, with some studies showing negative effects and others positive. Interestingly, in addition to the cAMP and PKA pathway, additional cAMP effectors have been identified, including cAMP-GEFs and the small GTPases Rap1 and Rap2 (Ohmstede et al. 1990; Altschuler et al. 1995; Kawasaki et al. 1998; Richards 2001). These effectors form the basis of cAMP interaction with the MAP kinase module. Initial studies on the interaction of these two pathways documented an inhibitory effect of cAMP on ERK 1/2 module activation by growth factors (Houslay and Kolch 2000), but cAMP has since been shown to stimulate ERK 1/2 activity in several cells types including 3T3-F442A adipocytes, ovarian granulosa cells, pituitary and neuronal cells, and the commonly used HEK and CHO-K1 cell lines (Houslay and Kolch 2000). This ability of cAMP to activate ERK 1/2 depends on which isoform of Raf (the Mekk) is expressed in the cell of interest (Vossler et al. 1997). In this manner, cAMP has been shown to regulate either transient or sustained activation of ERK 1/2 in the neuronal PC12 cell line (York et al. 1998).

The regulation of p38 MAPK and JNK modules by GPCRs has received less attention, and is still mainly unresolved. There is evidence describing the interaction of $G\alpha_q$, $G\alpha_{13}$, and $G\beta\gamma$ with MKK3/6 and the p38 MAPK pathway (Marinissen and Gutkind 2001). Recently, there are also data illustrating that cAMP can couple activation of p38 MAPK with stimulation of TSH, dopamine, and FSH receptors (Pomerance et al. 2000), implying a role for $G\alpha_s$ in regulating this MAPK module. Family B GPCRs such as those for PACAP and GLP-1 have both been implicated in regulating p38 MAPK phosphorylation in neuronal and pancreatic β-cells respectively (Buteau et al. 1999, 2001; Sakai et al. 2002). In fact, the role of p38 MAPK regulation by GLP-1 seems to be important in mediating the proliferative effects of this incretin (Buteau et al. 1999, 2001).

1.8.4 GPCRs and CREB transcription factor signaling

The first transcription factor family shown to be functionally regulated by phosphorylation was the cAMP response element (CRE) binding protein (CREB) family, including CREB, ATF-1 (activating transcription factor 1) and CREM (cAMP response element modulator). As its name implies, CREB was identified by its ability to bind the CRE element of the somatostatin gene, and is activated and phosphorylated in response to cAMP signals. The paradigm of CREB signal transduction includes GPCR-mediated increases in cAMP, activation of PKA, and subsequent phosphorylation of nuclear CREB at serine 133, which then regulates the trans-activating ability of CREB in mediating effects on CREs (TGACGTCA) in cAMP-responsive genes. Biochemical understanding of the phosphorylation-dependent events mediated by CREB are well established, however, the physiological role of this transcription factor in all tissues is not (reviewed in Mayr and Montminy 2001).

Similar to the MAPK pathways, numerous signals can feed into CREB phosphorylation and activation, making it difficult to study its regulation. In addition to the aforementioned paradigm of cAMP/PKA signaling, CREB has been identified as a substrate for numerous protein kinases including p90RSK, PKC, AKT/PKB (protein kinase B), MSK-1 (mitogen and stess-activated kinase-1), MAPKAP-2 (MAPK activated protein-2), and calcium-calmodulin kinases II and IV (Mayr and Montminy 2001). All of these kinases are potential targets of GPCR signaling, however, most of the published literature has been concerned with growth factor regulation of CREB (reviewed in Shaywitz and Greenberg 1999; Mayr and Montminy 2001; Lonze and Ginty 2002).

Clearly, Family B $G\alpha_s$-coupled GPCRs present themselves as prime regulators of CREB, however, few studies have investigated this mechanism of action. Vasoactive intestinal polypeptide, PACAP, GLP-1, GLP-2, and GIP have been shown to mediate phosphorylation of CREB in various tissues (Schomerus et al. 1996; Yusta et al. 1999; Trumper et al. 2001, 2002; Kemp and Habener 2002). However, coupling of these receptors to CREB phosphorylation and the physiological relevance of CREB-mediated actions has not been well established in all cases.

1.9 REGULATION OF β-CELL GROWTH AND SURVIVAL

1.9.1 Beta-cell mass in Type 2 Diabetes

In adults, pancreatic β-cell growth is regulated by replication from existing β-cells and neogenesis of new β-cells from precursor stem cells (Bonner-Weir 2000). Overall β-cell mass can be regulated by variations in cell number (hyperplasia), cell size (hypertrophy), neogenesis/differentiation, and cellular apoptosis. Evidence has accumulated that β-cell mass exists in a dynamic state, and that changes in mass and function allow for maintenance of plasma glucose levels within a very narrow range (Bonner-Weir 2001).

In an animal model of type 2 diabetes, the obese diabetic Zucker fa/fa rat (ZDF), changes in β-cell mass have been well characterized (Lingohr et al. 2002). In young animals, there is an initial expansion of β-cell mass that compensates for early insulin resistance. However, as obesity and glucose intolerance worsens with age, the β-cell population can no longer meet the requirements of heightening insulin resistance (Lingohr et al. 2002). This decreased β-cell mass in another rodent model of type 2 diabetes, *Psammonmys obesus,* is correlated with an onset of type 2 diabetes, and has been attributed to an increase in β-cell apoptosis, likely due to glucotoxity and/or elevated fatty acids (Donath et al. 1999). Recently, this notion of increased apoptosis correlated to decreased β-cell mass has also been confirmed in human pancreatic sections of type 2 diabetics (Butler et al. 2003). Thus, the factors regulating β-cell mass have become an important determinant in the pathophysiology and the treatment of diabetes.

1.9.2 Signal transduction regulating β-cell growth/survival

Numerous β-cell growth factors have been identified for both precursor and adult β-cells, however, the best characterized include growth hormone (GH) and insulin-like growth factor 1 (IGF-1) (Lingohr et al. 2002). These hormones both act on tyrosine kinase receptors, signaling via JAK2 (Janus kinase)/STAT5a/b (signal transducers and activators of transcription) and IRS-2 (insulin receptor substrate-2) respectively, to regulate β-cell mitogenesis. IGF-1 can also increase phosphorylation of ERK1/2 MAP kinases, however, the biological implications of this in terms of β-cell proliferation or survival are still unclear. The known survival actions of GH are mediated via increased expression of the anti-apoptotic protein Bcl-xL and members of the

SOCS (suppressors of cytokine signaling). Survival actions of IGF-1 are via PI3K-dependent PKB signaling, which has been shown to act on numerous effectors important in cell survival including GSK-3 (glycogen synthase kinase-3), β-catenin, Bad, Mdm2, procaspase-9, Rb (Retinoblastoma) protein, and FKHR-1 (forkhead family transcription factor). If these downstream targets are indeed regulated by PKB in β-cells, further support will be given to the importance of this protein kinase in regulating cell proliferation and survival (reviewed in Lingohr et al. 2002).

Pituitary adenylate cylcase activating protein (PACAP) and GLP-2, both members of the VIP-secretin-glucagon family of peptides, have been convincingly shown to act as anti-apoptotic agents in neurons and baby hamster kidney cells, respectively (Yusta et al. 2000, 2002; Vaudry et al. 2002). These actions are accomplished by cAMP mediated inhibition of caspase activity; however, the role of PKA is somewhat controversial. The anti-apoptotic actions of both peptide receptors have been linked to downstream kinase cascades that promote cell survival; Mek1/2 in the case of PACAP, and PKB and GSK-3β in mediating GLP-2 effects (Vaudry et al. 2002; Yusta et al. 2002). However, the coupling between class II receptors and anti-apoptotic effects has been largely unexplored, and relatively little is known about anti-apoptotic signaling in the pancreatic β-cell.

Given the pleiotropic actions of $G\alpha_s$ coupled receptors, their effects on MAP kinase pathways, and the established actions of GLP-1, it was hypothesized that GIP could also regulate cell fate. The role of GLP-1 and GIP in regulating β-cell fate has only become apparent during the completion of this thesis, and is a rapidly growing area. GLP-1 was first shown to be mitogenic in INS-1 cells acting via PI3K and p38 MAP kinase signaling (Buteau et al. 1999). This has since been confirmed *in vivo* (Xu et al. 1999; Stoffers et al. 2000) and further elucidation has implicated roles for PKCξ and transactivation of the EGF receptor (Buteau et al. 2001; Buteau et al. 2003). Recently, anti-apoptotic actions mediated via the GLP-1 receptor have been confirmed *in vivo* with exendin-4 administration in STZ (streptozotocin)-induced diabetic mice (Li et al. 2003). However, little was clarified in terms of mechanism of action. GIP growth and anti-apoptotic actions were also published during the period of research leading to this thesis (Trumper et al. 2001, 2002) supporting our notion of the importance of GIP in β-cell survival.

1.10 REGULATION OF RAT INSULIN GENE TRANSCRIPTION

1.10.1 Overview of the rat insulin promoter

Most species express only one insulin gene, however, in the mouse and rat, two non-allelic insulin genes exist. Insulin is highly regulated in pancreatic β-cells, from gene transcription events to insulin exocytosis. The importance of the promoter region of the insulin gene in driving expression was demonstrated early on with the establishment of the β-cell line, βTC-3. These mouse β-cells were transfected with the rat insulin II promoter (RIP2) driving the expression of simian virus 40 T-antigen, and causing tumourogenicity. Numerous cis-acting elements of the RIP1, RIP2, and human insulin promoter have since been characterized, and have been shown to interact with β-cell specific transcription factors (reviewed by Melloul et al. 2002).

Regulatory cis-acting elements include the critical and well studied E-boxes, A-boxes, CRE, and C2 element. E-boxes bind transcription factors of the basic helix-loop-helix (bHLH) family, including the insulin gene and pancreatic development transcription factor BETA2/NeuroD. A-boxes are characterized by their AT rich sequence, and their ability to bind the pancreatic duodenal homeobox (PDX-1) transcription factor also identified as IPF-1, STF-1, IDX-1, GSF, and IUF-1. The importance of PDX-1 extends beyond regulation of the insulin gene, by controlling the transcription of numerous islet-specific genes and pancreas development. The CRE sequence (TGACGTCA consensus, TGACGTCC in RIP1) mediates genetic responses to elevations in cAMP by binding the CREB family of transcription factors, members of the basic region leucine zipper (bZIP) family. Hormones that elevate β-cell cAMP levels such as the incretin GLP-1 are able to stimulate RIP1 promoter activity via the CRE element. Despite evidence suggesting the importance of the CRE element in basal and stimulated RIP1 activity, the binding of endogenous CREB was reported to have low affinity compared to exogenous CREB. Indeed, additional CRE binding proteins have been identified as activators or repressors of the insulin gene. Finally, the C2 element binds PAX DNA binding proteins containing a paired domain and homeodomain. As with Beta2/NeuroD and PDX-1, PAX4 and PAX6 are also critical for pancreatic development in addition to regulating the insulin gene (reviewed by Melloul et al. 2002). One can envision that CREB also contributes to the dynamic regulation of pancreatic development.

1.10.2 Regulation of the rat insulin gene by incretins

Both GIP and GLP-1 have been shown to stimulate promoter activity of the rat insulin 1 gene (Drucker et al. 1987; Fehmann and Göke 1995) and to have effects on insulin protein expression (Fehmann and Habener 1992; Wang et al. 1996). In addition, GLP-1 has been documented to increase insulin mRNA stability in β-cells (Wang et al. 1995). These effects are clearly desirable given the overall insulinotropic nature of the incretins. However, despite studies on GIP by Fehmann and colleagues, there has been no further elucidation of the signals coupling the GIP receptor to rat insulin gene promoter activity. In contrast, GLP-1 actions have recently been studied extensively (Skoglund et al. 2000; Chepurny et al. 2002; Lawrence et al. 2002).

Despite controversy regarding the importance of the CRE in the rat insulin 1 promoter, recent mutagenesis studies clearly illustrate that GLP-1/exendin-4 actions on RIP1 activity are via the CRE, and independent of A-boxes (PDX-1 binding motif) (Chepurny et al. 2002). However, the importance of PKA in mediating these events is unresolved (Skoglund et al. 2000; Chepurny et al. 2002; Lawrence et al. 2002). A wide screen of protein kinase inhibitors revealed a role for PKC, RSK, or MSK in transmitting the signal to transcriptional effects. Further, the involvement of a CREB family member in mediating promoter actions is required, however, the identity of this bZIP transcription factor remains enigmatic (Chepurny et al. 2002).

1.11 THESIS INVESTIGATION

There exists a potential role for the incretin hormone, GIP, in the pathophysiology of diabetes mellitus, obesity, and food-dependent Cushing's syndrome. GIP acts on its cognate GPCR on target tissues to promote specific physiological responses such as insulin secretion. However, the intracellular mechanisms involved in the actions of GIP are not well understood. Considering the limited knowledge available on GIP receptor stimulated intracellular signal transduction, it is of great importance to elucidate these mechanisms and apply them to further biomedical research. Elucidation of these intracellular signals forms a greater basic science knowledge base from which to apply strategies for therapeutic intervention in the treatment of diabetes, obesity, and food-dependent Cushing's syndrome. The present thesis investigation was undertaken to further elucidate intracellular signaling cascades mediated by the GIP receptor in

β-cell lines, with the hope of highlighting novel functional roles for the hormone. The following hypotheses were tested:

Hypothesis 1: Stimulation of the GIP receptor with GIP results in the activation of PLA_2 and the subsequent release of arachidonic acid that is functionally coupled to β-cell insulin secretion.

Hypothesis 2: The GIP receptor can regulate insulin secretion in a K^+_{ATP} channel-independent manner.

Hypothesis 3: Stimulation of the GIP receptor with GIP results in activation of the ERK1/2 MAP kinase module that is functionally coupled to tissue specific GIP actions.

Hypothesis 4: Stimulation of the GIP receptor with GIP regulates β-cell fate via regulation of MAP kinase signaling.

Hypothesis 5: Stimulation of the GIP receptor with GIP promotes rat insulin promoter activity via cAMP/CREB signaling.

CHAPTER 2: METHODOLOGY

2.1 REAGENTS

All chemicals, of reagent or molecular biology grade were from Amersham Pharmacia Biotech (Mississauga, ON), BDH Inc. (Toronto, ON), Fisher Scientific International (Pittsburgh, PA, USA), Gibco Life Technologies Inc. (now Invitrogen Canada, Burlington, ON), Merck (Darmstadt, Germany), Perkin-Elmer/Mandel Scientific/NEN Life Scientific Co. (Guelph, ON), Sigma (Oakville, ON, Canada) or VWR Canlab (Mississauga, ON). All tissue culture disposables were from BD Falcon (San Jose, CA, USA) and serum was from Cansera (Rexdale, Ont., Canada). Specific sources for other chemicals are indicated in brackets in the following sections describing experimental methodology.

2.2 PLASMID DNA CONSTRUCTS

The cDNA for the rat pancreatic islet GIP receptor was previously isolated by RT-PCR by Dr. R. Gelling (Wheeler et al. 1995). This construct is contained in the mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA), subcloned into the *HindIII/XhoI* (Gibco) restriction endonuclease sites. The empty mammalian expression plasmid pRK5 and the plasmid pRK-βARKct (encoding the C-terminus of β-adrenergic receptor kinase; bp 495~689) were kindly provided by Dr. R. J. Lefkowitz for experiments targeted at investigating a role for Gβγ signaling (Koch et al. 1994). The Rap1 GTPase constructs and vector were a kind gift from Dr. D. Altschuler (University of Pittsburgh, PA, USA), and the Src constructs (wt, Y527F and src RF) were all donated by Dr. J. Brugge (Harvard University, MA, USA). PI3K constructs, Δp85 and p110CAAX (dominant negative and constitutively active), were donated by Dr. G. Rutter (Bristol, UK). CREB constructs were obtained from Dr. C. Vinson (National Institutes of Health, MD, USA) and Dr. M. Montminy (The Salk Institute, CA, USA). The dominant negative construct, A-CREB, was from Dr. Vinson, and the M1-CREB and F/Y CREB constructs were donated by the Montminy laboratory. Finally, the rat insulin 1 gene promoter (RIP1) was donated by Dr. M. German (University of California, San Fransisco, CA, USA) and the rat insulin 2 gene promoter (RIP2) was kindly provided by Dr. C. B. Verchere (Children's

Hospital, University of British Columbia, Canada) with permission from Dr. R. Palmiter (University of Washington, Seattle, WA, USA).

2.3 CELL TISSUE CULTURE AND TRANSFECTION

Four cell types were used in experiments reported in this thesis: Chinese hamster ovary (CHO) fibroblast cells (strain K1; American type tissue collection: CCL-61) and three β-cell insulinoma cell models. βTC-3 cells, a clonal mouse β-cell insulinoma derived from transgenic mice expressing a hybrid insulin/oncogene (Efrat et al. 1988), BRIN-D11 cells from a rat β-cell insulinoma, were created by and obtained from Dr. P.B. Flatt (University of Ulster, Belfast, N. Ireland) (McClenaghan et al. 1996) and INS (832/13) cells, from a rat β-cell insulinoma transfected with the human insulin gene (Asfari et al. 1992; Hohmeier et al. 2000), were obtained from Dr. C.B. Newgard (University of Texas, USA).

Chinese hamster ovary cells (CHO-K1), cultured in DMEM/F12 (DMEM, Dulbecco's modified eagle media; Gibco) supplemented with 10% newborn calf serum (Cansera), were stably transfected with the wild type rat GIP receptor as previously described (Wheeler et al. 1995; Gelling et al. 1997). The CHO-K1 cell line obtained by pooling clones was termed rGIP-15, and has previously been shown to express receptors at levels similar to high level expressing clones (Gelling et al. 1997). In experiments targeted at investigating a role for Gβγ signaling, rGIP-15 clones were transiently transfected with plasmid DNA encoding the C-terminus of β-adrenergic receptor kinase (βARKct) (Koch et al. 1994) or the empty vector (pRK5). Briefly, 40-60% confluent monolayers in 10 cm culture plates (Becton Dickenson) were transfected using Superfect™ (Qiagen, Valencia, CA, USA) transfection reagent according to the manufacturers' protocol for all arachidonic acid release experiments. Cells were harvested 18 to 24 h post transfection and passaged into 24-well plates for subsequent arachidonic acid release experiments. Passages 20-40 of rGIP-15 cells were used in all experiments.

Transfections in all subsequent experiments used the reagent Lipofect2000™ (Gibco). In experiments targeted at investigating a role for Rap1 signaling in the regulation of the ERK1/2 cascade, rGIP-15 clones were transiently transfected with plasmid DNA encoding the wild type Rap1b small GTPase, or the empty vector (pCGN) (Altschuler et al. 1995). Briefly, 80-90 % confluent monolayers in 6-well culture plates (Becton Dickenson) were transfected with a 2:1 ratio of Lipofect2000™ to plasmid DNA (μL:μg DNA) according to the manufacturers'

protocol. Single wells in 6-well plates were generally transfected with 5 μg of DNA. Transfections were allowed to proceed for 5 h in DMEM media in the absence of serum, and thereafter regular growth medium was added to the cells overnight. Regular growth medium was replaced the following day. All transfections were performed concurrently with green fluorescent protein to ensure transfection and approximate efficiency (~ 40% for CHO-K1 cells). When increasing amounts of construct DNA were transfected, empty vector was added to ensure the same amount of total DNA was used in all cases.

βTC-3 cells were cultured in low glucose (5.5 mM) DMEM (Gibco Laboratories) supplemented with 12.5 % horse serum (Cansera) and 2.5 % fetal bovine serum (Cansera). Passages 20-30 were used for all experiments. It is important to maintain low passage number for clonal β-cells, as the age-related decline in glucose-responsiveness and insulin content is well characterized (Poitout et al. 1996), although the βTC-3 cells may be responsive to insulin secretagogues up to passage 39 (Kieffer et al. 1994), or even higher (Efrat et al. 1988). Initial screening experiments provided evidence for a reduced GIP responsiveness in older passaged βTC-3 cells (> passage 30), with a rightward shift in the cAMP concentration-response profile (EC_{50}).

INS-1 cells were cultured in 11 mM glucose RPMI (Sigma Laboratories) supplemented with 2 mM glutamine, 50 μM β-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, and 10 % fetal bovine serum (Cansera). In experiments targeted at investigating a role for Rap1 signaling in INS-1 (832/13) cells, cells were transiently transfected with plasmid DNA encoding the wild type Rap1b small GTPase, constitutively active (G12V), dominant negative Rap1 (N17), or the empty vector (pCGN). Briefly, 80-90 % confluent monolayers in 6-well culture plates (Becton Dickenson) were transfected using Lipofect2000™ (Gibco) transfection reagent as described above for rGIP-15 cells (using DMEM media). Regular growth medium was replaced 5 h after transfection. All transfections were performed concurrently with green fluorescent protein to ensure transfection and to estimate efficiency (~ 20-30 % for INS-1 cells). Passages 45-70 were used for all experiments.

2.4 IODINATION OF GIP AND BINDING ANALYSIS

Synthetic porcine GIP (5μg) was iodinated by the chloramine-T method, and the ^{125}I-GIP was further purified by reverse phase high performance liquid chromatography to a specific

activity of 250-300 μCi/μg (Wheeler et al. 1993). Aliquots were subsequently lyophilized and stored at -20 ^{0}C until use. Competitive binding analyses were performed as previously described with minor modifications (Wheeler et al. 1993). Briefly, CHO-K1 cells, plated two days prior in 24-well plates at 5 x 10^4 cells/well (βTC-3 cells and INS-1 cells plated at 5 x 10^5 cells/well, were washed twice with 4 ^{0}C Krebs-Ringer (115 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 10 mM $NaHCO_3$, 1.28 mM $CaCl_2$, 1.2 mM $MgSO_4$) containing 10 mM HEPES and 0.1 % bovine serum albumin (BSA), pH 7.4, (KRBH) and incubated in triplicate for 14-18 h at 4 ^{0}C with ^{125}I-GIP (50 000 cpm/well) in the presence or absence of unlabelled GIP (synthetic human GIP_{1-42}; Bachem, Torrence, CA, USA; concentrations ranging from 10^{-12}-10^{-5} M). After two consecutive washes in ice cold buffer, cells were solubilized with 0.1 M NaOH and transferred to test tubes for counting. Nonspecific binding was defined as that measured in the presence of an excess of human GIP (1 μM), and specific binding was expressed as a percentage of maximum binding (%B/B_o). Non-linear regression analysis of competitive-binding curves followed algorithms included with the Prism 3 software package (GraphPad, San Diego, CA), where the IC_{50} is the concentration of unlabelled competitor that displaces 50% of bound label. GLP-1 competitive binding was performed using ^{125}I-GLP-1, kindly provided by Novo Nordisk (Denmark).

Saturation binding isotherm experiments were also performed in some cases to determine experimentally the number of GIP binding sites (an approximation of receptor number). In these studies, cells in 24-well plates were prepared similar to above, except that serial dilutions of ^{125}I-GIP were added to wells in the presence or absence of 1 μM unlabelled peptide (in triplicate). Total label concentrations were calculated using the specific radioactivity of the label and measured cpm values of added label and specific binding. Data were fitted to a binding isotherm using the Prism 3 software (Graphpad), allowing for calculation of B_{max} (converted to sites/cell) and K_d.

2.5 CYCLIC AMP PRODUCTION AND INSULIN SECRETION

Intracellular cAMP and insulin release experiments were conducted with cells passaged into 24-well culture plates at 5 x 10^4 cells/well for CHO-K1 clones and 5 x 10^5 cells/well for βTC-3 and INS-1 cells. One hour prior to study, cells were washed with and preincubated in modified Krebs-Ringer buffer (KRBH) containing the indicated glucose concentrations. For cAMP studies, cells were washed twice with KRBH and then stimulated for 30 min with GIP in

the presence of the phophodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mM IBMX; RBI/Sigma, Natick, MA, USA). Following stimulation, reactions were stopped, and cells lysed in 70% ice cold ethanol, cellular debris removed by centrifugation, and cAMP subsequently quantified by radioimmunoassy (RIA) (Biomedical Technologies Inc., Stoughton, MA, USA). Ca^{2+}-free experiments were conducted in KRBH containing equimolar Mg^{2+} and supplemented with 10 mM EGTA. All insulin release experiments were performed over 60 min in KRBH, in the absence of IBMX, and insulin secreted into the media was quantified by RIA as previously reported (Hinke et al. 2000). All insulin measurements are therefore representative of total immumoreactive insulin, which may include proinsulin and insulin quantification. In figures where glucose and GIP are concomitantly added, "+ GIP" often denotes the addition of GIP in the presence of glucose.

In K^+_{ATP} channel-independent studies, a dose response profile was initially generated to determine which concentration of diazoxide to use. In agreement with published literature (Sato and Henquin 1998), cells were incubated for 30 min in 250 μM diazoxide prior to and during stimulation with test buffer. Buffer containing 30 mM K^+ was prepared by replacing NaCl with equimolar KCl. Ca^{2+}-free experiments were conducted as mentioned above. The role of PKA was assessed by incubating cells for 15 min with 5 μM H89, or 200 μM Rp-cAMPS prior to and during stimulation with GIP. Buffer containing released insulin was collected over 1 hour and assayed by RIA; thus, all reported insulin concentrations refer to immunoreactive (IR) insulin which may include the measurement of proinsulin due to antibody specificity.

2.6 ARACHIDONIC ACID RELEASE

Arachidonic acid (AA) release was determined by methods adapted from Shuttleworth and Thompson (Shuttleworth 1996). Cells were harvested and passaged into 24-well culture plates at 4 x 10^4 cells/well for CHO-K1 clones and 2 x 10^5 cells/well for βTC-3 cells. Respective media were replaced with media containing 0.125 μCi/mL [^{3}H]-AA (specific activity of 98.6 Ci/mmol; New England Nuclear, Boston, MA, USA) 18 to 24 hours following passaging, and the plates were incubated for an additional 36-48 h. Prior to the addition of experimental agents, the wells were washed twice with 0.5 mL of KRBH, and allowed to equilibrate for 1 h. Ca^{2+}-free experiments were conducted in KRBH containing equimolar Mg^{2+} and supplemented with 10 mM EGTA. Agonists were dissolved in Krebs-Ringer buffer, added in triplicate (0.5 mL

total volume per well), and incubated for the length of time shown in the figure legends. As a positive control, adenosine-5-triphosphate (ATP) was added at a final concentration of 5 μM. When used, the inhibitor haloenolactone suicide substrate (HELSS, Calbiochem, La Jolla, CA, USA) was added for 30 min prior to washing and addition of agonists. After incubation, 0.4 mL aliquots were placed into scintillation vials followed by the addition of 10 mL of Econo 2 scintillation fluid (Fisher, Nepean, Ontario, Canada) and radioactivity was determined by liquid scintillation spectrometry. AA released from cells was generally between 2-6 % of total ^{3}H-AA incorporated into cells.

2.7 KINEXUS IMMUNOBLOT ANALYSIS

For initial profiling of protein kinases and phosphatases expressed in rGIP-15, βTC-3 and INS-1 (832/13) cells, cells were harvested and plated into 10 cm culture dishes. After confluency was reached (2 days), cells were serum starved overnight in order to establish metabolic quiescence. Removed serum was replaced with 0.1 % BSA in media. Cells were washed with KRBH and protein was extracted with cellular lysis buffer (0.5% Triton X100, 60 mM β-glycerophosphate, 20 mM MOPS pH 7.2, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 1% Trasylol, and 1 mM PMSF). Thereafter, samples were sonicated (30 s), centrifuged (12 000 rpm for 30 min), and protein content was quantified using the BCA reagent (Pierce, Rockford, Il, USA) in order to ensure equal loading of gels for subsequent Western blotting.

Kinetworks™ KPKS 1.0 Western blotting analysis of the expression of 75 different protein kinases was initially performed by Kinexus Bioinformatics Corporation, with 500 μg of cell lysate protein following centrifugation (Pelech and Zhang 2002; Pelech et al. 2002 and www.kinexus.ca). The Kinetworks™ KPSS 1.1 screen of 31 different phosphorylation sites with phospho-site-specific antibodies was also performed by Kinexus with 300 μg of cell lysate protein. The Kinetworks™ KPPS screen detects 25 different cellular phosphatases and the KAPS apoptosis screen detects 25 different apoptosis related proteins (see www.kinexus.ca for details). Quantification of the immunoreactive bands on the Kinetwork blots (trace quantity) with ECL detection was performed with a Bio-Rad FluroS Max Imager and Bio-Rad Quantity One software.

2.8 ERK1/2 MODULE IMMUNOBLOT ANALYSIS

Studies investigating the ERK1/2 module kinase phosphorylation states of Raf-1, Mek 1/2, p90 RSK, and Elk-1 were conducted using the following antibodies: Phospho T202, Y204-ERK1 (p-ERK 1/2) was purchased from Santa Cruz Biotechnologies (California, USA), while p-Raf (S259), p-Mek (S217, S221), p-p90 RSK (S380), and p-Elk-1 (S383) were obtained from Cell Signaling Technology (Sampler kit discontinued, New England Biolabs). Total ERK 1/2 was assessed using a C-terminal targeted antibody from Santa Cruz Biotechnologies. Briefly, cells were harvested and plated into 6-well plates 2 days prior to overnight serum starvation and subsequent stimulation was performed on day 3. Cells were preincubated for 1 h at 37 ^{0}C in modified Krebs ringer solution prior to the addition of agonists or pharmacological inhibitors. Following the elapsed stimulation period, cells were washed once with ice-cold KRBH and lysed on ice. Pharmacological inhibitors (H89, GF109203x, PD 98059, U0126 (Calbiochem) were added for 15 min prior to agonist addition and maintained in the presence of agonists. Protein samples (50 μg protein/well) were separated on a 13 % SDS/polyacrylamide gel and transferred onto nitrocellulose (BioRad, Mississauga, Ont., Canada) membranes. Probing of the membranes was performed with the above mentioned antibodies and bands visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) using horseradish peroxidase-conjugated IgG secondary antibodies. For quantification of band density indicative of phosphorylation, films were analyzed using densitometric software (Eagle Eye; Stratagene, La Jolla, CA, USA).

2.9 P38 MODULE IMMUNOBLOT ANALYSIS

For determination of MKK3/6, p38 MAPK and ATF-2 phosphorylation, INS-1 (832/13) cells were harvested and plated into 6-well plates (2 x 10^{6} cells/well) 2 days prior to overnight serum starvation (3 mM glucose RPMI media with 0.1 % BSA) and subsequent stimulation was performed on day 3. Cells were stimulated in RPMI media containing the indicated glucose concentration and 0.1 % BSA. Following the elapsed stimulation period, proteins from both floating and adherent cells were extracted with cellular lysis buffer. Thereafter, samples were sonicated (30 s), centrifuged (12 000 rpm for 30 min), and protein content was quantified using the BCA reagent (Pierce) in order to ensure equal loading of gels for subsequent Western blotting. Pharmacological inhibitors (Wortmannin and SB202190; Calbiochem) were added for

15 min prior to agonist addition and maintained in the presence of agonists. Protein samples (50 μg protein/well) were separated on a 13 % SDS/polyacrylamide gel and transferred onto nitrocellulose (BioRad, Mississauga, Ont., Canada) membranes. Probing of the membranes was performed with antibodies from the p38 MAPK sampler kit (Cat # 9913), including Phospho T180, Y182-p38 MAPK (p-p38 MAPK), Phospho S189/207 MKK3/6 (p-MKK3/6), and Phospho T71 ATF2 (p-ATF2) obtained from Cell Signaling Technology, New England Biolabs. Total protein was assessed using β-tubulin antibody from Santa Cruz Biotechnologies. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) using horseradish peroxidase-conjugated IgG secondary antibodies. For quantification of band density, as a measurement of phosphorylation state, films were analyzed using densitometric software (Eagle Eye (Stratagene)).

2.10 CREB, CREM, AND ATF-1 IMMUNOBLOT ANALYSIS

For determination of CREB, CREM, and ATF-1 phosphorylation, INS-1 (832/13) cells were harvested and plated into 6-well plates (2×10^6 cells/well) 2 days prior to overnight serum starvation and subsequent stimulation was performed on day 3. Cells were stimulated in RPMI media containing the indicated glucose concentration and 0.1 % BSA. Pharmacological inhibitors (H89, U0126, SB203580; Calbiochem) were added for 15 min prior to agonist addition and maintained in the presence of agonists. Probing of the membranes was performed with Phospho S133-CREB 1B6 Monoclonal (p-CREB), which recognizes phospho S133 CREB, phospho S117 CREM, and phospho S63 ATF-1 (Kemp and Habener 2002), obtained from Cell Signaling Technology (New England Biolabs). Total CREB protein was also assessed (Cat # 9192, Cell Signaling Technology) to ensure protein phosphorylation was not due to differences in total CREB levels. Immunoreactive bands were visualized and quantified as described above.

2.11 DETERMINATION OF CELL GROWTH AND SURVIVAL

INS-1 (832/13) cells were seeded into 96-well plates (5×10^4 cells/well) prior to experimentation. In the proliferation experiments, after establishing metabolic quiescence in the absence of serum for 24 h (3 mM glucose RPMI with 0.1 % BSA), cells were cultured in RPMI

media (with 0.1 % BSA) with agents (glucose, glucose + GIP/GLP-1/GH) for an additional 24 h. Thereafter, cells were washed with KRBH and frozen at – 70 ^{0}C until assayed. Cells were quantified using the CYQUANT™ assay system (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. Fluorescence was measured using a microplate fluorescence reader (Bio-tek FL600, Winooski, VT, USA) with excitation/emission set at 400/500 nm. Final cell numbers were always greater than the initial number plated (5 x 10^4 cells/well) when assessing cellular proliferation.

Cell survival was assessed in the presence of prolonged serum starvation and glucose deprivation. Cells were initially deprived of serum and glucose for 24 h (RPMI with 0.1 % BSA); thereafter, GIP or forskolin were added for an additional 24 h, and cell number was quantified. Thus, cells had been deprived of serum and glucose for 48 h, resulting in 50 % cell death in the absence of GIP or forskolin (see Figure 2c). Final cell numbers were always less than the initial number plated (5 x 10^4 cells/well) in assessing cell survival.

2.12 CASPASE ACTIVITY

Cells seeded into 6-well plates were serum starved for 12-18 h (3 mM Glucose RPMI with 0.1 % BSA), and then subjected to glucose deprivation (RPMI with 0.1 % BSA) or treatment with wortmannin, or 2 mM streptozotocin (STZ). In studies examining the effects of glucose deprivation on caspase activity, 100 nM GIP was added concomitantly with 0 mM glucose containing media. In studies where inhibitors were utilized, all inhibitors (H89, UO126, Wortmannin, or SB202190) were added 15 min prior to glucose deprivation +/- GIP. Concentrations of inhibitors used were based on previous studies, and H89 has been shown to inhibit insulin secretion, ERK1/2 phosphorylation (Ehses et al. 2002A) and rat insulin promoter activity at 5 μM. In STZ studies, GIP and GLP-1 were added 10 min prior to 2 mM STZ and for 30 min during STZ. Following treatment, caspase-3 activity was determined after 2, 6, or 24 h according to the manufacturer's protocol (standard: 7-amino-4-methylcoumarin (AMC), substrate: Ac-DEVD-AMC, inhibitor: Ac-DEVD-CHO; Molecular Probes, Eugene, OR, USA). Caspase-3 activity/well was assessed using a microplate fluorescence reader (Bio-tek FL600, excitation/emission at 360/460 nM), and corrected for total protein content using the BCA protein assay (Pierce, Roxford, Il, USA). Caspase-9 like activity was determined using the standard 7-amino-4-(trifluoromethyl)coumarin (AFC), and the fluorescent substrate Ac-LEID-

AFC (Calbiochem), with excitation/emission at ~ 400/500 nm. The caspase-9 substrate can also be cleaved by caspases-4 and –5, hence the designation caspase-9 "like" activity. Caspase-3 activity was measured after 30 min at room temperature (manufacturer's instructions), whereas caspase-9 like activity was assessed after 2 h incubation at 37^0C.

Kinetworks™ KAPS 1.0 Western blotting analysis of the expression of 25 different apoptosis-related proteins was performed by Kinexus Bioinformatics Corp with 400 μg of INS-1 cell lysate subjected to wortmannin treatment in the absence and presence of GIP. Samples were confirmed to have elevated caspase-3 activity, which was reversed by GIP, based on substrate cleavage analysis prior to KAPS. This analysis detects caspases 1 α/β, 2, 3, 5, 6, 7, 8, 9, and 12, in addition to other apoptosis-related proteins.

2.13 RNA INTERFERENCE (RNAi)

In experiments targeted at knockdown of Mek1, cells were plated at 5 x 10^5 cells/well in 6-well plates or 3 x 10^6 cells/well in 10 cm plates the day before transfection of double stranded (ds)RNA oligonuceotides in media without Penicillin/streptomycin. Cells were transfected using Oligofectamine™ (Gibco) for 5 h as per the manufacturer's instructions. The following 21-mer oligoribonucleotide was used corresponding to nt 364-384 of the rat Mek1 gene; 5'-AAC TCC CCG TAC ATA GTG GGC- 3' with a 3' overhang of 2 nt. Designed RNA oligonucleotides were "blasted" against the GenBank®/EMBL database to ensure gene specificity. dsRNA was purchased from Dharmacon Research Inc. (Lafayette, CO, USA) along with the control scramble 21-mer oligonucleotide which was not present in mammalian cells as of January 23, 2002. Thus, the scramble duplex served as a negative control in RNAi experiments.

Protein levels of Mek1/2 were assessed over 4 days following transfection by immunoblot analysis, with day 3 showing peak knockdown. Thus, cells transfected in 10 cm dishes were plated into 6-well dishes the day following transfection, and caspase-3 experiments were carried out on day 3 post transfection. Antibodies directed against Mek 1 and Mek 2 were from BD Biosciences (Mississauga, ON, Canada) and had previously been screened for selectivity (Ehses et al. 2002A); total ERK1/2 and β-tubulin antibodies were used to correct for total protein loaded and were from Santa Cruz Biotechnologies.

2.14 RAT INSULIN PROMOTER ACTIVITY

The donated –410 rat insulin 1 promoter (Dr. M. German) was already expressed upstream of the firefly luciferase gene (pFoxluc-410RIP1). The rat insulin 2 promoter was cloned into the pGL2 and pGL3 luciferase vectors (Promega, Madison, WI, USA) in both the forward and reverse directions upstream of the luciferase gene. pGL3 basic and control (Promega) were used as negative and positive controls respectively for luciferase assays.

For transfection of INS-1 cells with RIP1 or RIP2 luciferase constructs, cells were plated in 6-well plates at 2 x 10^6 cells/well (day 1). Cells were transfected the next day with 5 μg DNA (10 μL Lipofect2000™) using DMEM low glucose media (1 mL total) without serum for 5 h. In experiments targeted at elucidating a role for CREB or Rap1 in GIP regulated RIP1 activity, RIP1 was cotransfected with the CREB constructs, A-CREB, M1-CREB, F/Y-CREB, Rap1 constructs, or empty vector (5 μg DNA each) on day 2. Thereafter, 3 mL of regular INS-1 media was added to the cells overnight. On day 3, cells were plated at 5 x 10^4 cells/well into 96-well plates using 3 mM glucose INS-1 media, and experiments were conducted on day 4 (36-48 h after transfection). Luciferase experiments were conducted in 50 μL/well RPMI media with 0.1 % BSA and the indicated glucose concentration. Experiments were terminated by the addition of 50 μL Steady-Glo® luciferase assay buffer (Promega) and cells were ensured to have lysed. Samples were transferred into 96-well white Costar plates (Corning, Acton, MA, USA) and light output was measured using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

2.15 DATA ANALYSIS

Data are expressed as means ± S.E.M. with the number of individual experiments presented in the figure legend. All data were analyzed using the nonlinear regression analysis program PRISM (Graphpad, San Diego, CA, USA), and significance was tested using Student's t-test and analysis of variance (ANOVA) with various posthoc tests: Dunnett's multiple comparison test, the Newman-Keuls post test, or the Tukey post test as indicated in figure legends. Statistical significance was set at 5 %.

CHAPTER 3: ALTERNATIVE PATHWAYS OF GIP POTENTIATED INSULIN SECRETION

3.1 BACKGROUND

3.1.1 Arachidonic acid as a signaling intermediate in the β-cell

The regulation of lipid metabolism by glucose in β-cells has been known since the 1980s. Glucose–stimulated insulin secretion has been correlated with an increase in free arachidonic acid release, via hydrolysis of *sn-2* substituents in β-cells. All isoforms of PLA_2 have been shown to be expressed in the pancreatic β-cell or cell lines, including Ca^{2+}-dependent cytosolic PLA_2, ATP-stimulatable Ca^{2+}-independent, and secretory PLA_2 isoforms. A recent study by Owada (Owada et al. 1999) has elucidated that glucose stimulated arachidonic acid promotes membrane depolarization (via the Na^+, K^+-ATPase) and hence insulin secretion.

Heterotrimeric G-proteins are activated by G-protein coupled receptors and undergo GDP/GTP exchange at the level of the Gα subunit, leading to dissociation of the trimer into Gα and the Gβγ subunit (Clapham and Neer 1997). The Gβγ subunit has recently been shown to act on a number of effector targets including ion channels, enzymes, and kinases (Clapham and Neer 1997). Recent data suggested that inactivation of free Gβγ completely abolished KCl, Ca^{2+}, and GTPγS-evoked insulin release from HIT-T15 cells (Zhang et al. 1998), establishing a role for these subunits in insulin secretion. A role for Gβγ has also been demonstrated in the coupling of PLA_2 and arachidonic acid production in rod outer segments (Jelsema and Axelrod 1987) and to the activation of cardiac potassium channels (Kim et al. 1989).

These observations provided the rationale for determining whether arachidonic acid and PLA_2 are involved in the glucose potentiating effects of GIP in the β-cell, with a focus on Gβγ subunits as a coupling mechanism for the GIP receptor.

3.1.2 K^+_{ATP} channel-independent pathway for insulin secretion

Until recently, the majority of studies on glucose-stimulated insulin secretion from pancreatic β-cells have focused on the role of changes in the ATP/ADP ratio in the closure of

K^+_{ATP} channels and the subsequent opening of voltage-activated Ca^{2+} channels (Ashcroft 2000). However, it is now clear that glucose-induced insulin secretion involves both K^+_{ATP} channel-independent and Ca^{2+}-independent mechanisms (Gembal et al. 1993; Sato et al. 1998; Aizawa et al. 2000; Henquin 2000). Additionally, K^+_{ATP} channel-independent actions and Ca^{2+}-independent mechanisms of the second messenger, cAMP, have also been demonstrated (Ämmälä et al. 1993; Yajima et al. 1999).

Glucose-dependent insulinotropic polypeptide regulates insulin secretion from pancreatic β-cells when prevailing glucose levels are elevated, such as following the ingestion of a meal (Pederson 1994). GIP has been shown to elevate cAMP levels and in this Chapter we provide evidence that GIP-stimulated arachidonic acid (AA) metabolism is an integral component of GIP-potentiated insulin secretion in βTC-3 cells (Ehses et al. 2001). Both of these second messengers are stimulated by GIP independently of glucose in βTC-3 cells (Hinke et al. 2000 and Figure 3C). Thus, we sought to determine whether insulin responses to these signals were dependent on Krebs cycle flux as are the actions of GIP. Furthermore, it was recently demonstrated that GIP-stimulated, PKA mediated, phosphorylation of serine-372 of KIR6.2 (inward rectifying K^+_{ATP} channel subunit) was paradoxically linked to an increase in channel activity (Béguin et al. 1999). Therefore, it appears that GIP is a physiological regulator of K^+_{ATP} channel-independent insulin secretion. The importance of cAMP and AA as signals in this GIP-mediated pathway has also not been explored.

Previous studies have led to the conclusion that glucose metabolism is necessary for GIP-mediated potentiation of insulin secretion, although the evidence for such glucose-dependence is largely based on *in vivo* and perfused organ studies (Brown et al. 1981; Mueller et al. 1982). Using βTC-3 cells, we sought to determine whether GIP was also capable of stimulating insulin secretion independently of the K^+_{ATP} channel, and whether this action was dependent on glucose metabolism. Glycolysis was bypassed by stimulating Krebs cycle turnover with the anaplerotic[1] substrates leucine, α-ketoisocaproate (KIC), and glutamine. These substrates are deemed "anaplerotic" as they can feed into Kreb's cycle intermediates (e.g. glutamine gets converted to α-ketoglutarate) via biochemical reactions to enhance Kreb's cycle flux and hence overall energy production. GIP was capable of stimulating insulin secretion in a concentration-dependent manner, independently from K^+_{ATP} channel closure.

[1] The strict definition of anaplerotic substrates includes only those which are converted directly into TCA cycle intermediates.

3.2 RESULTS

3.2.1 GIP stimulates arachidonic acid release

Initial studies were targeted at investigating GIP receptor signaling in an expression system, the rGIP-15 clone of CHO-K1 cells. Static incubations (45 min) revealed a concentration dependence to GIP-stimulated arachidonic acid production (Figure 3A). In agreement with published literature (Dickerson and Weiss 1995; Bymaster et al. 1999), ATP (5μM) increased AA release from rGIP-15 cells by greater than 200% ($p<0.01$, n=4). Parallel studies were performed in βTC-3 cells, a model of the pancreatic β-cell. These cells responded to arachidonic acid in a glucose-dependent manner (Figure 4). In the presence of glucose, AA potentiated insulin secretion at concentrations as low as 10 μM (Figure 4A), whereas 20-fold greater concentrations were required before a response was observed under glucose-free conditions (Figure 4C). The potentiation of insulin secretion elicited by 100 μM AA is comparable to that elicited by 100 nM GIP under 11 mM glucose conditions (compare Figure 4A vs. 10). Arachidonic acid had no effect on cAMP production in the presence of glucose while inhibiting it at higher concentrations in the absence of glucose (Figure 4B vs. D). GIP was found to stimulate AA release in a concentration dependent manner (Figure 3B & 3C). Interestingly, the EC_{50} value for GIP-stimulated AA release (1.4 nM ± 0.62 nM (n=3)) was similar to that for insulin release in these cells (data not shown; EC_{50} of 0.53 ± 0.23 nM (n=3)), in contrast to the 5-fold higher EC_{50} value for cAMP production (Hinke et al. 2000).

It is well established that the insulinotropic action of GIP is dependent on elevated glucose levels and that glucose induces activation of PLA_2 in pancreatic β-cells. However, we have recently shown that GIP receptor coupling to adenylyl cyclase in βTC-3 cells is independent of extracellular glucose concentrations (Hinke et al. 2000). In the current study, increases in ^{3}H-AA efflux stimulated by GIP were also found to be independent of extracellular glucose (Figure 3C), indicating that GIP-induced and glucose-induced increases in AA release were mediated via separate pathways.

Analysis of the time dependence of AA release in rGIP-15 cells demonstrated maximal release at 10 min (Figure 5A), which correlates well with that for GIP-stimulated cAMP production (data not shown). In contrast, GIP-induced AA release was not detected before 30 min of incubation in the βTC-3 cells (Figure 5B), and glucose-induced release was not observed until 60 min of incubation (Figure 5B).

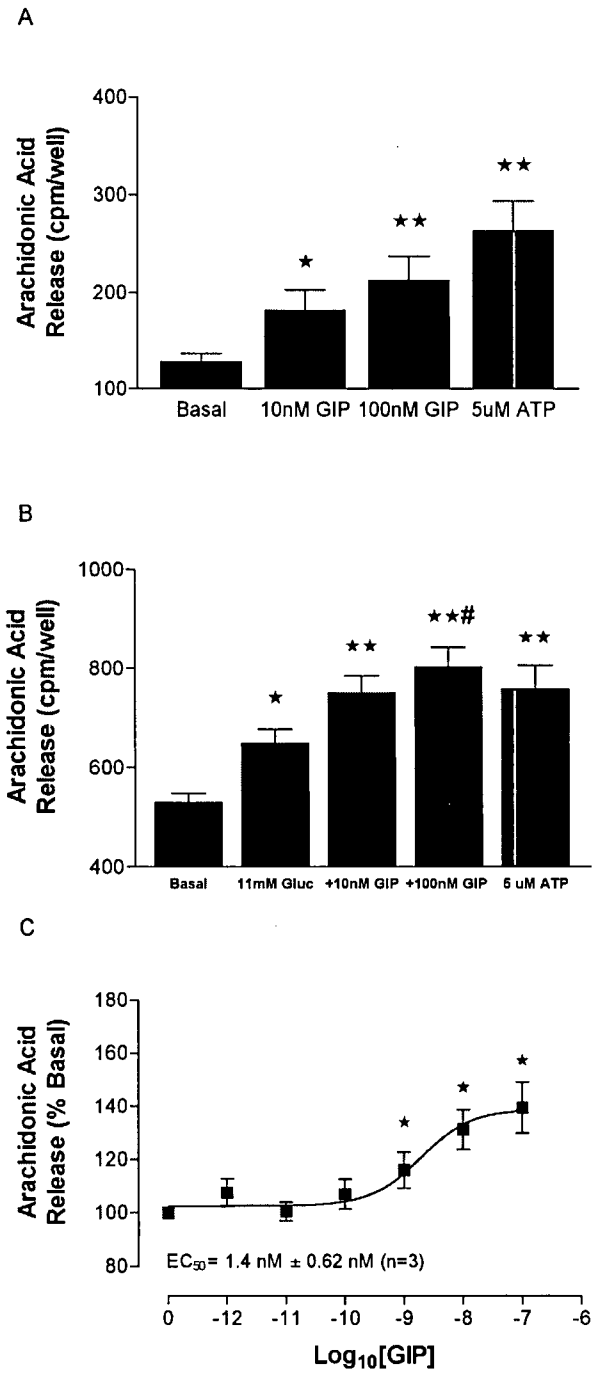


Figure 3: The effect of GIP on arachidonic acid release from rGIP-15 (A) and βTC-3 cells (B and C). Cells were prelabelled with [^{3}H]-AA for 36-48 h and preincubated in KRBH for 1 hour prior to the addition of agonists. Medium was removed at 45 min for (A) and 60 minutes for (B) and (C) and radioactivity measured by liquid scintillation counting. In (C), GIP stimulation was conducted under glucose-free conditions. For (A) n=4, (B) n=7-8, and (C) n=3-4, where * $p<0.05$, ** $p<0.001$ for basal vs. all; and # $p<0.05$ for 11 mM vs. 100 nM GIP as tested by ANOVA.

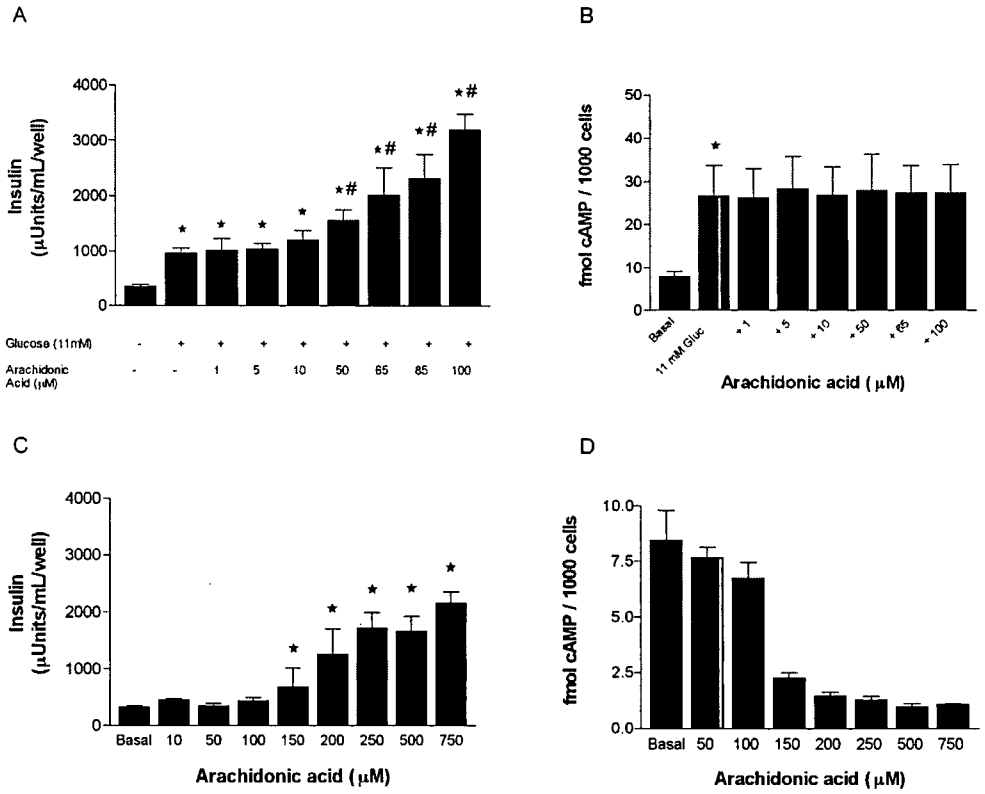


Figure 4: Effect of exogenous arachidonic acid on insulin release and intracellular cAMP under glucose-dependent (A, B) and independent (C, D) conditions in βTC-3 cells. Increasing concentrations of arachidonic acid were added to KRBH buffer containing zero (C, D, n=3-7) and 11 mM glucose (A, B; n=3-6). Insulin secretion and intracellular cAMP production were assessed by RIA, where * $p<0.05$ for basal vs. all; #$p<0.05$ for 11 mM vs. GIP as tested by ANOVA.

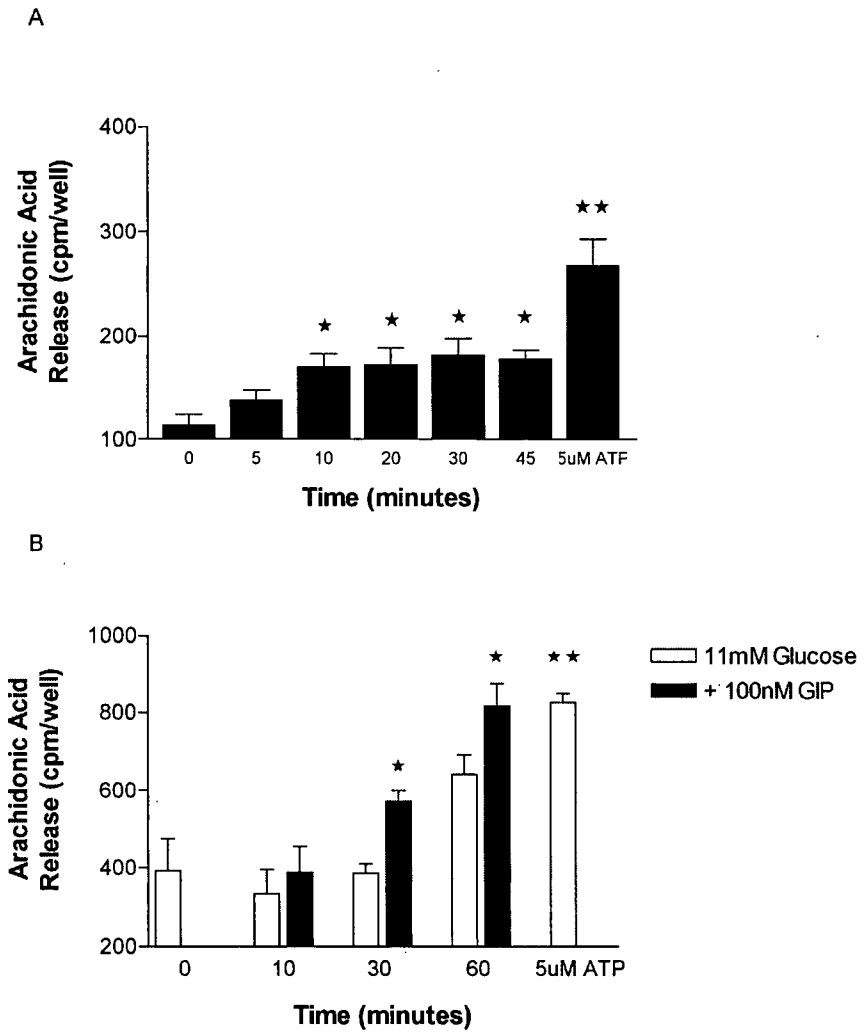


Figure 5: Time course analysis of GIP-stimulated arachidonic acid release in rGIP-15 (A) and βTC-3 cells (B). Media were removed at indicated time points and radioactivity was measured by liquid scintillation counting. Note that GIP stimulated AA release was evident by 30 min, however, no effect of glucose was observed by this point. For (A) n=4 and for (B) n=3-4, where * $p<0.05$.

3.2.2 GIP receptor coupling to arachidonic acid release

It was considered possible that these differences in onset of arachidonic acid release may reflect alternative GIP receptor-effector coupling systems in the two cell types. Since Gβγ has been previously implicated in the activation of phospholipase A_2 (Jelsema and Axelrod 1987), an inhibitor peptide of Gβγ, βARKct (β-adrenergic receptor kinase C-terminal tail), was transiently expressed in rGIP-15 cells. To confirm that cells had been transfected, GIP receptor internalization was monitored as Gβγ subunits have been shown to be required for G-protein receptor kinase (GRK) mediated GPCR internalization (Lin et al. 1998). Expression of βARKct was correlated with an inhibition of receptor internalization in these cells (data not shown). Initial experiments were conducted to examine GIP receptor binding and cAMP production in this expression model. βARKct expression was not found to have any significant effect on either receptor affinity for GIP or on activation of adenylyl cyclase (IC_{50} values for binding: 3.95 nM ± 0.91 (n=3) and 4.07 nM ± 0.97 (n=3) and EC_{50} values for cAMP production: 0.73 nM ± 0.12 (n=3) and 0.49 nM ± 0.09 (n=3) for vector and βARKct respectively). GIP receptors were shown, for the first time, to be capable of functionally coupling to AA production through Gβγ dimers, since the expression of βARKct significantly suppressed the GIP-mediated response by almost 70% (Figure 6, $p<0.05$). Purinergic receptors were also found to be coupled to AA production via Gβγ dimers, since βARKct expression reduced ATP-stimulated AA production by greater than 40%.

To characterize further the pathway by which AA is produced in the βTC3-cell by GIP, the effect of βARKct expression was investigated. To ensure transfection had occurred, cells were typically cotransfected with GFP (green fluorescent protein) as a marker of transfection efficiency. Inhibition of Gβγ action had no effect on glucose- or GIP-stimulated AA release (data not shown) or insulin secretion in βTC-3 cells (Figure 7). In addition, pertussis toxin (100 ng/mL and 500 ng/mL) had no effect on AA release, indicating that toxin sensitive G_α–proteins ($G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha q}$) do not play a role in glucose or GIP stimulated AA release in βTC-3 cells (data not shown). However, both the diterpene forskolin and the incretin GLP-1, agents that specifically elevate intracellular cAMP levels, were able to stimulate AA release (Figure 8), indicating that GIP may be acting on AA release via stimulation of adenylyl cyclase in the βTC3-cell. Interestingly, further examination using a specific PKA inhibitor, H89 (5 μM and 10 μM), has shown no effect on GIP or forskolin stimulated AA release (Figure 9). The possibility

that AA is a proximal intermediate to adenylyl cyclase activity can also be refuted since exogenous AA had either no effect or actually inhibited basal cAMP production in βTC3-cells (Figure 4B and 4C).

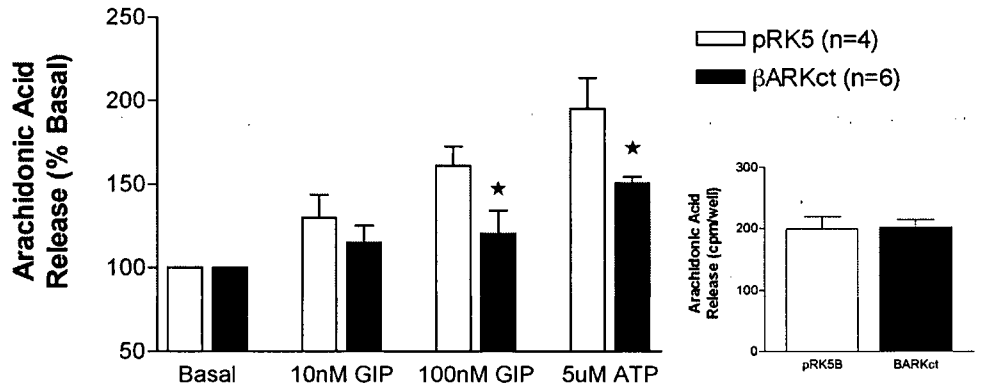


Figure 6: Effect of G-protein βγ inhibition on GIP-mediated arachidonic acid release in rGIP-15 cells. rGIP-15 cells expressing the GIP receptor were transiently transfected with 10 μg pRK5 vector or βARKct cDNA construct using Superfect™ reagent. Arachidonic acid efflux was quantified by liquid scintillation counting. The inset illustrates basal levels of AA release, where * $p<0.05$.

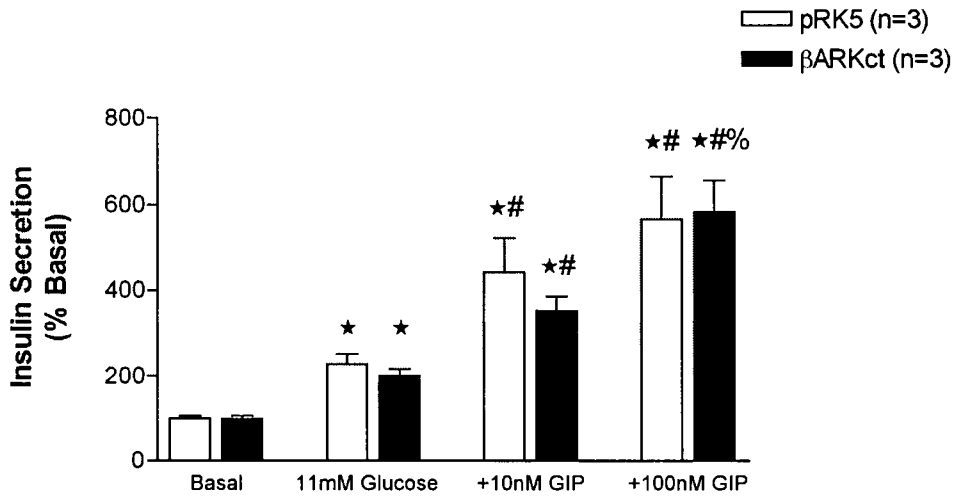


Figure 7: Effect of G-protein βγ inhibition on glucose and GIP-potentiated insulin secretion in βTC-3 cells. βTC-3 cells expressing the GIP receptor were transiently transfected with 10 μg pRK5 vector or βARKct cDNA construct using Superfect™ reagent. Insulin secretion was assessed by RIA and corrected for cell number by representation as % basal, where n =3 and * $p<0.05$ for basal vs. all; #$p<0.05$ for 11 mM vs. GIP, %$p<0.05$ for 10 nM GIP vs. 100 nM GIP as tested by ANOVA.

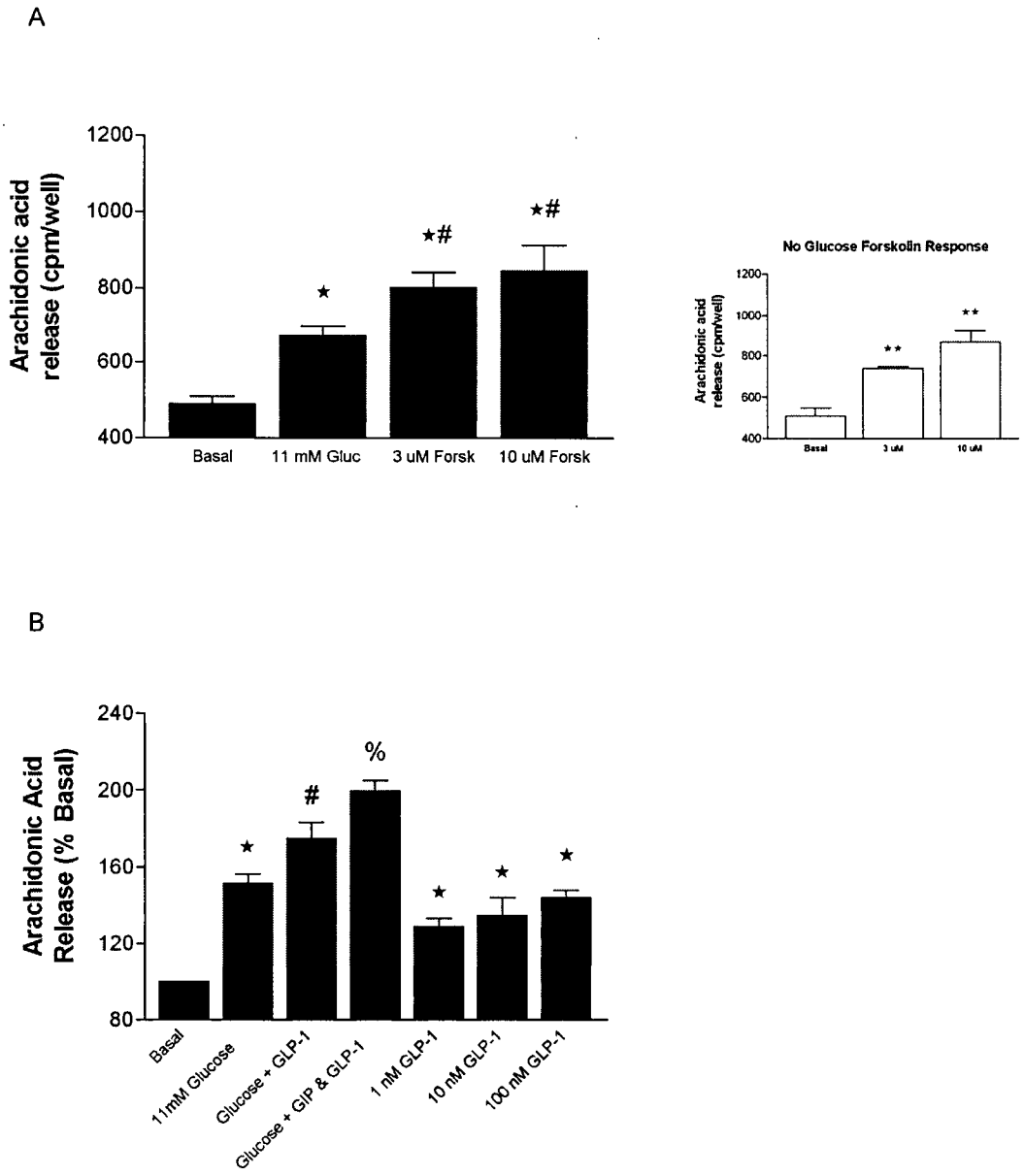


Figure 8: A role for cAMP signaling as a mediator of arachidonic acid release in βTC-3 cells. The ability of the diterpene forskolin, and the incretin GLP-1 to elicit arachidonic acid release was examined under glucose dependent (A; n=3-4, B; n=3-8) and independent (A inset; n=3, B; n=3-8) conditions, where * $p<0.05$, ** $p<0.001$ for basal vs. all, #$p<0.05$ for 11 mM vs. glucose + GLP-1, and %$p<0.05$ for glucose + GLP-1 vs. glucose + GLP-1 + GIP as tested by ANOVA. 100 nM GIP and GLP-1 were added concomitantly with 11 mM glucose.

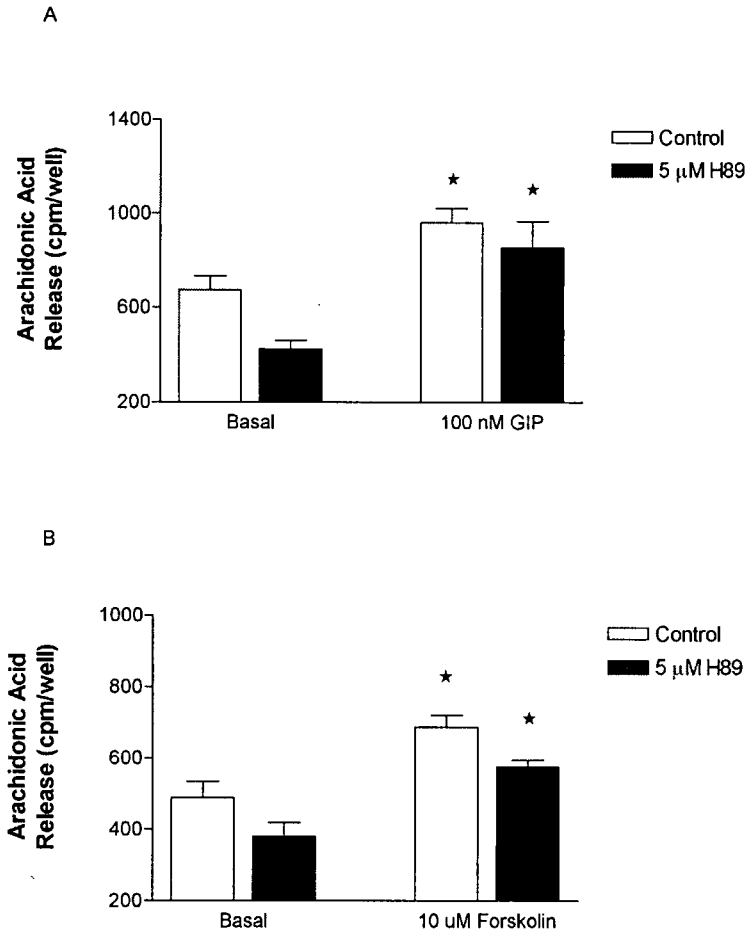


Figure 9: Effect of PKA Inhibition on GIP (A) and forskolin (B) mediated arachidonic acid release in βTC-3 cells. Cells were preincubated for 15 min in 5 μM H89 prior to and during experiments. Arachidonic acid efflux was quantified by liquid scintillation counting (n≥3), where * $p<0.05$.

3.2.3 A role for Ca^{2+}-independent PLA_2 in GIP-mediated insulin secretion from βTC-3 cells

Reduction of extracellular Ca^{2+} was found to have no affect on GIP stimulated AA release in βTC-3 cells, implying that a Ca^{2+}-independent mechanism was involved in the production of AA (Figure 10A). As predicted, neither glucose nor GIP were able to stimulate insulin secretion from βTC3-cells under stringent Ca^{2+}-free conditions (Figure 10B). However, GIP was clearly still capable of elevating cAMP levels despite a reduction in basal cAMP production (Figure 10C). The cAMP levels resulting from GIP-stimulation under Ca^{2+}-free conditions were, however, significantly suppressed compared to control conditions ($p<0.05$). The ability of GIP to release AA under Ca^{2+} free conditions suggested that a Ca^{2+}-independent PLA_2 was involved. An inhibitor specific for $iPLA_2$, HELSS (haloenol lactone suicide substrate), has previously been shown to inhibit glucose stimulated AA production and insulin secretion in several β-cell models (Gross et al. 1993; Ramanadham et al. 1993, 1997). In the present study, HELSS was found to inhibit GIP-stimulated AA production as well as glucose- and GIP-stimulated insulin secretion (Figure 11), supporting the aforementioned hypothesis, that the enzyme coupled to GIP receptor signaling is a Ca^{2+}-independent PLA_2.

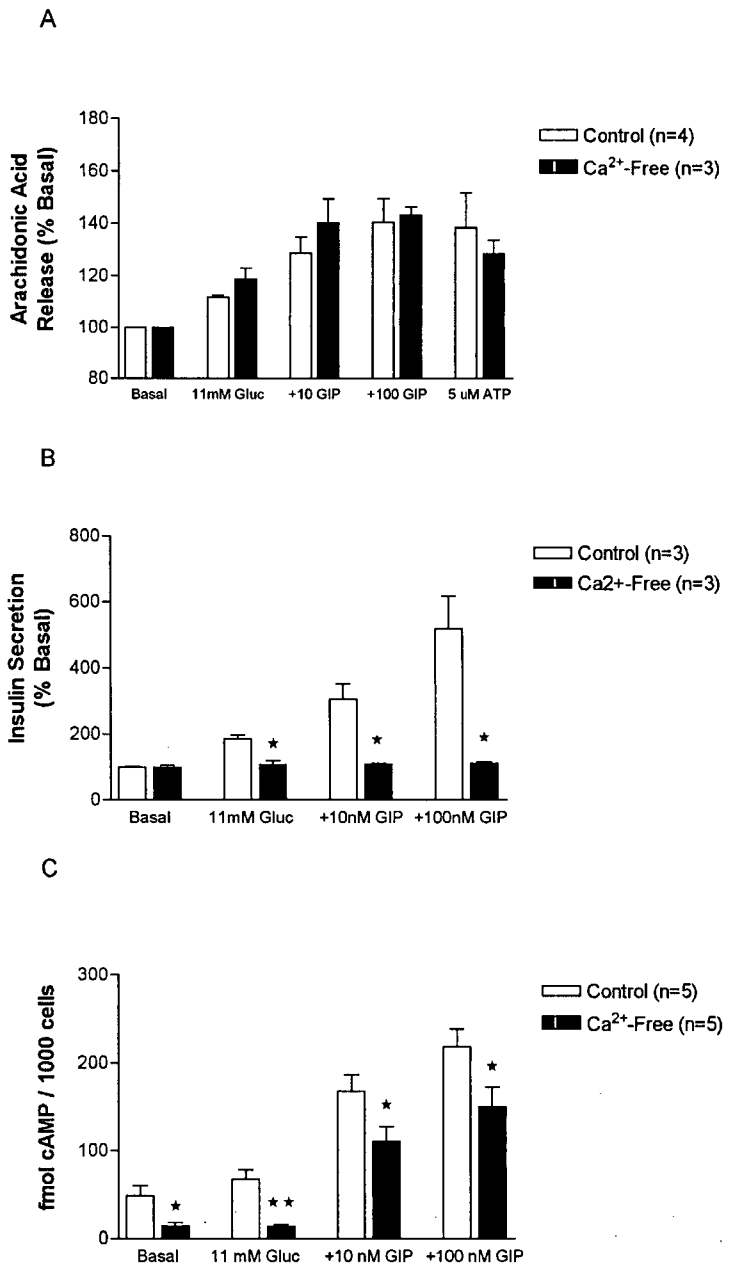


Figure 10: Effect of Ca^{2+}-free extracellular media on GIP-mediated arachidonic acid release (A), insulin secretion (B), and cAMP production (C) in βTC-3 cells. Ca^{2+}-free Krebs-Ringer buffer contained equimolar $MgCl_2$ to replace $CaCl_2$, and was supplemented with 10 mM EGTA. Arachidonic acid efflux was quantified by liquid scintillation counting (n=3-4), and insulin (n=3) and cAMP levels (n=5) were determined by RIA, where * $p<0.05$, ** $p<0.001$.

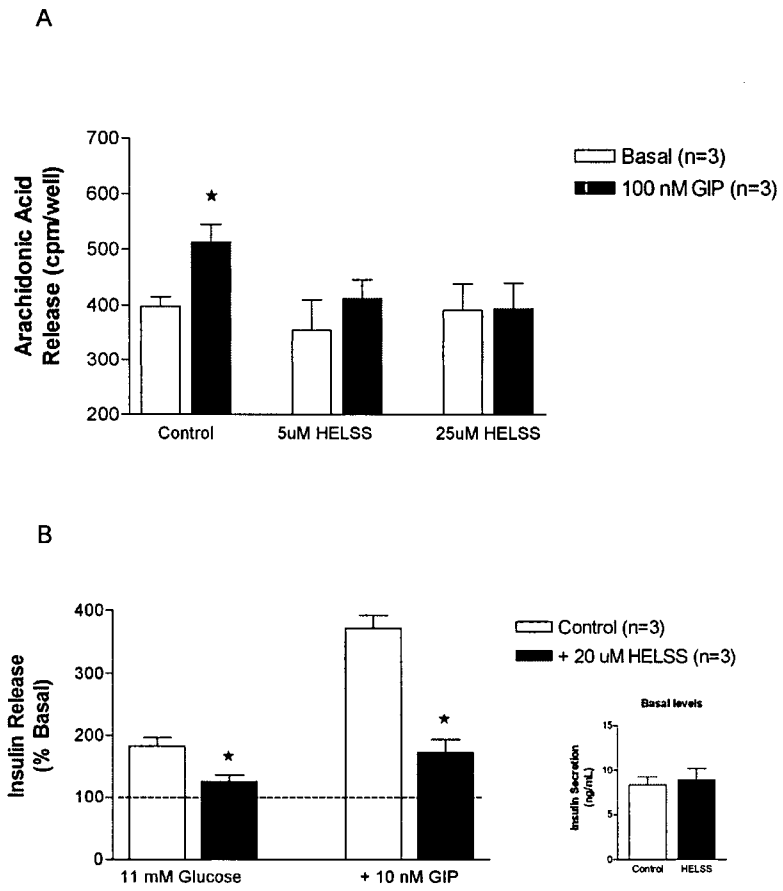


Figure 11: Effect of Ca^{2+}-independent PLA_2 inhibition on GIP-mediated arachidonic acid release (A) and insulin secretion (B) in βTC-3 cells. Cells were preincubated with the inhibitor, HELSS, for 30 min prior to stimulation and washed with KRBH before the addition of glucose and GIP. The inset (B) represents basal insulin secretion levels under control and test conditions, where * $p<0.05$.

3.2.4 GIP stimulates insulin secretion independent of K^+_{ATP} channel closure in βTC-3 cells

Prior to studying K^+_{ATP} channel-independent effects in βTC-3 cells, it was established that diazoxide completely inhibited both glucose-stimulated insulin secretion (IC_{50} = 27.9 ± 6.2 μM (n=3), Figure 12A) and GIP potentiation of responses to glucose (Figure 12B). Thus, based on the measured IC_{50}, and the current literature (Sato and Henquin 1998), 250 μM diazoxide was used in all subsequent experiments to ensure K^+_{ATP} channels remained open.

Membrane depolarization, by elevation of extracellular K^+ (K^+_o) to 30 mM, in the presence of diazoxide increased insulin secretion 4.3 ± 0.3 fold (n=3) from a basal level of 0.48 ± 0.13 μU/1000 cells (n=3). Figure 13 illustrates that, under these conditions, concentration-dependent responses to GIP were still sigmoidal for insulin secretion (EC_{50} = 7.3 ± 1.6 nM (n=4)) and cAMP production (EC_{50} = 15.7 ± 3.4 nM (n=3)). These findings therefore demonstrate that GIP potentiates insulin secretion independently of the K^+_{ATP} channel, by a pathway that may involve cAMP. It is interesting to note that 11 mM glucose was unable to potentiate insulin secretion in a K^+_{ATP} channel-independent manner in βTC-3 cells (Figure 13A). However, we assumed that glucose metabolism was still necessary for GIP to potentiate insulin secretion independently of the K^+_{ATP} channel.

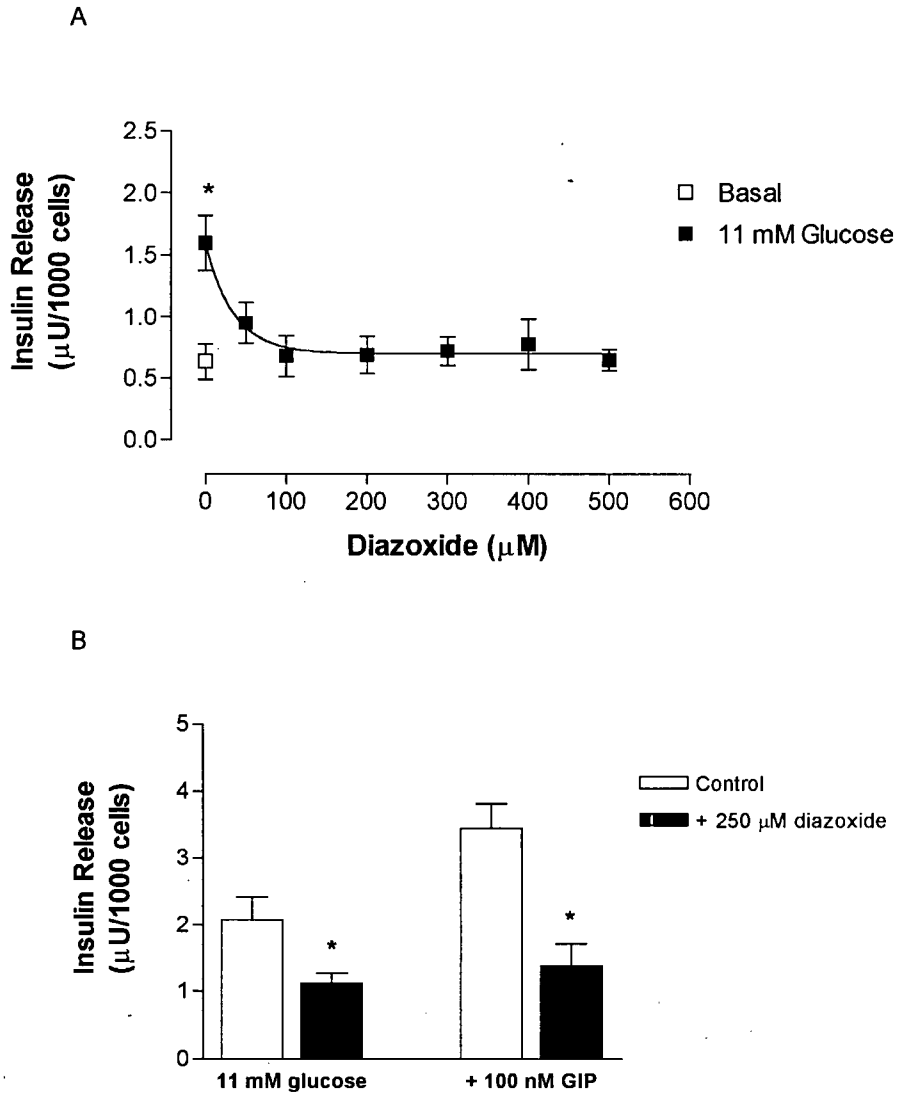


Figure 12: Diazoxide inhibits 11 mM glucose (A) and GIP stimulated (B) insulin secretion in βTC-3 cells. A concentration-response curve for diazoxide was generated to determine the concentration necessary to inhibit K_{ATP}-channel mediated insulin secretion (A). Based on the IC_{50} (27.9 ± 6.2 μM (n=3)) and previous studies, cells were preincubated with 250 μM diazoxide in all subsequent experiments. The effects of diazoxide were also found to inhibit GIP potentiation of glucose-stimulated insulin secretion (B). Data are expressed as mean ± S.E.M. for experiments performed in triplicate; A (n=3) and B (n=3).

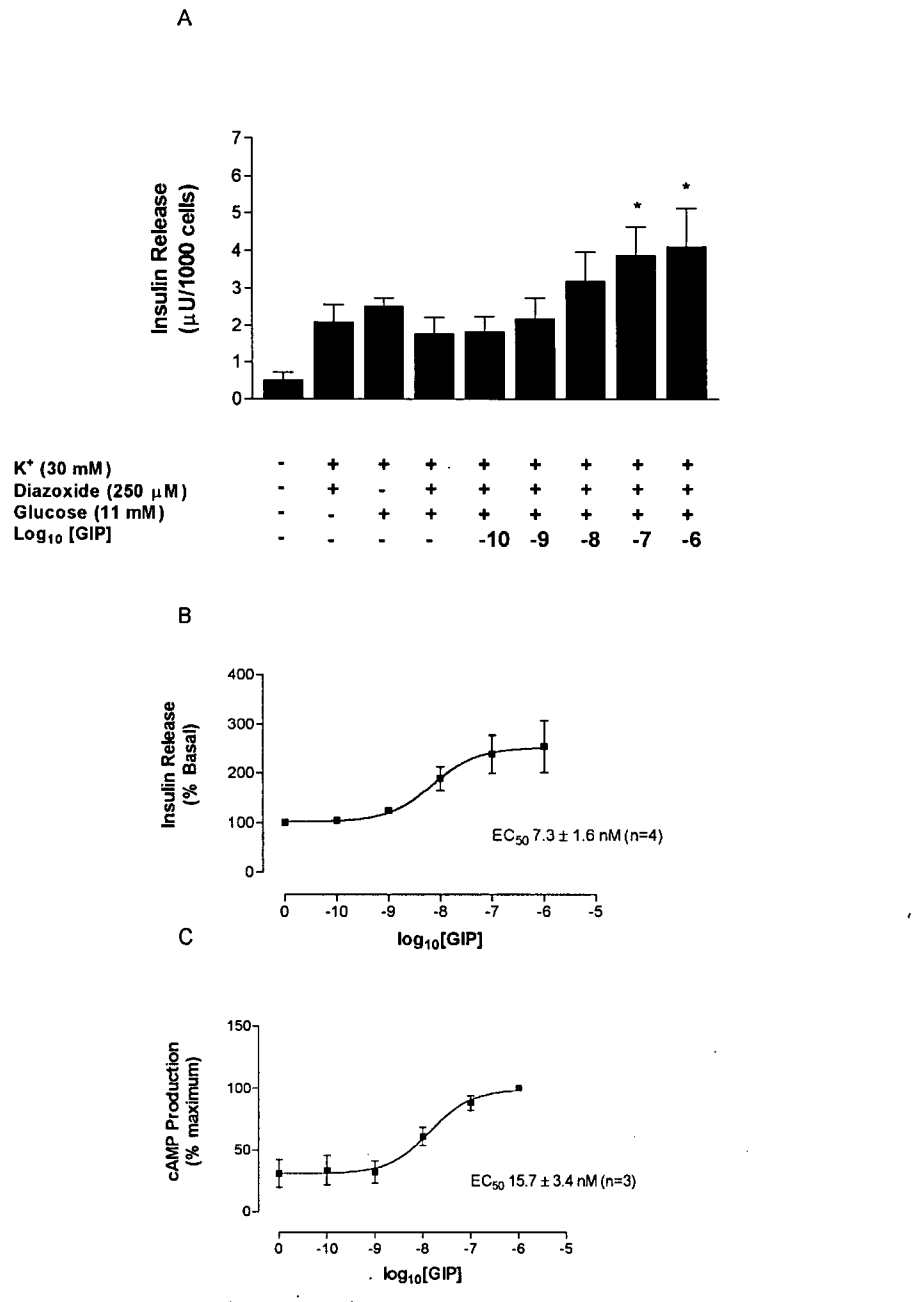


Figure 13: K^+_{ATP} channel-independent pathway of GIP potentiated insulin secretion (A, B) and cAMP production (C). A GIP concentration response curve (B) was constructed from (A), where basal ("0") is representative of insulin release in the presence of 30 mM K^+_o, 250 μM diazoxide, and 11 mM glucose. cAMP data were normalized to % maximum and data presented as mean ± S.E.M. for experiments performed in triplicate; A (n=4), B (n=4), and C (n=3).

3.2.5 GIP and Krebs cycle flux in regulating insulin secretion from βTC-3 cells

Subsequent experiments were targeted at identifying whether GIP could stimulate insulin secretion in the absence of glucose, but in the presence of anaplerotic secretagogues; and ultimately, whether Krebs cycle turnover was required for the K^+_{ATP} channel-independent actions of GIP. Investigations were therefore conducted in zero glucose modified Krebs ringer (KRBH) and in the presence of the anaplerotic substrates leucine, α-ketoisocaproic acid (KIC) or glutamine in various combinations. This necessitated the preincubation of βTC-3 cells for 1 hour in 0 mM glucose KRBH in order to make the cells metabolically (glycolytically) quiescent and thus ablate responses to GIP over a nM to μM range (data not shown).

Concomitant addition of 1 mM leucine and 1 mM glutamine to βTC-3 cells previously incubated in 0 glucose KRBH increased insulin secretion to levels similar to those achieved with 11 mM glucose (2.3 ± 0.3 fold basal (n=6) vs. 2.6 ± 0.2 fold basal (n=3) respectively). As illustrated in Figure 14A, GIP potentiated leucine- and glutamine-induced insulin release in a concentration-dependent manner, with an EC_{50} of 2.4 ± 1.3 nM (n=6) and maximum increase of 2.7 ± 0.4 fold basal (n=6). A further glycolysis-bypassing secretagogue that feeds into the Krebs cycle, KIC, was used instead of leucine to confirm these findings (Figure 15A). In the presence of 1 mM KIC, GIP (10 nM) potentiated insulin secretion 2.1 ± 0.6 fold (n=3). Similar levels of potentiation were achieved when glutamine (1.9 ± 0.6 fold (n=3)) or KIC plus glutamine (2.2 ± 0.8 fold (n=3)) were used as secretagogues.

The effects of leucine, glutamine and KIC on cAMP formation, in the presence and absence of GIP were concomitantly studied. As depicted in Figures 14C and 15B, only glutamine alone (2.8 ± 0.2 (n=5) fold basal of 12.8 ± 2.9 fmol cAMP/1000 cells) or in combination with leucine or KIC was able to stimulate cAMP production in clonal βTC-3 cells. Such levels of stimulation were also obtained with 11 mM glucose in previous studies (Ehses et al. 2001). Most notably, the potentiation of cAMP production by GIP was completely nutrient-independent.

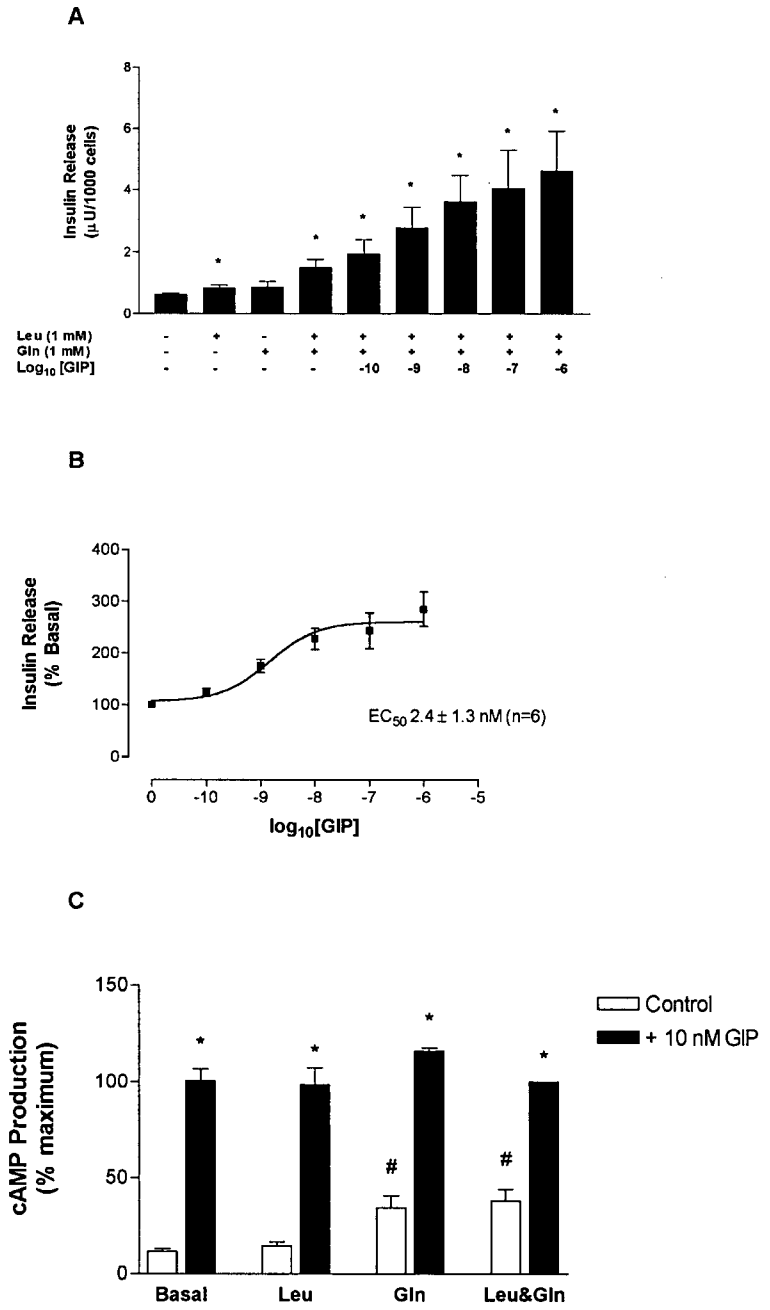


Figure 14: Leucine (Leu) and/or glutamine (Gln) potentiated insulin release (A, B) and cAMP production (C) by GIP in βTC-3 cells. Cells were stimulated with 1 mM Leu and/or 1 mM Gln with or without increasing concentrations of GIP for 60 min as outlined in Materials and Methods. Graph (B) was constructed from (A) with the "0" point representing insulin secretion in the presence of 1 mM Leu and Gln. The cAMP data were normalized to % maximum, where * $p<0.05$ compared to respective controls and # $p<0.05$ compared to respective basal control. Data represent mean ± S.E.M. for experiments done in triplicate; A (n=3-6), B (n=6), and C (n=5).

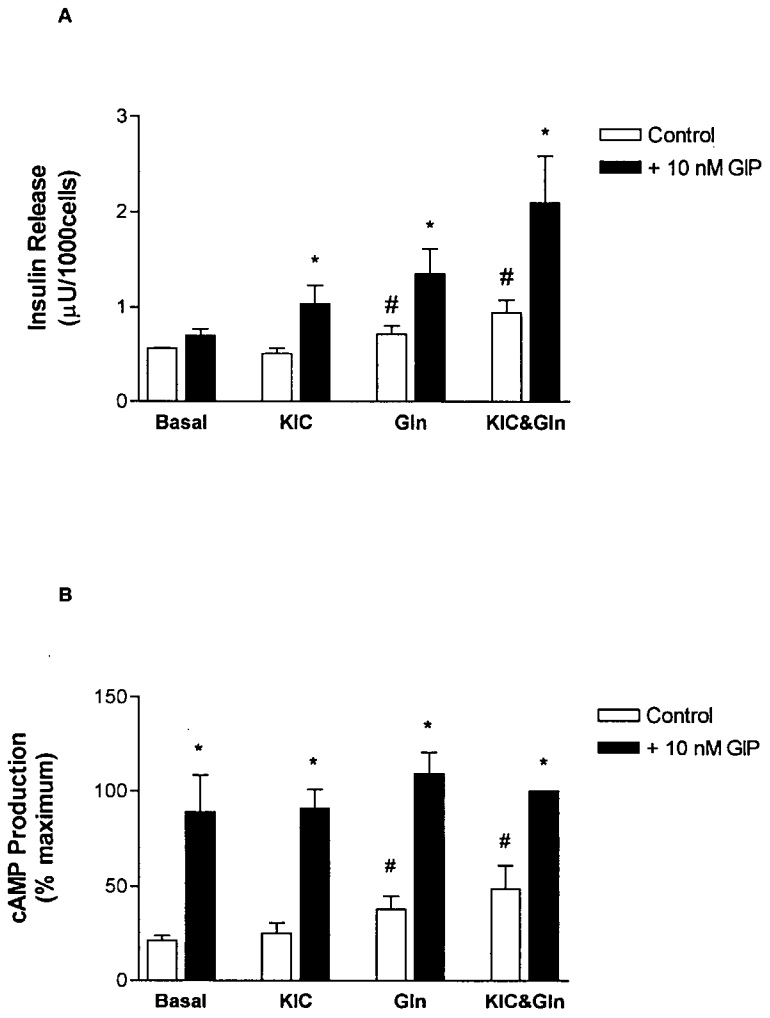


Figure 15: α-Ketoisocaproate (KIC) and/or glutamine (Gln) potentiated insulin release (A) and cAMP production (B) by GIP in βTC-3 cells. Cells were stimulated with 1 mM KIC and/or 1 mM Gln with or without 10 nM GIP as outlined in Materials and Methods. * $p<0.05$ compared to respective controls and # $p<0.05$ compared to respective basal control, where data represent mean ± S.E.M. for experiments performed in triplicate; A (n=3) and B (n=3-4).

3.2.6 GIP can potentiate insulin secretion in a K^+_{ATP} channel and nutrient independent manner from βTC-3 cells

Our final aim was to establish which proximal signals were responsible for GIP-mediated, K^+_{ATP} channel-independent insulin secretion and investigate their dependence on Krebs cycle flux. Since the production of both cAMP and arachidonic acid are stimulated by GIP independently of glucose in βTC-3 cells (Hinke et al. 2000; Ehses et al. 2001), we sought to determine whether insulin responses to these second messengers were dependent on Krebs cycle flux (as were GIP actions; Figures 14 & 15). Forskolin and arachidonic acid potentiated insulin secretion was minimal in the absence of either glutamine, or glutamine plus leucine or KIC (Figure 16). Therefore, Figure 16 clearly demonstrates that the ability of both forskolin and exogenous arachidonic acid to potentiate insulin secretion under normal conditions is dependent on Krebs cycle turnover. These data support the proposal that both cAMP and arachidonic acid are integral to GIP-mediated insulin release.

Finally, it was investigated whether Krebs cycle turnover was necessary for GIP to potentiate K^+_{ATP} channel-independent insulin secretion. Intriguingly, 10 nM GIP was found to stimulate insulin release in the complete absence of nutrients, when K^+_{ATP} channels were held open with diazoxide and Ca^{2+} influx was restored with high extracellular K^+ (Figure 17 "Basal"). These actions were mimicked by 1 μM forskolin, however they were not evident with arachidonic acid (Figure 17). These data, suggest that cAMP is the main second messenger responsible for nutrient and K^+_{ATP} channel-independent actions of GIP. Although arachidonic acid was capable of amplifying insulin secretion independently of K^+_{ATP} channels in the presence of anaplerotic substrates, it was unable to elicit release in the absence of nutrients (Figure 17), and clearly requires Krebs cycle flux to elicit insulin secretion under these conditions. Interestingly, similar to glucose, the anaplerotic substrates tested did not consistently stimulate insulin secretion in the absence of K^+_{ATP} channel closure (Figure 17). Furthermore, leucine and KIC alone were actually found to suppress the ability of GIP to potentiate insulin exocytosis under these conditions ($p<0.05$; $n=4$), while also slightly affecting forskolin actions (Figure 17).

GIP was also capable of stimulating insulin secretion and cAMP production in a concentration-dependent manner in the absence of nutrients, under K^+_{ATP} channel-independent conditions (EC_{50} values for insulin and cAMP 5.2 ± 3.6 nM ($n=3$) and 6.4 ± 5.5 nM ($n=3$) respectively). Since cAMP actions have previously been shown to amplify the exocytotic effects

of elevated intracellular Ca^{2+} (Ämmälä et al. 1993), we investigated whether the present actions of GIP were also Ca^{2+}-dependent, and whether they could be blocked by administration of the PKA inhibitors H89 and Rp-cAMPS (adenosine 3,5-cyclic phosphorothioate-Rp). In agreement with this proposal, GIP-induced insulin secretion was found to be Ca^{2+}-dependent (Figure 18A) and mediated by activation of PKA (Figure 18A & B), thus establishing a nutrient-independent mechanism of action for GIP stimulated insulin secretion in mouse βTC-3 cells.

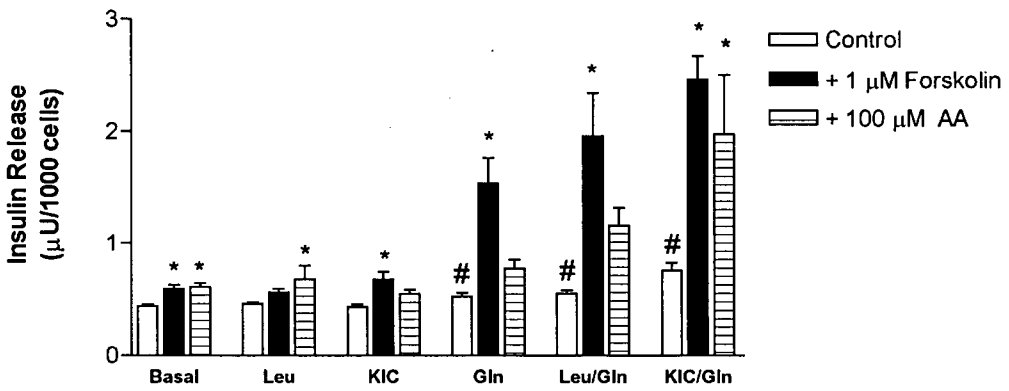


Figure 16: The role of Krebs cycle flux in cAMP and arachidonic acid potentiated insulin secretion. All anaplerotic substrates were added at a concentration of 1 mM concurrently with forskolin or arachidonic acid. Data represent mean ± S.E.M. for experiments done in triplicate, where * $p<0.05$ compared to respective controls and # $p<0.05$ compared to respective basal control; n=3-15.

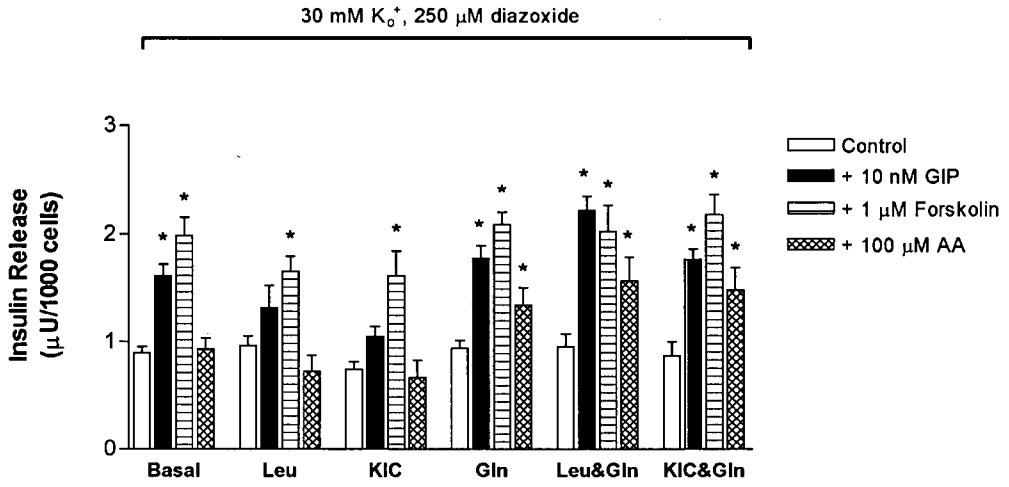


Figure 17: The role of Krebs cycle flux in K^+_{ATP} channel-independent stimulation of insulin secretion by GIP, cAMP, and arachidonic acid. Cells were preincubated in diazoxide, which was replaced by test buffer containing high K^+_o and diazoxide for subsequent assessment of insulin release. All anaplerotic substrates were added at a concentration of 1 mM, and data are representative of experiments done in triplicate and presented as mean ± S.E.M (n=3-16); * $p<0.05$ compared to respective controls.

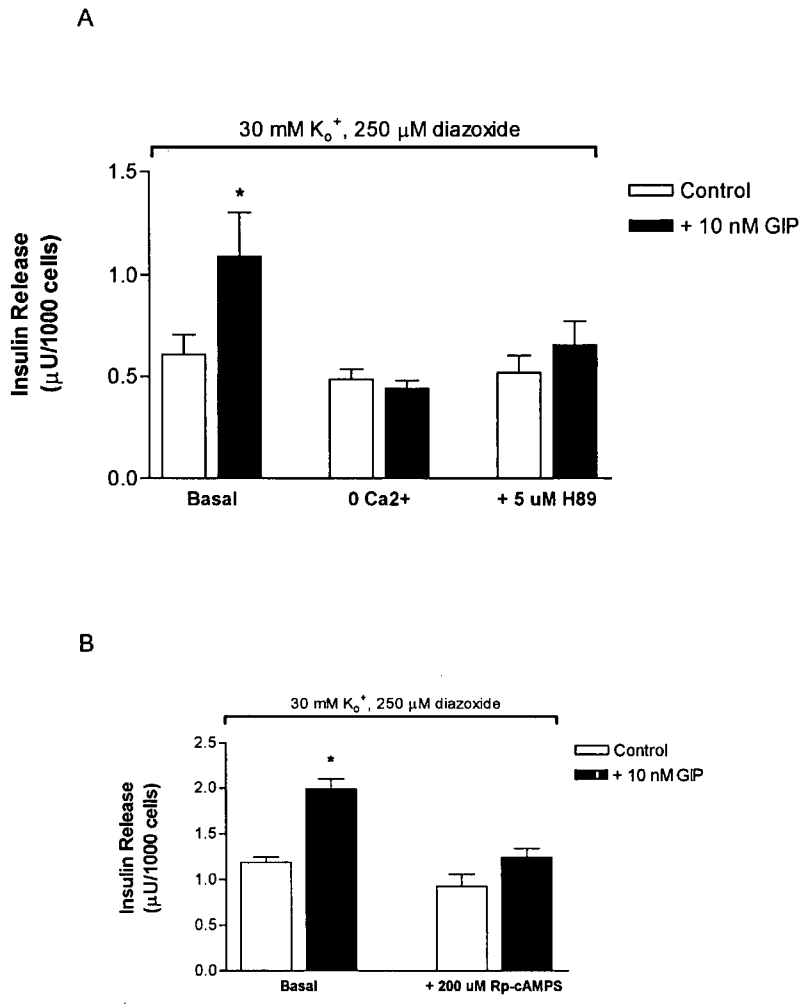


Figure 18: The role of extracellular Ca^{2+} (A) and PKA (A, B) in the nutrient-independent effects of GIP on insulin secretion. Basal refers to control test buffer (modified KRBH containing high K^+_o and diazoxide), whereas 0 Ca^{2+} was achieved by replacing $CaCl_2$ with equimolar $MgCl_2$ and supplementing with 10 mM EGTA. Cells were preincubated for 15 min in 5 μM H89 (A) and 200 μM Rp-cAMPS (B) to inhibit PKA activity, and the inhibitor was also present during the stimulation. Data represent experiments done in triplicate expressed as mean ± S.E.M (n=4).

3.3 DISCUSSION

3.3.1 GIP-stimulated arachidonic acid release

In human type 2 diabetes there is a decreased insulin response to GIP that is of unknown etiology. One possible underlying defect is in the normal signal transduction pathways by which GIP stimulates insulin secretion in β-cells. At the time this study was conducted, it been established that GIP stimulates adenylyl cyclase (Siegel and Creutzfeldt 1985), increases iCa^{2+} (Lu et al. 1993B) and activates MAP kinase (Kubota et al. 1997). The study outlined in this chapter was undertaken to identify alternate mechanisms by which GIP regulates beta cell function. We have shown that GIP receptors in βTC-3 cells and transfected CHO-K1 cells are capable of coupling to transduction systems that release arachidonic acid from membrane lipids via activation of a Ca^{2+}-independent phospholipase A_2. Additionally, this signaling pathway was shown to involve G-protein βγ coupling in CHO-K1 cells whereas a cyclic AMP-mediated pathway is probably involved in βTC-3 cells (Figures 6 and 7).

Initial studies of GIP-stimulated AA release revealed a marked difference in the time dependence of AA release between CHO-K1 and βTC-3 cells. The much more rapid release evident in rGIP-15 cells is in agreement with previously observed AA production rates observed with rhodopsin and muscarinic receptors expressed in CHO-K1 cells (Dickerson and Weiss 1995; Bymaster et al. 1999) and other cell types (Shuttleworth 1996; Sauvadet et al. 1997; Balsinde et al. 2000). However, coupling of GIP to AA release was much slower in βTC-3 cells suggesting a unique GIP receptor-AA coupling mechanism. There was also a difference between GIP- and glucose-induced AA release in βTC-3 cells, with GIP initiating release by 30 min, whereas glucose had no effect by this time (Figure 5). This suggests that separate mechanisms couple glucose and the GIP receptor to AA production. Extensive studies have established that the glucose-induced AA production involved in beta cell insulin secretion (Turk et al. 1993; Simonsson and Ahren 2000) involve activation of an ATP sensitive, Ca^{2+}-independent PLA_2 (Ramanadham et al. 1994; Simonsson and Ahren 2000). This enzyme has been identified in a number of insulinoma cell lines, including βTC-3 cells (Ramanadham et al. 1997), and further studies were therefore performed to determine whether GIP-induced AA release also resulted from its activation.

The C-terminal fragment of the β-adrenergic receptor kinase protein (βARKct or G-protein receptor kinase 2; GRK2) was utilized to study the role of Gβγ signaling. Jelsema and

Axelrod (1987) first suggested that activation of PLA_2 can be performed by $G\beta\gamma$ subunits (Jelsema and Axelrod 1987). In the present study it was found that the GIP receptor can couple to PLA_2 via G-protein $\beta\gamma$ subunits in CHO-K1 cells, whereas neither glucose nor GIP-stimulated arachidonic release or insulin secretion were dependent on $G\beta\gamma$ subunit signaling in βTC-3 cells (Figure 6). This is in contrast to their involvement in K^+ and bombesin-stimulated insulin secretion in HIT-T15 cells (Zhang et al. 1998). Further studies are needed to determine whether $G\beta\gamma$ subunits are involved in GIP receptor–effector coupling in other targets such as the stomach, fat or adrenal gland (McIntosh et al. 1981; Lacroix et al. 1992; McIntosh et al. 1999; Miyawaki et al. 2002).

Glucose-, GIP-, and ATP-stimulated AA release were all shown to be independent of extracellular Ca^{2+}, indicating that they are likely acting on a similar $iPLA_2$ isoform. Recently cholecystokinin, also an insulinotropic peptide, was also shown to activate islet PLA_2 independently of extracellular Ca^{2+} (Simonsson et al. 2000). Despite a complete ablation of insulin release under Ca^{2+} free (extracellular) conditions, intracellular cAMP levels were still stimulated by GIP (Figure 10C), implying this may be the proximal messenger to AA release. A reduction in basal cAMP production is likely attributable to a decrease in basal Ca^{2+}-activated adenylyl cyclase activity, therefore accounting for the reduction in GIP stimulated cAMP levels. From these observations and those previously reported by our laboratory, it can thus be concluded that both GIP stimulated cAMP and AA production are proximal signaling events independent of glucose and extracellular Ca^{2+}, but insufficient to elicit insulin exocytosis. However, these signaling intermediates may play a more direct role in the actions of GIP under euglycemic conditions, such as those in the adipocyte (McIntosh et al. 1999; Miyawaki et al. 2002). In fact, we also have evidence for the stimulation of ^{3}H-AA release by GIP from 3T3-L1 cells, and 3T3-L1 cells overexpressing the GIP receptor (EC_{50} = 4.1 ± 3.4 nM for endogenous and 0.78 ± 0.19 nM for overexpression model, n=3, $p<0.05$).

In islets, glucose stimulation can elevate endogenously generated AA from the micromolar range to cellular concentrations of 50 to 200 μM, as measured by mass spectrometry (Simonsson and Ahren 2000). In agreement with work published by Metz (Metz 1988), exogenous AA over this range was able to stimulate insulin release from βTC-3 cells in the presence of glucose. However, in its absence, responsiveness to AA was reduced at least 10-fold. Interestingly, application of exogenous AA has been shown to elevate iCa^{2+} concentrations in pancreatic islets (Metz 1988) and there is considerable evidence suggesting a role for

arachidonic acid itself or its metabolites in the regulation of capacitive and non-capacitive Ca^{2+} influx in a number of cellular systems (Rzigalinski et al. 1999; Osterhout and Shuttleworth 2000). Thus, it is tempting to speculate that fluxes in free endogenous AA, brought about by GIP, may play an integral role in regulating iCa^{2+} concentrations and thereby influence insulin secretion.

The studies in this Chapter indicate that the ability of GIP to stimulate PLA_2 activity probably occurs via cAMP actions in the beta cell. Since the specific $iPLA_2$ inhibitor HELSS ablated insulin responses to glucose and thus the potentiating effect of GIP (Figure 11), the converging actions on insulin secretion of these two secretagogues may occur distally to the formation of cAMP (by GIP) and arachidonic acid (by glucose and/or GIP). Arachidonic acid and/or its metabolites may therefore be mainly involved in the fine tuning of the insulin response. The actions of cAMP could be direct, via activation of small G-proteins (e.g. Rap), or through a guanine nucleotide exchange factor (GEF), however the involvement of PKA is unlikely (Figure 9). These results are in contrast to a recent study demonstrating an inhibitory effect of cAMP and incretins (GIP and GLP-1) on CCK-8 stimulated arachidonic acid production and insulin release in the rodent islet (Simonsson et al. 2000). However, implicit in studies conducted with isolated islets is the existence of paracrine and endocrine interaction between α-, δ-, and PP cells that contribute to a functional response. This may account for the different responses observed in the clonal cell line used in the present study.

Finally, these studies assessed the production of AA by measuring total radioactivity secreted from βTC-3 cells. Although it has been shown in studies on tumor β-cell lines that a surprisingly small percentage of released radioactivity consists of metabolites (Simonsson and Ahren 2000), a recent study suggested a role for lipooxygenase-12 metabolites in β-cell function (Owada et al. 1999). Further studies need to be conducted to discriminate between AA and its metabolites produced by GIP stimulation of the beta cell.

We have recently observed that both GIP-stimulated cAMP (Hinke et al. 2000) and AA production in βTC-3 cells are independent of extracellular glucose containing media (Figure 3). Thus, generation of these messengers is glucose-independent, however, both are glucose-dependent in their ability to potentiate insulin secretion. Therefore, further studies were initiated to clarify the role of Krebs cycle flux in GIP actions on insulin secretion, with a focus on these two second messengers. Moreover, the role of these proximal signals in the K^+_{ATP} channel-independent actions of GIP on insulin secretion were also investigated.

3.3.2 K^+_{ATP} channel and glucose-independent insulin secretion by GIP

Following the discovery of an ATP sensitive K^+ channel in the pancreatic β-cell (Cook and Hales 1984), it has been widely considered to be the central component by which glucose stimulates insulin secretion (Ashcroft 2000). However, it was demonstrated subsequently that when the K^+ channel was held open with diazoxide, and intracellular Ca^{2+} levels were elevated by depolarizing the cell with a high concentration of KCl, glucose was still capable of stimulating insulin secretion (Gembal et al. 1992; Sato et al. 1992). These results were supportive of earlier studies demonstrating that glucose was still capable of stimulating insulin secretion even in the presence of sulfonylureas, drugs that close the K^+_{ATP} channel (Loubatieres-Mariani et al. 1973; Henquin 1980). The physiological importance of K^+_{ATP} channel-independent glucose actions in the β-cell was clearly demonstrated in mice lacking either the Kir6.2 (Miki et al. 1998) or SUR1 (Seghers et al. 2000) subunits of the K^+_{ATP} channel, since both maintain some ability to secrete insulin in response to glucose, and glucose intolerance never develops under regular feeding in these animals.

Discussion concerning the triggering versus amplifying (potentiation) effects of glucose on insulin release has recently come to the forefront after being proposed by Grodsky some 30 years ago (Grodsky 1972; Henquin 2000). The triggering action of glucose and nutrients on insulin secretion is considered to be due to the closure of K^+_{ATP} channels and the subsequent elevation of $[Ca^{2+}]_i$. This K^+_{ATP} channel-dependent event is regarded as the physiological stimulus for first phase insulin secretion. Non-fuel secretagogues (such as incretins), are then able to augment insulin release further through second-messenger mediated actions which are dependent on this $[Ca^{2+}]_i$ increase. These events may represent the mechanism of second phase insulin release, and are furthermore considered to be K^+_{ATP} channel-independent (Komatsu et al. 2002).

Despite the strong evidence for a K^+_{ATP} channel-independent action of glucose, it was found that in βTC-3 cells, glucose (and anaplerotic substrates) stimulates insulin secretion mainly via its effects on the K^+_{ATP} channel (Figure 12 and 13). This may be due to the elevated hexokinase, versus glucokinase, expression levels in βTC-3 cells influencing downstream glucose metabolism and signaling (D'ambra et al. 1990; Liang et al. 1996). Alternatively, it may also reflect an exaggerated constitutive pathway of insulin secretion in βTC-3 cells (Nagamatsu and Steiner 1992).

It has been proposed that incretin stimulation of the β-cell accounts for 50-70% of the postprandial insulin response to glucose (Nauck et al. 1993A). Both GIP and glucagon-like peptide-1 (GLP-1) are known to increase intracellular cAMP production and they have therefore been proposed to potentiate glucose-induced insulin secretion by acting at the level of the K^+_{ATP} channel via activation of PKA (Ding and Gromada 1997; Gromada et al. 1998). A recent report, however, demonstrated that PKA phosphorylation of the β-cell K^+_{ATP} channel (Serine 372 of Kir6.2 subunit) mediated by GIP was paradoxically correlated with an increase in channel activity, suggesting that GIP must also act via K^+_{ATP} channel-independent pathways. The current study on mouse βTC-3 cells establishes that such actions do indeed exist, and a similar situation may exist in humans since sulfonylurea administration was shown to potentiate the effects of GIP on insulin secretion in patients with type 2 diabetes mellitus (Meneilly et al. 1993). This study is analogous to those showing that glucose could still stimulate insulin secretion in the presence of maximal K^+_{ATP} channel block via sulfonylureas (Loubatieres-Mariani et al. 1973; Henquin 2000). Thus, GIP controls insulin secretion by a mechanism not restricted to the inhibition of the K^+_{ATP} channel.

GIP receptor activation also potentiates insulin secretion when glucose is replaced by the anaplerotic substrates leucine, KIC, glutamine, or combinations thereof (Figure 13). Leucine and KIC are metabolized into acetyl-CoA and thereby feed into the Krebs cycle, where they increase the cytoplasmic ATP:ADP ratio (see Figure 19; (Prentki 1996). Both leucine and KIC can activate glutamate dehydrogenase to increase the rate at which glutamate, derived from glutamine, is converted to the Krebs cycle intermediate α-ketoglutarate (Malaisse et al. 1982; MacDonald 1990). Thus, all three metabolites serve as fuels for the Kreb's cycle, and may act synergistically to further enhance insulin secretion (as seen in Figures 14 or 15). These results, therefore, support a role for Krebs cycle flux in the potentiating effects of GIP on insulin exocytosis. Thus, the glucose-dependence of GIP can be bypassed if the cellular ATP:ADP ratio is increased by alternative means.

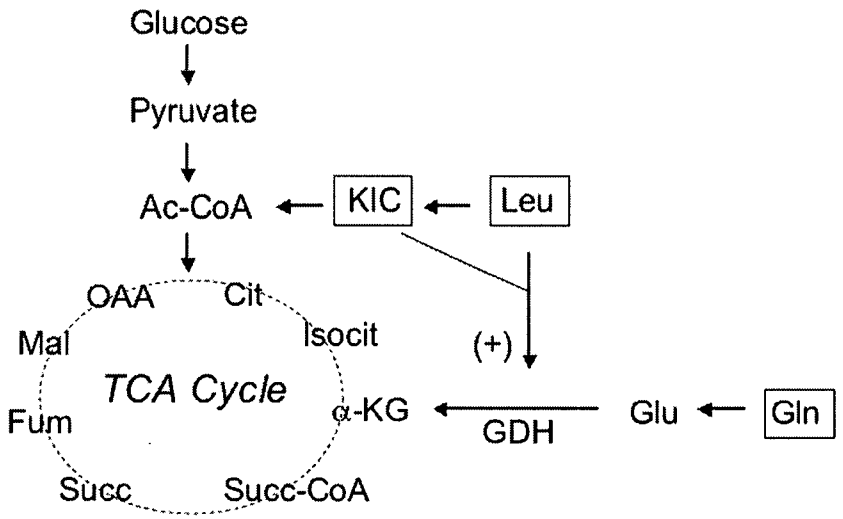


Figure 19: Anaplerosis: the process by which Leu, KIC, and Gln feed into the TCA cycle. Leucine (Leu) is metabolized into α-ketoisocaproic acid, which feeds into Krebs/TCA cycle via conversion to Acetyl CoA (Ac-CoA). Glutamine (Gln) is metabolized into the Kreb's cycle intermediate α-ketoglutarate (α-KG) by the enzyme glutamate dehydrogenase (GDH). Both Leu and KIC are both known to increase the activity of GDH. Cit = citrate, Isocit = isocitrate, Succ-CoA = succinyl-CoA, Succ = succinate, Fum = fumarate, Mal = malate, OAA = oxaloacetate, Glu = glutamate.

The present results (Figures 13-15), and those already published (Hinke et al. 2000; Ehses et al. 2001), clearly demonstrate that GIP stimulation of cAMP and arachidonic acid production is independent of nutrients and extracellular Ca^{2+} in βTC-3 cells. Cyclic AMP has been proposed to increase the rate of insulin exocytosis by mediating effects on granule mobilization and by increasing the readily releasable pool of insulin granules (Ämmälä et al. 1993; Gromada et al. 1998), whereas arachidonic acid has been shown to elevate intracellular Ca^{2+} levels in islets (Metz 1988). Thus, under normal conditions, GIP acts to amplify the triggering effects of nutrients on insulin secretion via cAMP and arachidonic acid or its metabolites. However, when considering K^{+}_{ATP} channel-independent actions, GIP-stimulated cAMP production is sufficient to amplify insulin exocytosis in the absence of nutrients (Figure 17, 18). Thus, we propose that in mediating the K^{+}_{ATP} channel-independent actions of GIP on insulin secretion, cAMP acts in a nutrient–independent manner, while arachidonic acid actions are dependent upon Krebs cycle flux.

In agreement with previous demonstrations that cAMP enhances K^+_{ATP} channel-independent insulin secretion in a glucose-dependent manner in rat islets (Yajima et al. 1999), the present results illustrate that in individual β-cells (βTC-3 cells), cAMP/PKA is also a key determinant for stimulating insulin release in the absence of nutrients. Such a role has not previously been described in islet studies, possibly due to complex intra-islet interactions. Additionally, isolated islets may require a minimal glucose level for hormonally-induced cAMP responses to be detected, but this is not the case for the beta cell lines that we have tested to date (βTC-3 cells and INS-1). This glucose-independence of signaling in beta cell models therefore allows the investigation of effects mediated via cAMP and glucose metabolism independently.

Membrane depolarization of β-cells is normally provided by metabolic flux increasing the cytoplasmic ATP:ADP ratio, and subsequently inhibiting K^+_{ATP} channel activity (Ashcroft 2000). From the present study one can conclude that the concept of *glucose-dependence* with respect to GIP in βTC-3 cells is simply based on the ability of the nutrient (i.e. glucose or anaplerotic substrates) to affect membrane potential. This is in support of earlier work on RIN 1046-38 insulinoma cells, wherby the glucose requirement for GLP-1 actions could also be replaced by membrane depolarization (Montrose-Rafizadeh et al. 1994). In agreement with Henquin's proposal for new terminology (Henquin 2000), glucose evokes the triggering mechanism which supplies the beta cell with an influx of Ca^{2+}, while GIP amplifies these effects via signals acting on pathways distal to this increase in Ca^{2+}. The current study extends those of Ding and Gromada (1997), by removing the influence of GIP on the K^+_{ATP} channel, and by eliminating any need for glucose in initiating exocytosis.

In summary, GIP was shown to amplify insulin secretion via K_{ATP} channel-independent means in the presence and absence of nutrients. The glucose-dependence of GIP-stimulated insulin exocytosis under normal conditions could be bypassed by increasing Krebs cycle turnover using anaplerotic secretagogues. Furthermore, in βTC-3 cells, this glucose-dependence could be alleviated by depolarizing the cell (while maintaining K_{ATP} channels open). These actions of GIP on nutrient and K_{ATP} channel-independent insulin exocytosis were found to be mediated by cAMP and PKA. Thus, it is apparent that glucose-dependence is solely related to the ability of nutrients to elicit membrane depolarization in βTC-3 cells.

CHAPTER 4: REGULATION OF MAPK SIGNALING AND CELL SURVIVAL BY GIP

4.1 BACKGROUND

The GIP receptor was previously shown to be coupled to the activation of MAPK (ERK 1/2) in CHO-K1 cells by Kubota and colleagues in 1997 (Kubota et al. 1997). However, at the time the present study was initiated, no further elucidation of the coupling mechanism linking the GIP receptor, or other family B GPCRs to ERK 1/2 activation had been investigated. Given the importance of this mitogenic signal, we sought to investigate its regulation in the pancreatic β-cell. Thus, the aim of studies described in the first part of this Chapter was to further elucidate GIP receptor signal transduction with a focus on the regulation of the ERK 1/2 module (Raf1→Mek1/2→ERK1/2→p90 RSK). We hypothesized that the GIP receptor is coupled to the regulation of the entire ERK 1/2 module via cAMP signaling.

Based on the regulation of the mitogenic ERK1/2 module by GIP, and the previously established role for GLP-1 in stimulating INS-1 β-cell growth, we further sought to investigate a role for GIP in the regulation of cell fate. This was initiated with an underlying goal of determining the importance of GIP-stimulated MAP kinase signaling in regulating these processes. The GLP-1 receptor was recently shown to stimulate beta (INS-1) cell growth via regulation of p38 MAPK (Buteau et al. 1999), however the role of the ERK1/2 module was not studied. Thus, we hypothesized that the GIP receptor is coupled to the regulation of INS-1 cell fate via control of MAPK signaling.

4.2 RESULTS

4.2.1 Expression profiling of protein kinases and phosphatases

The expression levels of protein kinases and phosphatases are cell-type specific. Our approach to delineating the signaling pathways responding to GIP receptor activation included an initial assessment of the various protein kinases (Figure 20; Tables 1-3) and phosphatases expressed (Table 4-5), with a comparison between a heterologous GIP receptor expression system (rGIP-15) and the two β-cell lines (βTC-3 and INS-1 (832/13)). With respect to MAPK cascades, it is evident that four modules (Raf1/B→Mek1/2→ERK1/2, Cot/Tpl-

2→Mek4/7→SAPK, PAKα→Mek6→p38α, and ERK3) were all present in rGIP-15, βTC-3, and INS-1 cells (Table 1; ERK5 was not blotted for). Furthermore, a pathway regulating cell survival (PDK1→PKB→GSK3), the multiple isoforms of PKC (α, β, δ, ε, γ, λ, μ, θ, ζ), and the ubiquitous cyclin-dependent kinases (Cdk 1, 2, 4, 5, 6, 7, 9) regulating cell cycle progression are also potential regulatory effector molecules for GIP in the cell lines (Table 1-2).

On comparing the kinase expression profiles of rGIP-15, βTC-3, and INS-1 cells, there were few major differences in expression levels of the ubiquitous kinases of the MAPK modules mentioned above. Differences were confined mainly to the relative expression level of the upstream Meks (Mek 1, 2, 4, 6, 7; Mek3 was not blotted for), the Mekks (Mos, Cot, GCK, Hpk, Mst1), and the numerous PKC isoforms (Table 1-3). The most reassuring finding was that 50-70 % of the protein kinases probed for were expressed at similar levels in the three cell lines (48 % across all three; 59 % for rGIP-15 vs. INS-1; 61 % for βTC-3 vs. INS-1; 73 % for rGIP-15 vs. βTC-3).

Intrinsic regulation of protein kinase networks is achieved via the equilibrium between protein kinase-mediated phosphorylation events and protein phosphatase-mediated dephosphorylation events. Thus, in addition to profiling protein kinases, we also assessed relative protein phosphatase levels in rGIP-15 and INS-1 cells (Table 4-5). Interestingly, the only MAPK phosphatase (MKP) expressed in our CHO and INS-1 cells was MKP-2, implicating it as a principal regulator of the ERK module. Various subfamily members of the PPP family of serine/threonine protein phosphatases were also detected. These include the PPP1 (PP1), the PPP2A (PP2A, PP4 (PPX)), the PPP2B (PP2B), and the PPP5 (PP5) subfamilies. Many of the protein tyrosine phosphatases (PTPs) probed for were not expressed (CD45, LAR, PTP1b, PTP1c, PTP-PEST).

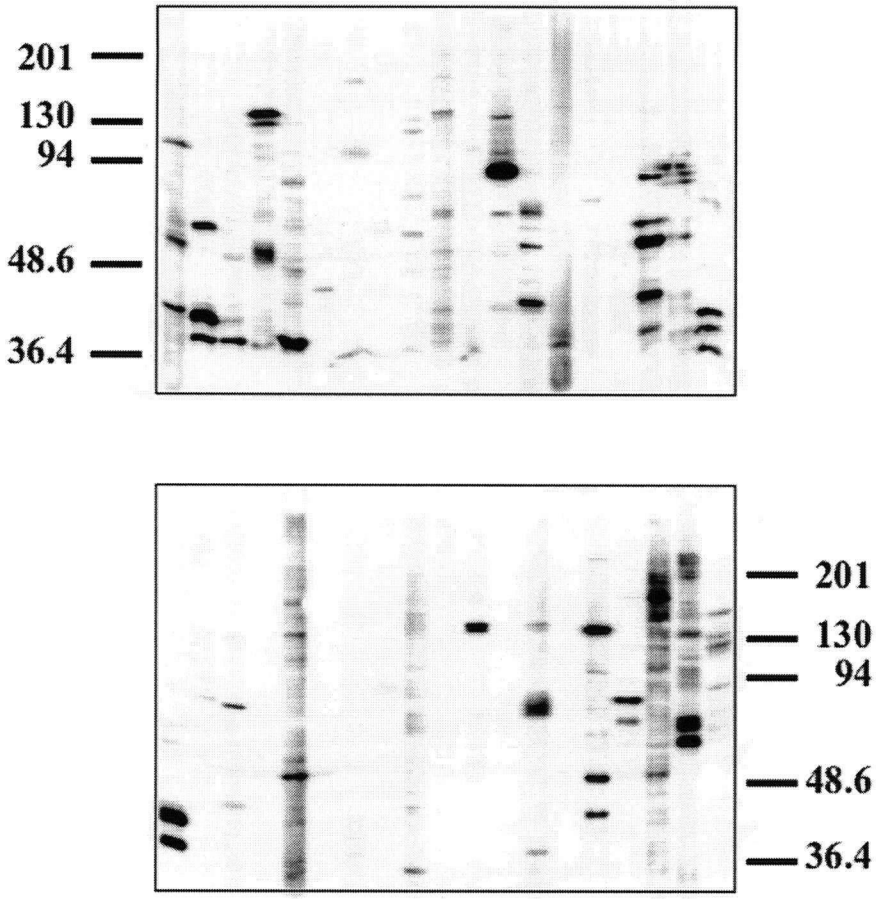


Figure 20: Representative blots from a protein kinase profile in rGIP-15 cells. The identical rGIP-15 cell lysate was loaded into each lane (20 μg of protein loaded/lane) for Western blotting with 75 different protein kinase antibodies. The molecular weight markers (in kDa) are shown. Quantitative assessment (trace quantities) was based on band densities.

Table 1: Highly expressed protein kinases in rGIP-15, βTC-3, and INS-1 (832/13) cells. Proteins were extracted in ice-cold lysis buffer, followed by sonication prior to centrifugation. Protein was quantified using the BCA assay, and 20 μg protein were loaded/lane for Western blotting. Quantitative assessment (trace quantities) was based on band densities; highlighted kinases represent those whose expression level was similar in the three cell lines investigated (n=3). Abbreviations are as follows: bone marrow X kinase (BMX), Bruton tyrosine kinase (Btk), calmodulin-dependent kinase (CaMK), cyclin-dependent kinase (Cdk), casein kinase (CK), cancer Osaka thyroid oncogene (Cot, Tpl-2), C-terminus Src kinase (CSK), death-associated protein kinase (DAPK), DNA-activated protein kinase (DNAPK), extracellular regulated kinase (ERK), focal adhesion kinase (FAK), Fyn oncogene related to SRC (Fyn), germinal center kinase (GCK), G protein-coupled receptor kinase (GRK), glycogen synthase kinase 3 (GSK3), hematopoetic progenitor kinase (Hpk), inhibitor NF-κB kinase (IKK), Janus kinase (JAK), kinase suppressor of Ras 1 (Ksr), lymphocyte specific protein-tyrosine kinase (Lck), oncogene Lyn (Lyn), MAPK kinase (Mek), MAPK interacting kinase (Mnk), v-mos Moloney murine sarcoma viral oncogene homolog 1 (Mos), mammalian sterile 20-like 1 (Mst1), NIMA (never in mitosis) related kinase 2 (Nek), p38 MAPK (p38), p21 activated kinase (PAK), 3-phosphoinositide-dependent kinase (PDK), protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), cGMP-dependent protein kinase (PKG), dsRNA-dependent protein kinase (PKR), protein tyrosine kinase (Pyk), oncogene Raf1 (Raf1), v-Raf murine sarcoma viral oncogene homolog B1 (RafB), RhoA kinase (ROK), 90 kD ribosomal S6 kinase (RSK), ribosomal S6 kinase (S6K), stress-activated protein kinase (SAPK/JNK), oncogene Src (Src), spleen tyrosine kinase (Syk), Yamaguchi sarcoma viral oncogene homolog 1 (Yes), Zeta-chain (TCR) associated protein kinase (ZAP70), ZIP kinase (ZIPK).

Highly Expressed Protein Kinases					
CHO cells with GIPR	Trace Quantity	βTC-3 cells	Trace Quantity	INS-1	Trace Quantity
BMX	5219 +/- 854	***CaMK4***	*3825 +/- 468*	***CaMK4***	*5374 +/- 1252*
CaMK4	*4965 +/- 380*	**Cdk 2**	5949 +/- 194	***Cdk 5***	*13169 +/- 957*
Cdk 5	*4210 +/- 819*	***Cdk 5***	*7357 +/- 833*	***Cdk 7***	*9882 +/- 1306*
Cdk 6	6781 +/- 1875	***Cdk 7***	*9520 +/- 656*	***CK 2 α***	*4946 +/- 644*
Cdk 7	*4588 +/- 805*	***CK 2 α***	*6243 +/- 2437*	***CK 2 α'***	*22371 +/- 2553*
CK 2 α	*8829 +/- 1878*	***CK 2 α'***	*19357 +/- 3056*	**CK 2 α"**	17283 +/- 2564
CK 2 α'	*11170 +/- 3900*	***ERK 1***	*42835 +/- 4096*	Cot (Tpl2)	8011 +/- 861
CK 2 α"	6170 +/- 1460	***ERK 2***	*17907 +/- 1176*	***ERK 1***	*30573 +/- 4908*
ERK 1	*26058 +/- 3058*	***ERK 3***	*6205 +/- 1446*	***ERK 2***	*10092 +/-1206*
ERK 2	*12901 +/- 3408*	***FAK***	*6882 +/- 1163*	***ERK 3***	*7648 +/- 819*
ERK 3	*6679 +/- 1376*	***GRK 2***	*13105 +/- 2244*	***FAK***	*5281 +/- 609*
FAK	*13642 +/- 4632*	***GSK 3 α***	*11655 +/- 2294*	***GRK 2***	*7400 +/- 1456*
Fyn	11057 +/- 1948	GSK 3 β	21203 +/- 3624	***GSK 3 α***	*4734 +/- 346*
GRK 2	*9585 +/- 2229*	Hpk 1	9405 +/- 1074	IKK α	6741 +/- 506
GSK 3 α	*10560 +/- 3460*	JAK 1	5925 +/- 835	***Mek 2***	*4092 +/- 346*
GSK 3 β	6637 +/- 2365	JAK 2	9751 +/- 326	***Mek 4***	*24364 +/- 1721*
Hpk 1	8122 +/- 2859	***Mek 2***	*7035 +/- 1485*	Mos	11847 +/- 2466
JAK 1	5444 +/- 2335	***Mek 4***	*19320 +/- 3621*	***P38 α***	*21497 +/- 3615*
JAK 2	4364 +/- 913	***P38 α***	*19196 +/- 3587*	***PKB α***	*6436 +/- 1599*
Mek 2	*8179 +/- 1733*	p70 S6K	11776 +/- 1641	PKC β	4573 +/- 1332
Mek 4	*4219 +/- 921*	PAK α	14718 +/- 5307	PKC δ	12211+/- 2241
P38 α	*26243 +/- 1831*	***PKB α***	*6343 +/- 1295*	***PKC μ***	*6954 +/- 1383*
p70 S6K	10663 +/- 1834	PKC α	6824 +/- 1295	***PKC ζ***	*15117 +/- 3993*
PAK α	7912 +/- 2954	PKC β	12036 +/- 1063	***Raf B***	*8726 +/- 1791*
PKB α	*8831 +/- 907*	PKC ε	18832 +/- 2631	***Raf 1***	*17931 +/- 4748*
PKC μ	*11420 +/- 1364*	PKC λ	4987 +/- 972	***RSK 1***	*19662 +/- 2319*
PKC ζ	*37137 +/- 3665*	***PKC μ***	*11153 +/- 243*		
Raf B	*4982 +/- 476*	***PKC ζ***	*34330 +/- 3633*		
Raf 1	*16495 +/- 4985*	PKR	29921 +/- 4795		
RSK 1	*12123 +/- 3684*	***Raf B***	*8290 +/- 749*		
ZIPK	7087 +/- 1977	***Raf 1***	*10445 +/- 501*		
		RSK 1	*39525 +/- 6400*		
		Syk	5339 +/- 780		
		ZIPK	8358 +/- 2388		

Table 2: Minimally expressed protein kinases in rGIP-15, βTC-3, and INS-1 (832/13) cells. Protein kinases were assessed as described for Table 1.

Minimally Expressed Protein Kinases					
CHO cells with GIPR	Trace Quantity	βTC-3 cells	Trace Quantity	INS-1	Trace Quantity
CaMKK	2559 +/- 650	CaMK1	1390 +/- 125	CaMK1	636 +/- 90
Cdk 1	1682 +/- 117	Cdk 1	2521 +/- 62	***Cdk 4***	*993 +/- 150*
Cdk 2	2581 +/- 683	***Cdk 4***	*4029 +/- 207*	Cdk 6	1078 +/- 253
Cdk 4	*2000 +/- 158*	Cdk 6	1982 +/- 947	***Cdk 9***	*1015 +/- 194*
Cdk 9	*1443 +/- 165*	***Cdk 9***	*1898 +/- 314*	***CK 1 δ***	*469 +/- 110*
CK 1 δ	*2787 +/- 187*	***CK 1 δ***	*1698 +/- 553*	ERK 6	982 +/- 76
Cot (Tpl-2)	1929 +/- 640	CK 2 α"	3263 +/- 528	GSK 3 β	2164 +/- 373
CSK	2784 +/- 862	Fyn	3434 +/- 762	KSR 1	692 +/- 84
GCK	4019 +/- 881	IKK α	1843 +/- 262	Lyn	758 +/- 275
IKK α	2265 +/- 797	Mek 6	2539 +/- 615	Mek 1	2052 +/- 313
Mek 1	2606 +/- 111	Mnk 2	1400 +/- 194	Mek 6	3245 +/- 332
Mek 7	3756 +/- 2649	Nek 2	2917 +/- 450	PAK α	2851 +/- 112
Mnk 2	1726 +/- 26	***PDK 1***	*3096 +/-1596*	p70 S6K	2105 +/- 159
Mos	1357 +/- 291	***PKA***	*3273 +/- 1051*	***PDK 1***	*1121 +/- 69*
PDK 1	*2514 +/- 86*	PKC δ	1895 +/- 1004	***PKA***	*3107 +/- 803*
PKA	*450 +/- 50*	PKG	1778 +/- 149	PKC α	3497 +/- 1304
PKC α	1831 +/- 170	ROK α	3751 +/- 775	PKC ε	3648 +/- 1135
PKC δ	1822 +/- 348	RSK 2	2200 +/- 1200	Pyk 2	2042 +/- 535
PKC ε	1646 +/- 732	*SAPK (JNK)*	*2655 +/- 231*	*SAPK (JNK)*	*1666 +/- 106*
PKC λ	3282 +/- 152			ZAP70	922 +/- 172
ROK α	2426 +/- 959			ZIPK	1347 +/- 298
SAPK (JNK)	*2458 +/- 595*				

Table 3: Protein kinases screened for that were not expressed in rGIP-15, βTC-3, and INS-1 (832/13) cells. Protein kinases were assessed as described for Table 1.

Protein Kinases NOT Detected		
CHO cells with GIPR	βTC-3 cells	INS-1
Btk	BMX	BMX
CaMK1	*Btk*	*Btk*
CK1 ε	CaMKK	CaMKK
DAPK	***CK 1 ε***	Cdk 1, 2
DNAPK	CSK	***CK 1 ε***
ERK 6	***DAPK***	CSK
KSR 1	***DNAPK***	***DAPK***
Lck	Cot (Tpl-2)	***DNAPK***
Lyn	ERK 6	Fyn
Mek 6	GCK	GCK
MST 1	KSR 1	Hpk 1
Nek 2	***Lck***	JAK 1, 2
Pim 1	Lyn	***Lck***
PKC β, γ, θ	Mek 1, 7	Mek 7
PKG	Mos	Mnk 2
PKR	***MST 1***	*MST 1*
Pyk 2	PKC γ, θ	Nek 2
RSK 2	***Pim 1***	***Pim 1***
Src	Pyk 2	PKC γ, λ, θ
Syk	***Src***	PKG
Yes 1	***Yes 1***	PKR
ZAP70	ZAP70	ROK α
		RSK 2
		Src
		Syk
		Yes 1

Table 4: Expression levels of protein phosphatases in rGIP-15 and INS-1 (832/13) cells. Proteins were extracted in ice-cold lysis buffer, followed by sonication prior to centrifugation. Protein was quantified using the BCA assay, and 20 μg protein were loaded/lane for Western blotting. Quantitative assessment (trace quantities) was based on band densities; highlighted kinases represent those whose expression level was similar in the three cell lines investigated (n=2). Abbreviations are as follows: leukocyte common antigen CD45 transmembrane phosphatase (CD45), cyclin-dependent kinase associated phosphatase (KAP), LCA antigen-related (LAR) transmembrane tyrosine phosphatase (LAR), MAP kinase phosphatase 1 (MKP-1), MAP kinase phosphatase 2 (MKP-2), MAP kinase phosphatase 3 (MKP-3), MAP kinase phosphatase PAC1 (PAC1), protein phosphatase 1 catalytic subunit alpha isoform (PP1/C-alpha), protein phosphatase 1 catalytic subunit beta isoform (PP1/C-beta), protein phosphatase 1 catalytic subunit gamma isoform (PP1/C-gamma), protein phosphatase 5 catalytic subunit (PP5 or PPT), protein phosphatase 2A, A regulatory subunit alpha and beta isoforms (PP2A/A-alpha/beta), protein phosphatase 2A catalytic subunit alpha isoform (PP2A/C alpha), protein phosphatase 2A catalytic subunit beta isoform (PP2A/C beta), protein phosphatase 2B catalytic subunit alpha isoform (PP2B/A alpha), protein phosphatase 2 catalytic subunit alpha isoform (PP2C alpha), protein phosphatase 2C catalytic subunit beta isoform (PP2C beta), protein phosphatase 2C catalytic subunit delta isoform (PP2C/delta), protein phosphatase X regulatory subunit (PPX/A'2), protein phosphatase 2X catalytic subunit (PP2X/C), protein phosphatase 6 catalytic (PPV/C), protein tyrosine phosphatase 1B (PTP1B), protein tyrosine phosphatase 1C (PTP1C), protein tyrosine phosphatase 1D (PTP1D), protein tyrosine phosphatase with PEST sequences (PTP-PEST), phosphatidylinositol 3'-phosphate and protein phosphatase (PTEN), signal regulatory protein substrate of PTP1D phosphatase (SIRP a1).

Protein Phosphatases Expressed			
CHO cells with GIPR	Trace Quantity	INS-1	Trace Quantity
MKP-2	*1547 +/- 766*	KAP	813 +/- 381
PTP1C	*1223 +/- 115*	LAR	886 +/- 16
PTP1D	*10411 +/- 6028*	***MKP-2***	*371 +/- 24*
PP1/C-α	*12168 +/- 1941*	PTEN	1358 +/- 431
PP1/C-β	*804 +/- 266*	***PTP1C***	*274 +/- 68*
PP1/C-γ	*7465 +/- 877*	***PTP1D***	*6028 +/-1748*
PP2A/A-αβ	*27377 +/- 4820*	***PP1/C-α***	*6916 +/- 1681*
PP2A/C	*2776 +/- 356*	***PP1/C-β***	*1187 +/- 339*
PP2B/A-α	*715 +/- 147*	***PP1/C-γ***	*11277 +/- 1189*
PP2C/α	*1526*	***PP2A/A-αβ***	*11480 +/- 622*
PP2C/β	*2824*	***PP2A/C***	*1030 +/- 20*
PP2X/C	*1480 +/- 247*	***PP2B/A-α***	*1076 +/- 575*
PP5 (PPT)	*3155 +/- 416*	***PP2C/α***	*4821 +/- 836*
PPV/C	*13250 +/- 171*	***PP2C/β***	*3435 +/- 1042*
PP2X/A'2	*952 +/- 62*	**PP2C/δ**	2023 +/- 192
		PP2X/C	*7080 +/- 1097*
		PP5 (PPT)	*2502 +/- 1653*
		PPV/C	*12697 +/- 1565*
		PP2X/A'2	*473 +/- 243*
		PTP1B	4084 +/- 1459

Table 5: Protein phosphatases screened for that were not expressed in rGIP-15 and INS-1 (832/13) cells. Protein phosphatases were assessed as described for Table 4.

Protein Phosphatases NOT Expressed	
CHO cells with GIPR	INS-1
KAP	*CD45*
LAR	***MKP-1***
CD45	***MKP-3***
MKP-1	***PAC1***
MKP-3	***PTP-PEST***
PAC-1	***SIRPa1***
PTEN	
PTP-PEST	
PP2C/δ	
PTP1B	
SIRPa1	

4.2.2 GIP-mediated activation of ERK1/2 in rGIP-15 cells and β-cell models

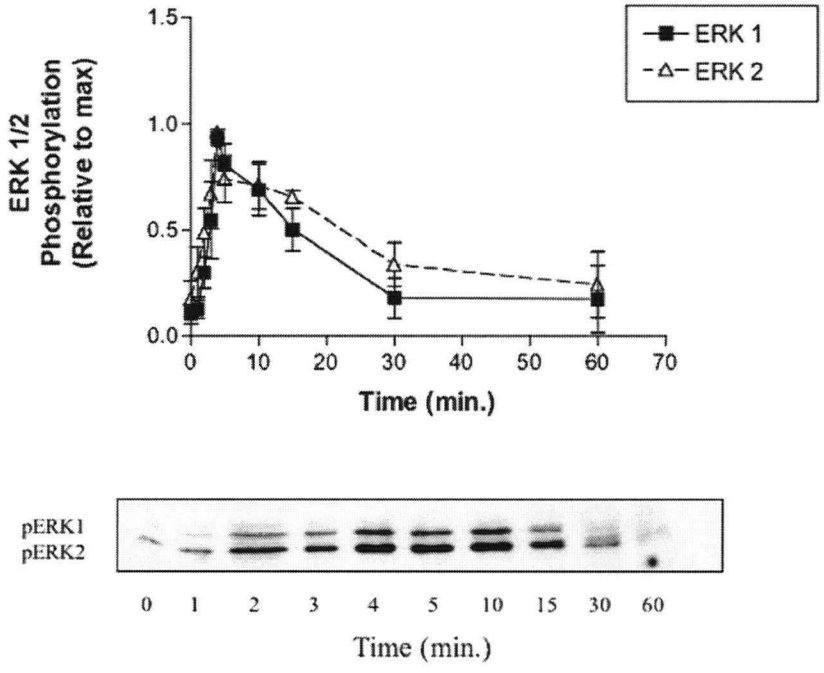
Parallel activations of ERK 1 (p44 MAPK) and 2 (p42 MAPK) were assessed using a phospho-specific antibody known to bind only the phosphorylated (T202 and Y204 in ERK1) and activated forms of these kinases (Chen et al. 2001). ERK 1 and ERK 2 exhibited transient phosphorylation kinetics, with maximal phosphorylation evident at 4-5 min and a return to basal by 30-60 min (Figure 21A). Figure 21B illustrates the concentration dependence of GIP mediated ERK 1/2 phosphorylation and activation, with ERK 1 and 2 phosphorylation increased significantly by nM and pM concentrations of GIP, respectively, and a maximal ERK 1/2 phosphorylation at least 4-times basal.

Concurrent analysis of ERK 1/2 phosphorylation in β-cell lines revealed similar GIP-mediated activation (Figure 22). Initially, studies were carried out on the βTC-3 cell line. However, glucose was found to have no effect on ERK 1/2 phosphorylation in this cell line (Figure 22A), and INS-1 cells were therefore utilized as a more representative physiological model (Khoo and Cobb 1997; Hohmeier et al. 2000). GIP activated ERK 1/2 in both the presence and absence of glucose (11 mM) in these cells. Glucose caused a distinct leftward shift in the concentration-response profile of GIP-mediated ERK 1/2 phosphorylation, without

enhancing the fold-maximal phosphorylation attained (Figure 22B and 22C). Supportive studies with a third β-cell line, Brin-D11, also mimic these actions of GIP (data not shown).

GIP signals via cAMP and arachidonic acid (AA) in rGIP-15 cells and β-cell lines (Wheeler et al. 1995; Ehses et al. 2001). To identify the proximal regulator of the ERK module we tested the ability of these two second messengers to modulate ERK 1/2 phosphorylation, while using the phorbol ester, PMA (phorbol myristic acid), as a positive control for ERK 1/2 activation (Figure 23). Both forskolin and AA increased ERK 1/2 activity in rGIP-15 cells. However, only forskolin was active in INS-1 cells. Forskolin also activated ERK 1/2 in βTC-3 cells (data not shown), implying that cAMP is a positive regulator of ERK 1/2 activity in β-cells.

A



B

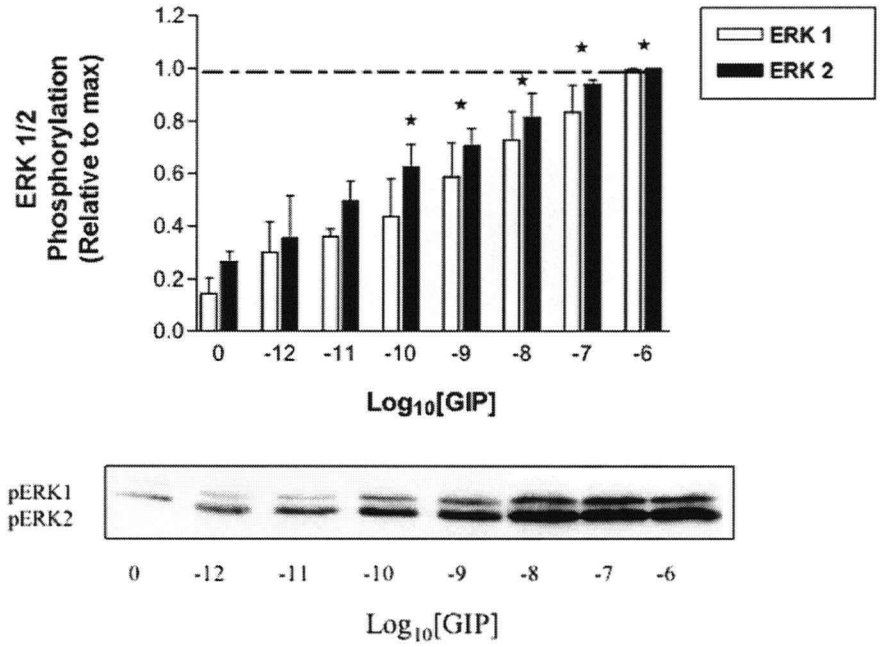
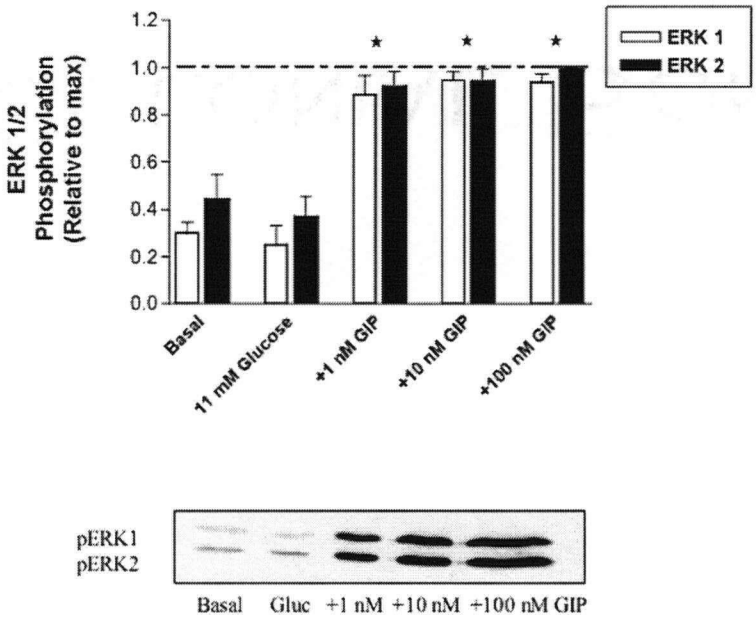
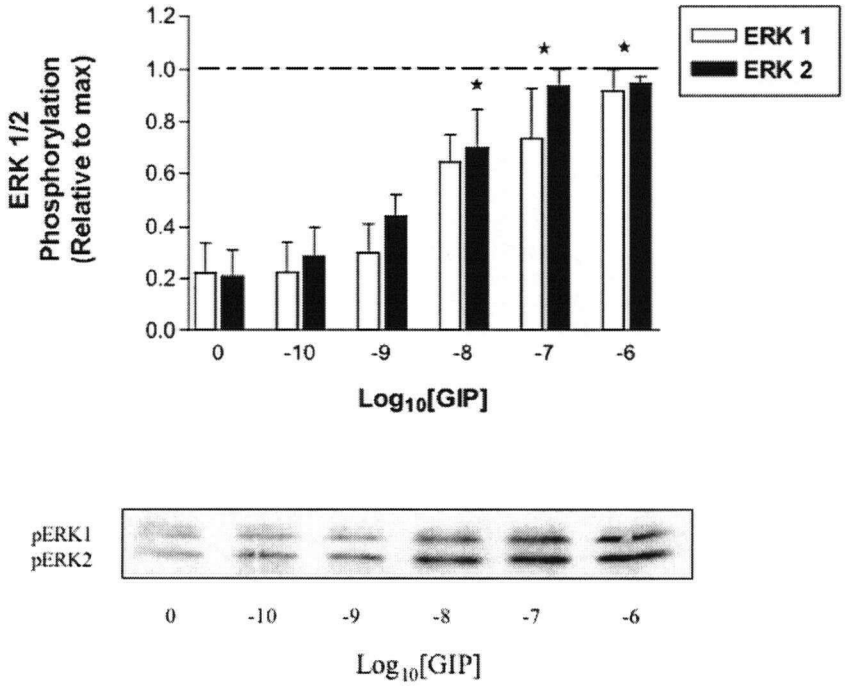


Figure 21: GIP stimulates ERK 1/2 phosphorylation in a transient (A) and concentration-dependent (B) manner in rGIP-15 cells. Cells were stimulated in Krebs ringer buffer for 5 min (B) or indicated times (A). Stimulation was stopped by addition of ice-cold lysis buffer, followed by sonication prior to centrifugation. Protein was quantified using the BCA assay, and 50 μg protein were loaded/lane for Western blotting. Membranes were blotted with Phospho T202, Y204-ERK (p-ERK) antibody, prior to densitometric analysis. Data represent mean ± S.E.M. (A, n=4-5; B, n=4), where * $p<0.05$ compared to respective controls (ANOVA, with Dunnett's multiple comparison test).

A


ERK 1/2
Phosphorylation
(Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ERK 1
ERK 2
Basal
11 mM Glucose
+1 nM GIP
+10 nM GIP
+100 nM GIP
pERK1
pERK2
Basal Gluc +1 nM +10 nM +100 nM GIP

B


ERK 1/2
Phosphorylation
(Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ERK 1
ERK 2
0
-10
-9
-8
-7
-6
Log10[GIP]
pERK1
pERK2
0
-10
-9
-8
-7
-6
Log10[GIP]

C

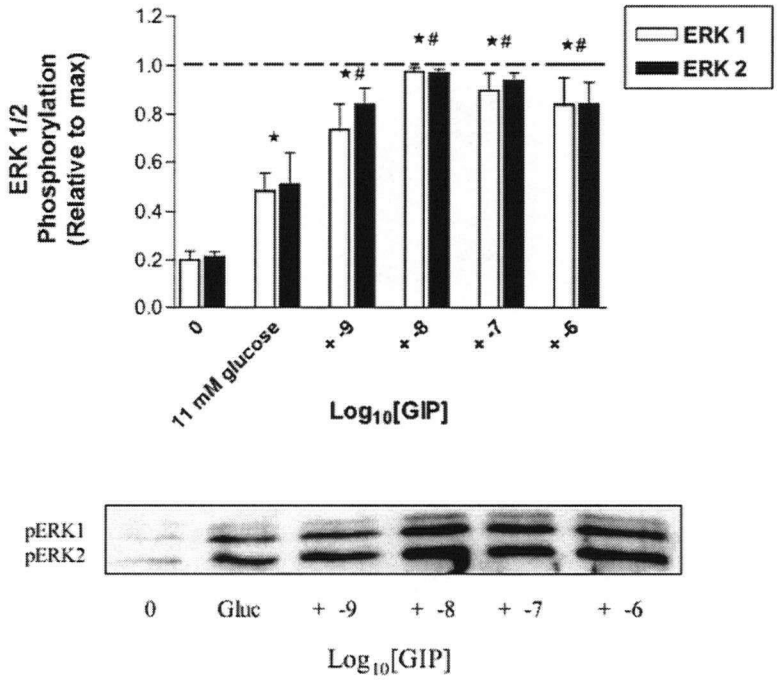


Figure 22: GIP stimulates ERK 1/2 phosphorylation in the presence of glucose in βTC-3 cells (A), and in both the absence (B) and presence (C) of glucose in INS-1 (832/13) cells. Cells were stimulated in Krebs ringer buffer for 5 min containing 11 mM glucose (A and C), or 0 mM (B). Fifty μg protein samples were separated by SDS-PAGE and membranes were blotted with Phospho T202, Y204-ERK (p-ERK) antibody, prior to densitometric analysis. Data represent mean ± S.E.M. (A, n=3; B, n=4; C, n=4). In panel C, * $p<0.05$ compared to basal controls and # $p<0.05$ compared to glucose control (ANOVA, with Newman-Keuls post hoc).

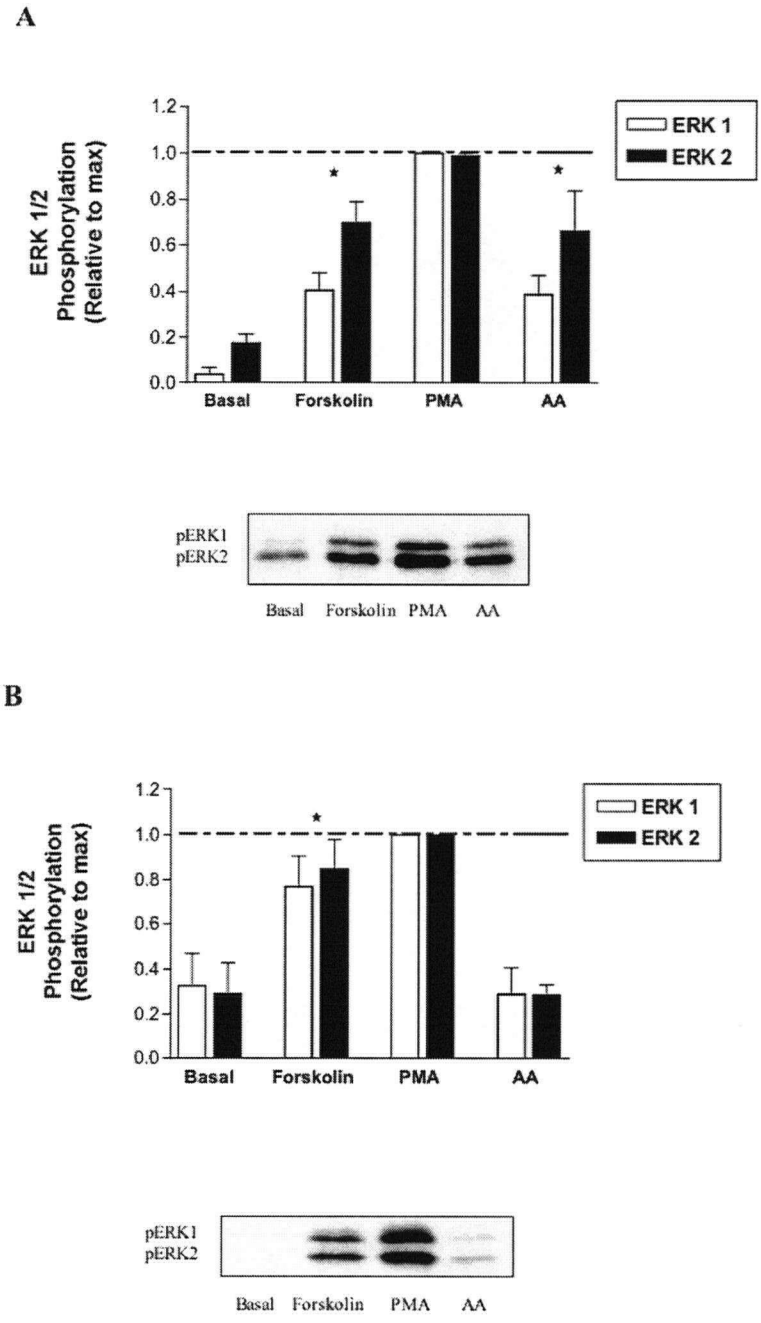


Figure 23: Forskolin, PMA, and arachidonic acid (AA) regulate ERK 1/2 in rGIP-15 (A) and INS-1 (832/13) cells (B). Cells were stimulated in Krebs ringer buffer for 5 min containing 0 mM glucose. Agonist concentrations were 10 μM forskolin, 4 μM PMA, and 100 μM AA. Fifty μg protein samples were separated by SDS-PAGE and membranes were blotted with Phospho T202, Y204-ERK (p-ERK) antibody, prior to densitometric analysis. Data represent mean ± S.E.M. (A, n=3; B, n=3; C, n=4), where * $p<0.05$ compared to respective controls (ANOVA, with Dunnett's multiple comparison test).

4.2.3 GIP regulates the ERK1/2 module upstream of Raf-1 via PKA

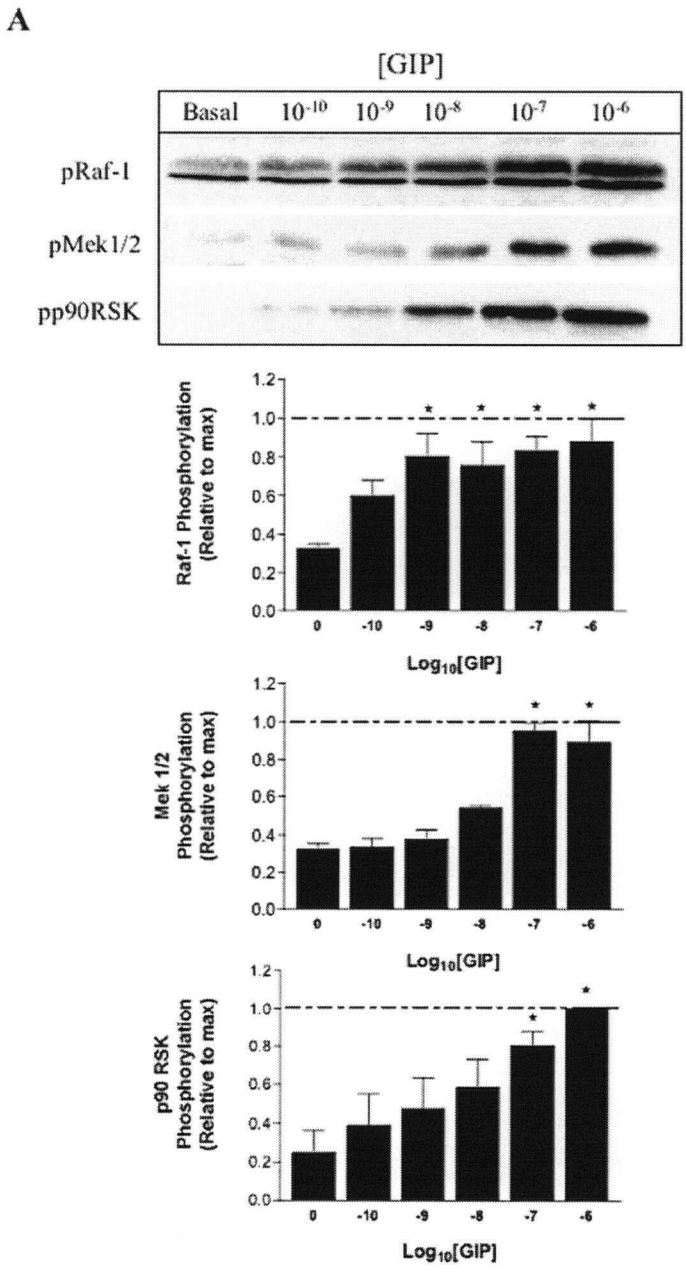
GIP has previously been shown to activate ERK 1/2 (Kubota et al. 1997; Trumper et al. 2001), but the other kinases in this module were not examined and no attempt was made to link ERK 1/2 activation to GPCR effector coupling. Figure 24A illustrates the concentration-dependent actions of GIP on phosphorylation of S259 of Raf-1, S217/221 of Mek 1/2, and S380 of p90 RSK, after a 5 min stimulation in rGIP-15 cells. There is a high correlation in responses among these 3 kinases, but we found no evidence for Elk-1 (S383) phosphorylation after 5 min (n=3). Evidence supporting a role for Raf-1 and p90 RSK in responses to GIP in the β-cell (INS-1 clone 832/13) is shown in Figure 24B, under 0 mM glucose conditions. Under these conditions the state of S217/221 phosphorylation of Mek 1/2 was already high, and further enhancement of activity by GIP could not be detected. However, the Mek 1/2 specific inhibitors, PD98059 and U0126, both completely abolished forskolin, PMA, and GIP-mediated ERK 1/2 phosphorylation, supporting a role for Mek 1/2 in the cascade (Figure 24C). Thus, GIP is able to regulate the Raf1→Mek1/2→ERK1/2→RSK module in both CHO-K1 and INS-1 β-cells.

The general PKC and PKA inhibitors, GF109203x (Bis) and H89 respectively, were used to evaluate the role of these protein-serine/threonine kinases in mediating GIP actions on the ERK module (Figures 25 and 26). These studies were conducted in rGIP-15 cells using forskolin, PMA, and AA as agonists representing 3 potential activation pathways (PKA, PKC, and AA, respectively). PKC inhibition (2 μM GF109203x) reduced basal ERK 1/2 phosphorylation, as well as significantly abrogating forskolin and PMA induced ERK 1/2 phosphorylation ($p<0.05$, n=4). The specificity of GF109203x for PKC is demonstrated by the complete ablation of PMA effects. Figure 25 clearly demonstrates that GIP-mediated activation of ERK 1/2 can be PKC-independent, as there was only a slight reduction in mean responses to GIP, which were not significant.

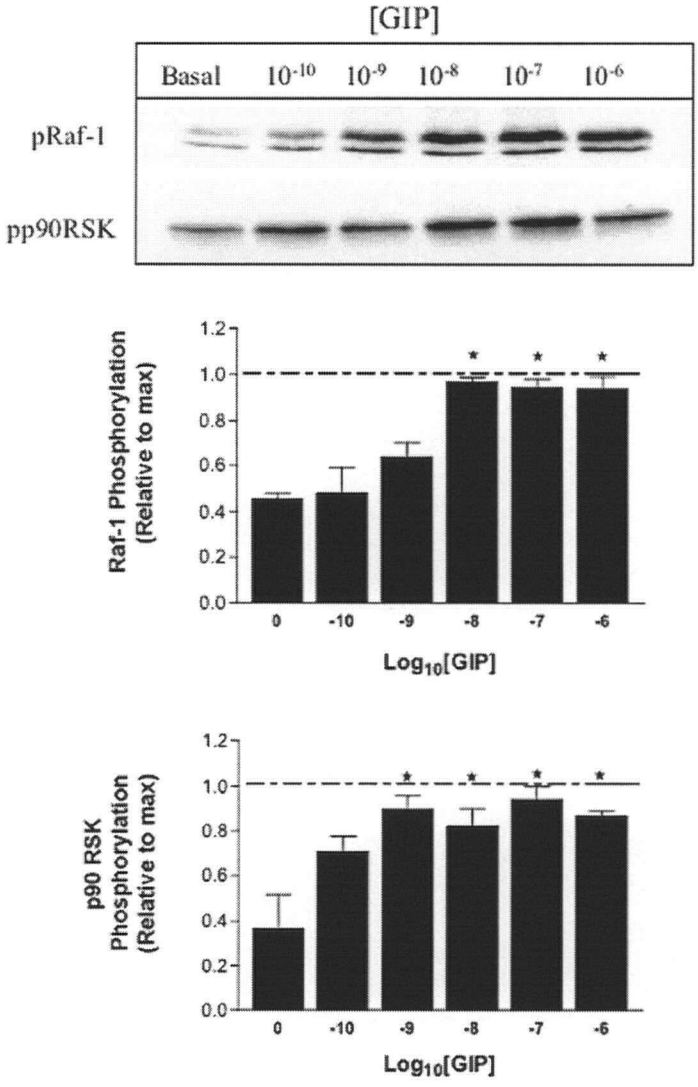
In contrast, PKA inhibition suppressed basal, forskolin-, AA-, and GIP-stimulated ERK 1/2 phosphorylation, while not altering PMA effects (Figure 26A and B). While both Bis and H89 had dramatic effects on basal ERK1/2 phosphorylation, only H89 was able to significantly suppress activation by GIP. We therefore concluded that GIP regulates ERK 1/2 activity through activation of PKA. To determine the step at which PKA exerts its regulation of the 3-tiered ERK 1/2 module, the effect of H89 on agonist-induced changes in phospho-Raf-1 and phospho-Mek 1/2 was studied (Figure 26). It is evident that PKA regulation occurred upstream of Raf-1, as H89 was able to block the phosphorylation of S259 of Raf-1 and S217/221 of Mek 1/2. Thus,

GIP seemingly inhibits Raf-1 activity via increased phosphorylation of S259 (Zimmerman and Moelling 1999), while positively regulating Mek 1/2, ERK 1/2, and p90 RSK.

It should also be noted that PMA reduced the phosphorylation of Raf-1, while activating both Mek 1/2 and ERK 1/2 (Figure 26). Furthermore, AA seemingly reduced Raf-1 phosphorylation while bypassing Mek 1/2 and activating ERK 1/2. These findings illustrate that PKC-mediates a reduction in Raf-1 S259 phosphorylation, while enhancing downstream Mek 1/2 and ERK 1/2 activity.



B


[GIP]
Basal
10-10
10-9
10-8
10-7
10-6
pRaf-1
pp90RSK
Raf-1 Phosphorylation (Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
-10
-9
-8
-7
-6
Log10[GIP]
p90 RSK Phosphorylation (Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
-10
-9
-8
-7
-6
Log10[GIP]

C

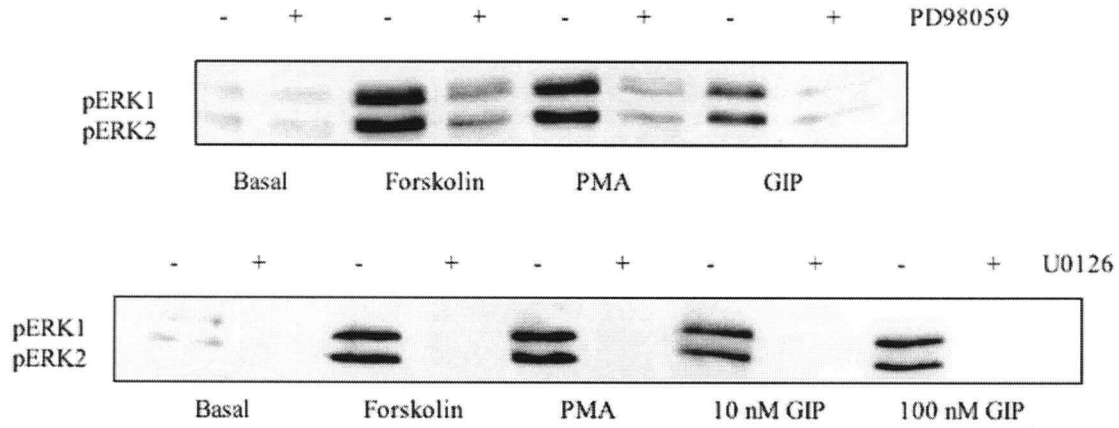


Figure 24: GIP regulates the ERK 1/2 module in CHO (A) and INS-1 (832/13; B and C) cells. rGIP-15 (A) or INS-1 (B and C) cells were stimulated in Krebs ringer buffer for 5 min containing 0 mM glucose. The Mek1/2 inhibitors, PD98050 (100 μM) or UO126 (10 μM), were added 15 min prior to and during stimulations (C). Fifty μg protein samples were separated by SDS-PAGE and membranes were blotted with antibodies against Phospho T202, Y204-ERK (p-ERK), p-Raf (S259), p-MEK (S217, S221), p-p90RSK (S380), and p-Elk-1 (S383). p-Elk-1 was not detected at 5 min. Data represent mean ± S.E.M. (A; n=3, B; n=3-4, C; blots representative of 3 and 2 independent experiments with similar results), where * $p<0.05$ compared to respective controls (ANOVA, with Dunnett's multiple comparison test).

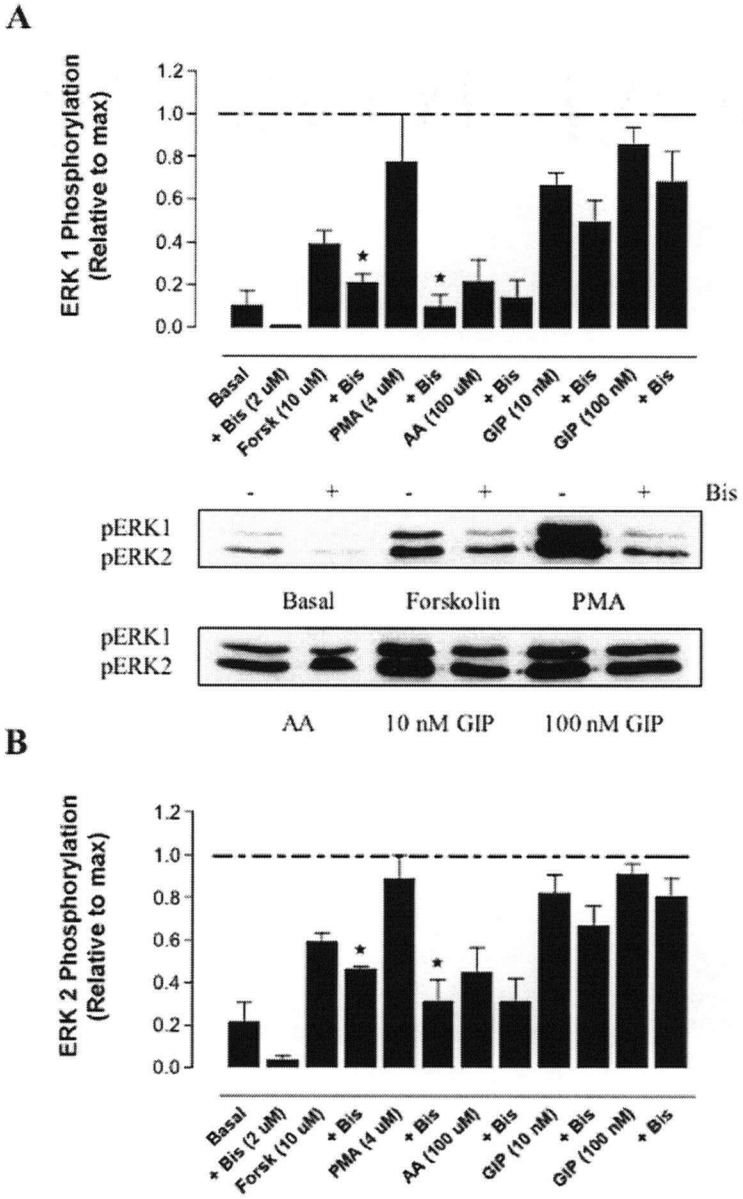
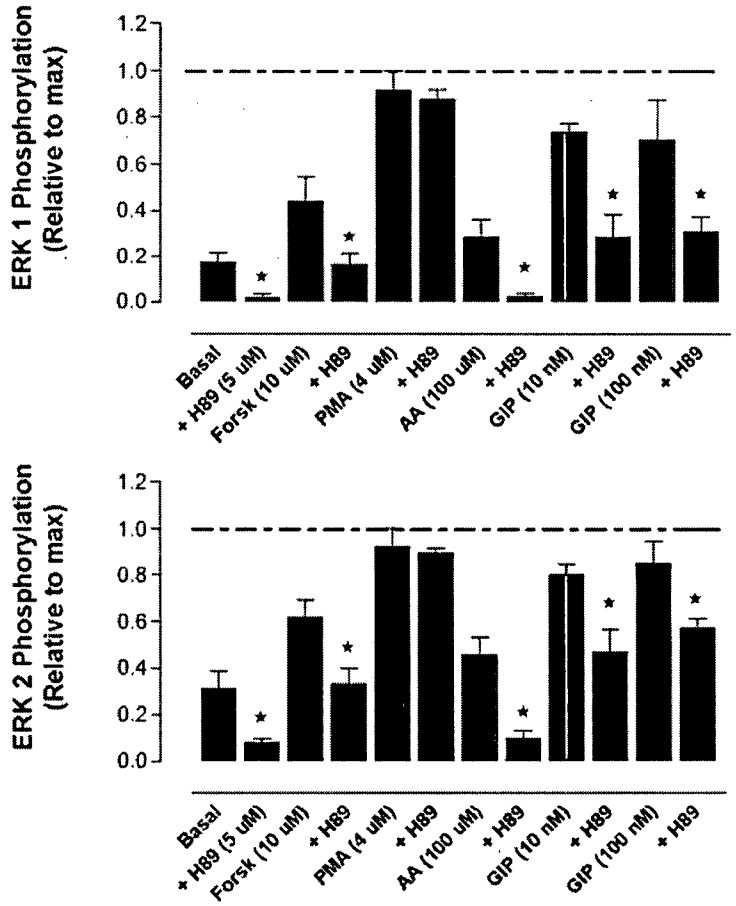


Figure 25: GIP regulation of ERK 1/2 is PKC-independent in rGIP-15 cells. Inhibitor, GF109203x (Bis), was added 15 min prior to and during 5 min stimulations. Fifty μg protein samples were separated by SDS-PAGE and membranes were blotted with Phospho T202, Y204-ERK (p-ERK) antibody, prior to densitometric analysis. Data represent mean ± S.E.M. (n=4), where * $p<0.05$ compared to respective controls (Student's t-test), and blots are representative.

A


ERK 1 Phosphorylation (Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Basal
+ H89 (5 uM)
Forsk (10 uM)
+ H89
PMA (4 uM)
+ H89
AA (100 uM)
+ H89
GIP (10 nM)
+ H89
GIP (100 nM)
+ H89
ERK 2 Phosphorylation (Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Basal
+ H89 (5 uM)
Forsk (10 uM)
+ H89
PMA (4 uM)
+ H89
AA (100 uM)
+ H89
GIP (10 nM)
+ H89
GIP (100 nM)
+ H89

B

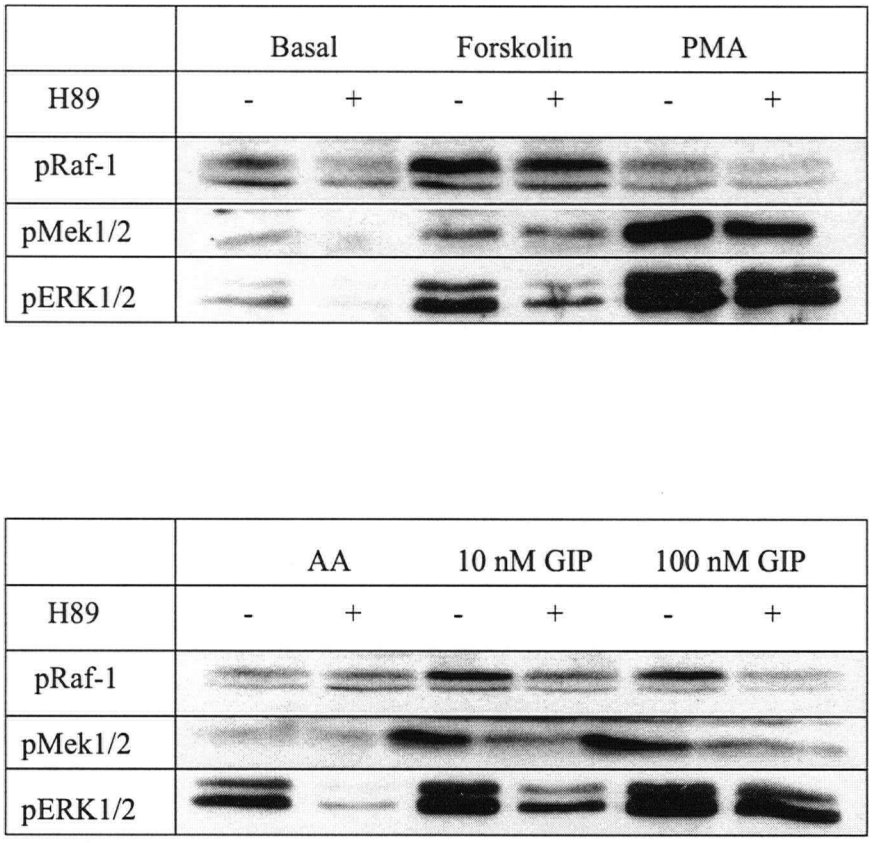


Figure 26: GIP regulation of ERK 1/2 (A, B), Raf-1 (B), and Mek 1/2 (B) is PKA-dependent in rGIP-15 cells. Inhibitor, H89, was added 15 min prior to and during 5 min stimulations. Fifty μg protein samples were separated by SDS-PAGE and membranes were blotted with antibodies against Phospho T202, Y204-ERK (p-ERK), p-Raf (S259), p-MEK (S217, S221), p-p90RSK (S380), and p-Elk-1 (S383). Data are representative blots of four (ERK 1/2; Panel A or B) and two (Raf-1, MEK 1/2; Panel B) independent experiments, where * $p<0.05$ compared to respective controls (Student's t-test).

4.2.4 GIP activates ERK1/2 via Rap1 independently of Gβγ and Src

The above data reveal a positive effect of GIP on the Mek1/2→ERK1/2→RSK module, in contrast to the inhibitory influence of GIP on Raf-1 activity through promotion of S259 phosphorylation (Kolch 2000; Dhillon et al. 2002A; Kubicek et al. 2002). It was hypothesized that another kinase must be responsible for the positive influence of GIP on ERK 1/2 activation. Previous studies have shown that the Raf isoform present in specific cell types determines whether cAMP affects ERK 1/2 activation positively or negatively (Houslay and Kolch 2000). From the protein kinase profile we were able to confirm the presence of an abundant amount of both Raf-1 and B-Raf in CHO-K1 (rGIP-15), βTC-3, and INS-1 (832/13) cells (Table 1 and Figure 27). B-Raf has been shown conclusively to regulate cAMP stimulation of ERK 1/2 in PC12 cells (Vossler et al. 1997). Thus, we transfected rGIP-15 cells with increasing amounts of the upstream regulator of B-Raf, the small GTPase Rap1 (Ohtsuka et al. 1996), and assessed GIP-mediated ERK1/2 activation. Figure 28A clearly shows that submaximal GIP concentrations were able to exert a greater influence on ERK1/2 phosphorylation in the presence of increased amounts of Rap1. This was without an effect on total ERK 1/2 expression levels (Figure 28A). Thus, we conclude that GIP regulation of the ERK 1/2 module occurs upstream of B-Raf, via PKA and Rap1.

In view of the fact that the β_2-adrenergic receptor was shown to switch from $G_{\alpha s}$ to $G_{\alpha i}$ coupling, and activate ERK1/2 via $G_{\beta\gamma}$ subunits (Daaka et al. 1997), we investigated whether a similar pathway was involved in regulating downstream ERK 1/2 signaling. An inhibiting peptide (βARKct) that we previously employed to investigate GIP signaling (Ehses et al. 2001), was expressed in increasing amounts in rGIP-15 cells, but it was unable to reverse 1 nM GIP-mediated activation of ERK 1/2 phosphorylation (Figure 28), thus providing evidence against a role for $G_{\beta\gamma}$ signaling in ERK1/2 regulation. We also found no role for the lipid kinase, PI3K, in regulating ERK1/2 in rGIP-15 cells (Figure 29A). Recent findings have implicated $G_{\alpha s}$ in the activation of the tyrosine kinase Src (Ma et al. 2000), and Src in the regulation of ERK1/2 (Schmitt and Stork 2002). We found no evidence for Src involvement in the GIP activation of ERK 1/2 using dominant negative and constitutively active contructs (Src RF and Src Y527F; n=3, Figure 29) (Schmitt and Stork 2002). This is consistent with the absence of detectable Src from our protein kinase profile data (Table 3).

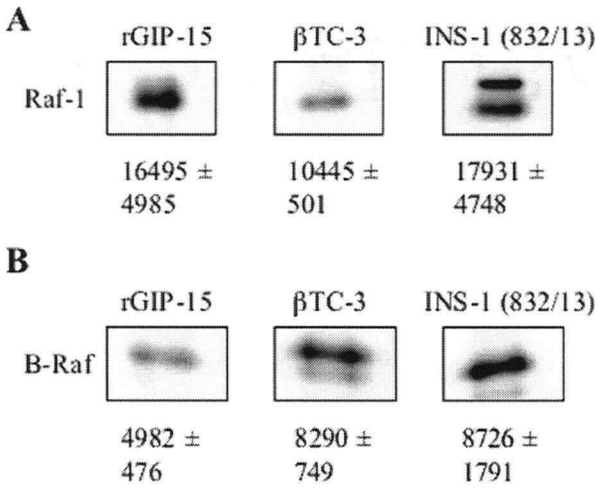
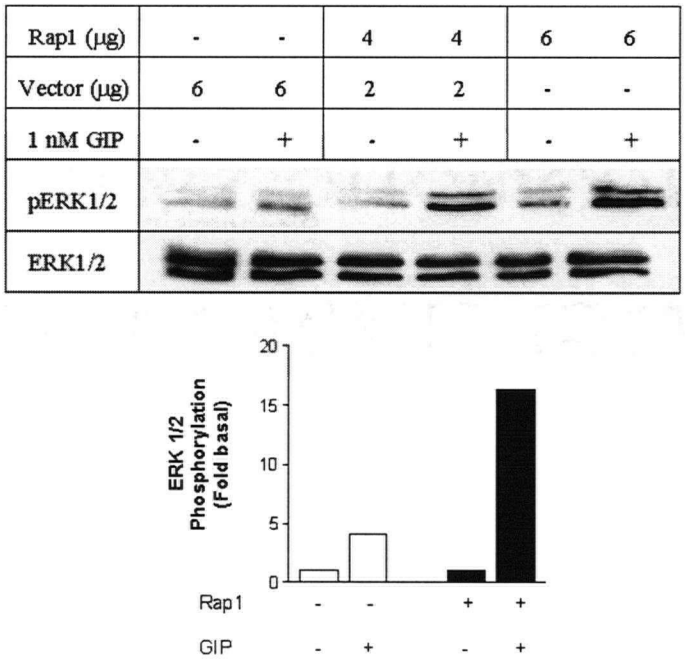


Figure 27: rGIP-15 cells and β-cells (βTC-3 and INS-1 (832/13)) express both 74 kD Raf-1 (A) and 95 kD B-Raf (B) isoforms. Twenty μg protein samples were separated by SDS-PAGE and membranes were blotted with Raf-1 (C-20) and B-Raf (H-145) from Santa Cruz Biotechnology. Multiple bands in A (rGIP-15 and INS-1 cells) are representative of decreased mobility due to the phosphorylation state of Raf-1. Blots are representative of three independent experiments (Trace quantity is reported below the respective blots; taken from Table 1A).

A



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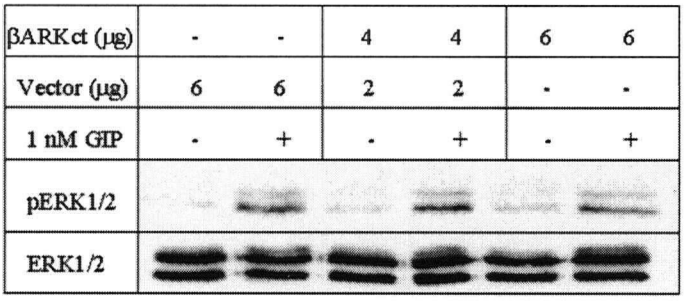
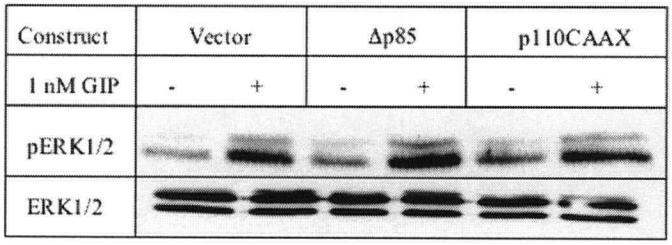
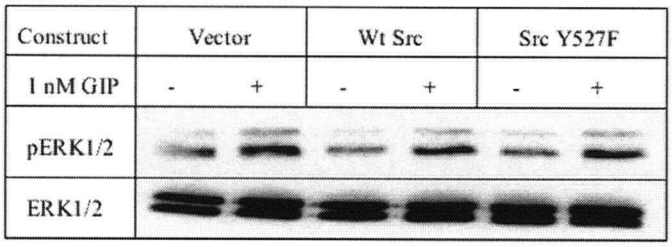


Figure 28: GIP mediated ERK 1/2 activation occurs via Rap1 (A) and independently of Gβγ subunits (B). rGIP-15 cells were transfected with vectors +/- constructs 2 days prior to experiments using Lipofect2000 (Gibco). Vector (pCGN) and wt Rap1b were a kind gift from Dr. D. Altschuler (Pittsburgh), and the C-terminus of β-adrenergic receptor kinase (βARKct) and the vector (pRK5) were provided by Dr. R. J. Lefkowitz (Duke University). Proteins were separated by SDS-PAGE and membranes were blotted with Phospho T202, Y204-ERK (p-ERK), and a C-terminal targeted ERK 1/2 antibody from Santa Cruz Biotechnology to assess total ERK 1/2. Blots are representative of two (A) or three (B) independent experiments with similar results.

A



B



C

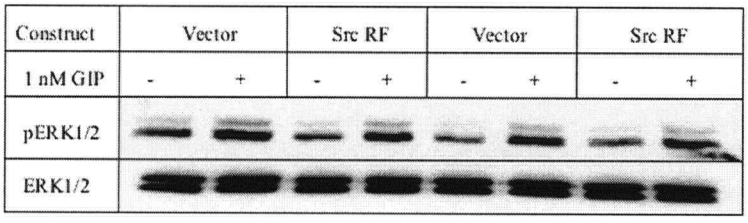


Figure 29: GIP mediated ERK 1/2 phosphorylation is independent of PI3K (A) and Src (B, C). rGIP-15 cells were transfected with vectors +/- constructs 2 days prior to experiments using Lipofect2000 (Gibco). PI3K constitutively active (p110 CAAX) and dominant negative (Δp85) constructs were a kind gift from Dr. G. Rutter (Bristol). Src wild type, constitutively active (Src Y527F), and dominant negative (Src RF) constructs were kindly donated by Dr. J. Brugge (Harvard). Proteins were separated by SDS-PAGE and membranes were blotted with Phospho T202, Y204-ERK (p-ERK), and a C-terminal targeted ERK 1/2 antibody from Santa Cruz Biotechnology to assess total ERK 1/2. Blots are representative of three independent experiments with similar results.

4.2.5 INS-1 (832/13) cells express functional GIP receptors which stimulate cell growth

Coupling of the GIP receptor to phosphorylation of the ERK1/2 module implies that this incretin can regulate cell fate. The studies presented in sections 4.2.5 and 4.2.6 of Chapter 4 were designed to identify a potential role for GIP in the control of cellular proliferation and survival of INS-1 β-cells. Since GIP receptors in the INS-1 clone 832/13 cell line had not been previously characterized, ligand binding, adenylyl cyclase stimulation, and insulin secretory responses to GIP were initially studied (Figure 30). Cells expressed receptors at a density of 1571 ± 289 binding sites/cell (n=3) with an IC_{50} for binding of 21.1 ± 2.49 nM (n=3) and a K_D = 531 ± 22 pM (n=3); cAMP production was stimulated by GIP with an EC_{50} of 4.70 ± 1.81 nM (n=4)); 5.5 mM glucose stimulated insulin secretion was potentiated by 10 nM GIP (1.63 ± 0.18 % total insulin secreted for 5.5 mM glucose vs. 2.44 ± 0.29 % total insulin secreted ($p < 0.05$, n=3)). These data compare well with other insulinoma cell lines tested for GIP binding, cAMP production, and insulin release (Amiranoff et al. 1984; Kieffer et al. 1993) (unpublished observations). Furthermore, we have also confirmed the presence of functional GLP-1 receptors on INS-1 (832/13) cells (IC_{50} for binding of 5.37 ± 0.62 nM (n=3) and an EC_{50} of 1.37 ± 0.62 nM (n=4) for cAMP production).

The INS-1 cell line has been previously established by Rhodes and co-workers as a cellular model for β-cell proliferation (Hugl et al. 1998; Dickson et al. 2001). Based on previous work presented in this Chapter demonstrating that GIP could regulate MAP kinase signaling in INS-1 cells (Ehses et al. 2002A), we wanted to establish that GIP was indeed a growth and survival factor. GIP was found to potentiate 11 mM glucose mediated β-cell proliferation over 24 h (Figure 31A) to levels comparable to those obtained with GH (GIP stimulated growth to 158 ± 16 % of that in 5.5 mM glucose; while GH promoted growth to 158 ± 9 % of control (n=3-5)). In a separate experiment (Figure 31B), 100 nM GIP stimulated cell growth to 131 ± 7 % of that measured in the presence of 5.5 mM glucose, similar to the proliferative responses to 100 nM GLP-1 (129 ± 4 %; n=4).

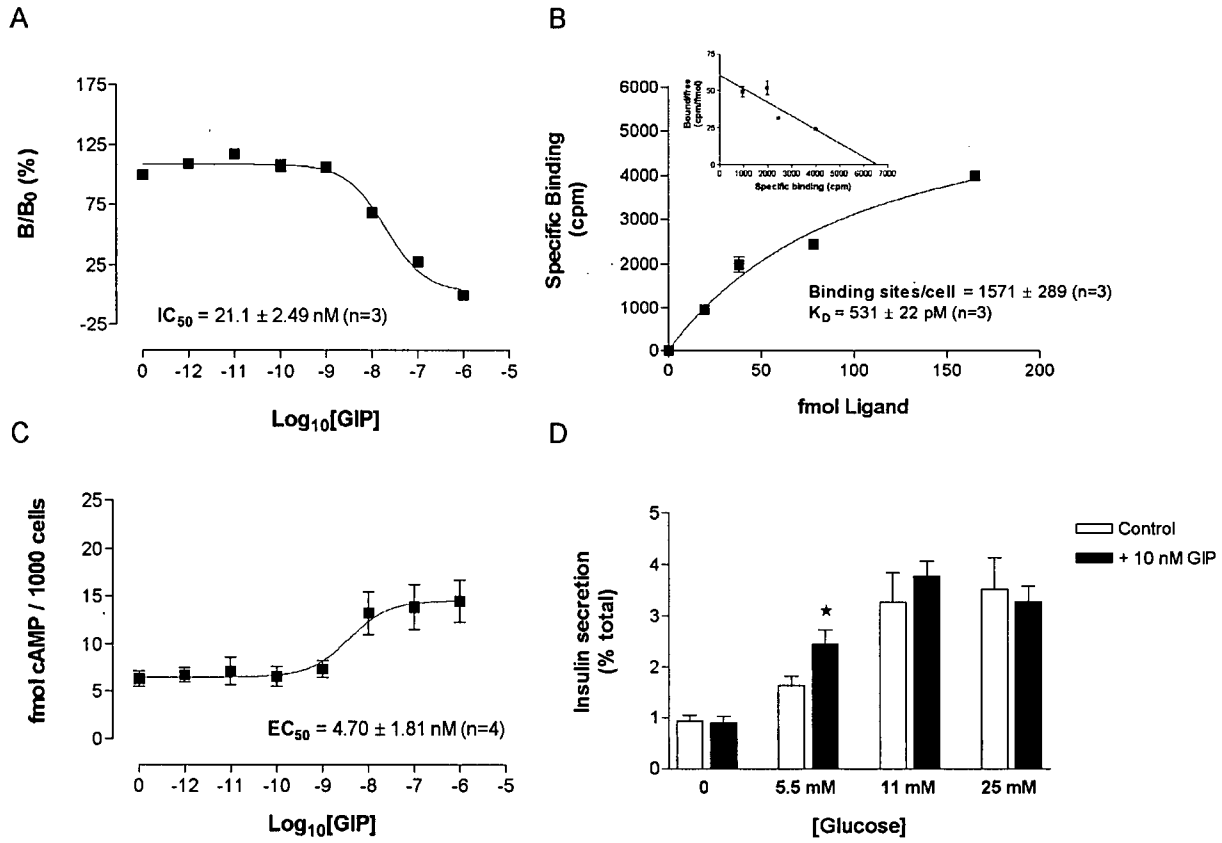


Figure 30: INS-1 (832/13) cells express functional GIP receptors coupled to cAMP production and insulin secretion. Cells were plated in 24-well plates at 5 x 10^5 cells/well two days prior to performing competitive binding (A), saturation binding (B), cAMP production (C), and insulin secretion (D) experiments. Protocols were as described in Experimental procedures with values representing means of 3 or 4 experiments performed in triplicate, where * represents $p < 0.05$ (Panel B is a representative plot).

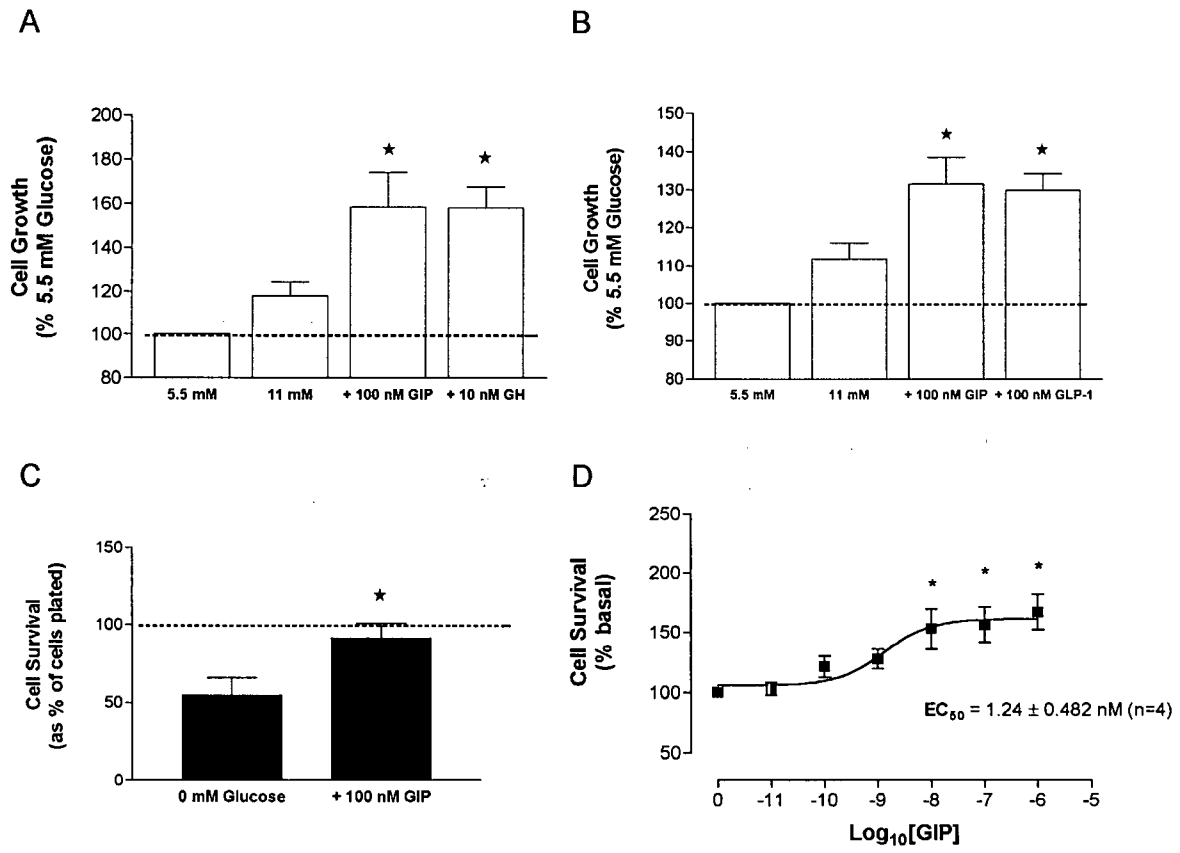


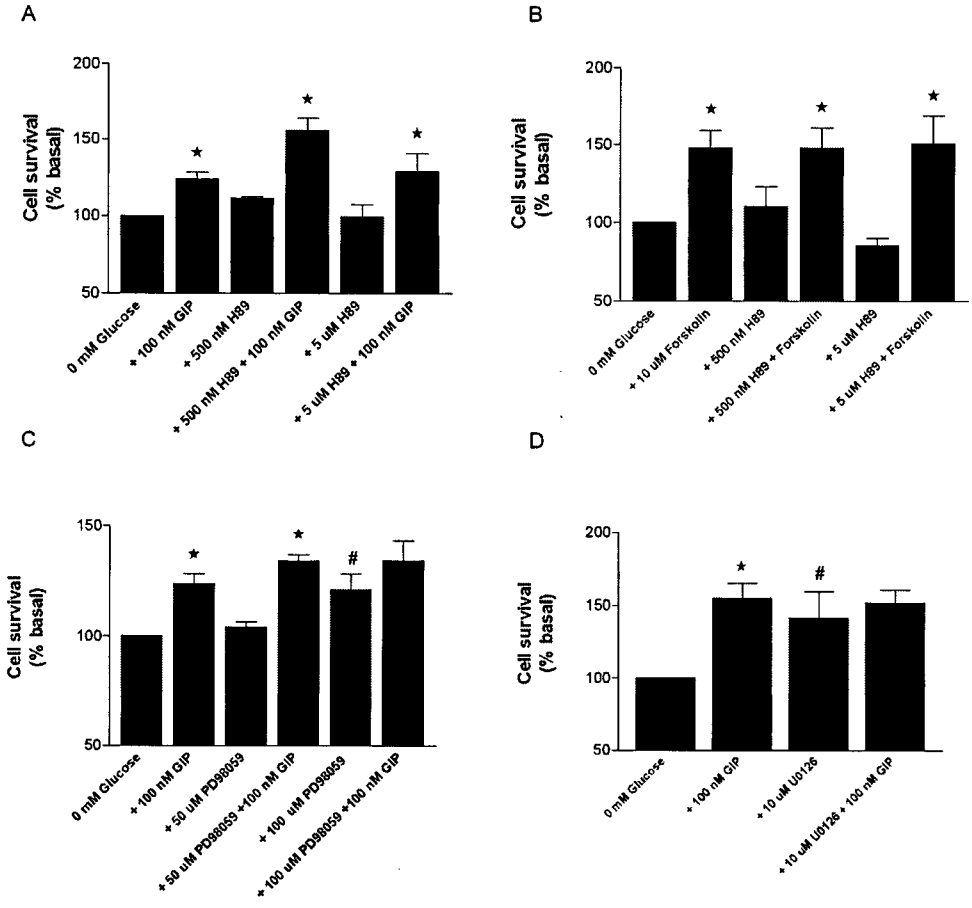
Figure 31: GIP potentiates 11 mM glucose induced cell growth (A, B) and promotes cell survival of INS-1 (832/13) cells (C, D). In all experiments, cells were serum starved before and during the course of the experiment. During cell growth studies (A and B), final cell numbers were always greater than initial plating densities, indicative of mitogenesis; where final cell numbers were quantified fluorometrically by CYQUANT™. In C and D, cells were serum and glucose starved for 48 h, with or without the addition of GIP for the final 24 h. Values are means of 5 (A), 4 (B), 3 (C), and 4 (D) individual experiments done in triplicate, where * represents $p < 0.05$ (Student's t-test or ANOVA with Dunnett's post hoc test).

4.2.6 GIP promotes cell survival via p38 MAP kinase

While determining the glucose-dependence of these growth promotive effects, it was observed that GIP was capable of reversing the detrimental effects of 0 mM glucose media (serum free) on cellular survival. Incubation of cells in the presence of 0 mM glucose media for 48 h resulted in approximately 50 % cell death (Figure 31C and 32A). Surprisingly, 91 ± 10 %

of the cells plated remained viable when the medium was supplemented with 100 nM GIP after 24 h. These cell survival effects of GIP were found to be concentration-dependent with an EC_{50} value of 1.24 ± 0.48 nM GIP (n=4; Figure 31D). Thus, GIP can indeed act as a growth and survival factor in INS-1 cells, even in the complete absence of glucose.

In order to establish which intracellular signaling pathways were involved in the GIP-induced cell survival (with a focus on MAP kinases), studies were performed with pharmacological inhibitors used at concentrations shown to exhibit selectivity for candidate protein kinases (Figure 32) (Ehses et al. 2002A). Stimulation of adenylyl cyclase with forskolin mimicked the effects of GIP on cell survival, but the failure to inhibit the effect of either agent with H89 (Figure 32A and B) indicates a PKA-independent mode of action. Neither of the Mek1/2 inhibitors PD98059 (50 and 100 μM) nor U0126 (10 μM) blocked the effects of GIP on cell survival (Figure 32C and D). The ability of GIP to promote cell survival was further supported by studies on the effect of the PI3Kinase-PKB pathway inhibitor, wortmannin (Figure 32E). Interestingly, cells were partially protected against wortmannin-induced cell loss by GIP treatment (n=3, $p<0.05$). The only compound tested that influenced GIP-mediated cell survival was the inhibitor SB202190 (Figure 32F), indicating that, as with GLP-1 (Buteau et al. 1999; Buteau et al. 2001), GIP can act via modulation of p38 MAPK activity.


A
Cell survival (% basal)
200
150
100
50
0 mM Glucose
+ 100 nM GIP
+ 500 nM H89
+ 500 nM H89 + 100 nM GIP
+ 5 uM H89
+ 5 uM H89 + 100 nM GIP
B
Cell survival (% basal)
200
150
100
50
0 mM Glucose
+ 10 uM Forskolin
+ 500 nM H89
+ 500 nM H89 + Forskolin
+ 5 uM H89
+ 5 uM H89 + Forskolin
C
Cell survival (% basal)
150
100
50
0 mM Glucose
+ 100 nM GIP
+ 50 uM PD98059
+ 50 uM PD98059 +100 nM GIP
+ 100 uM PD98059
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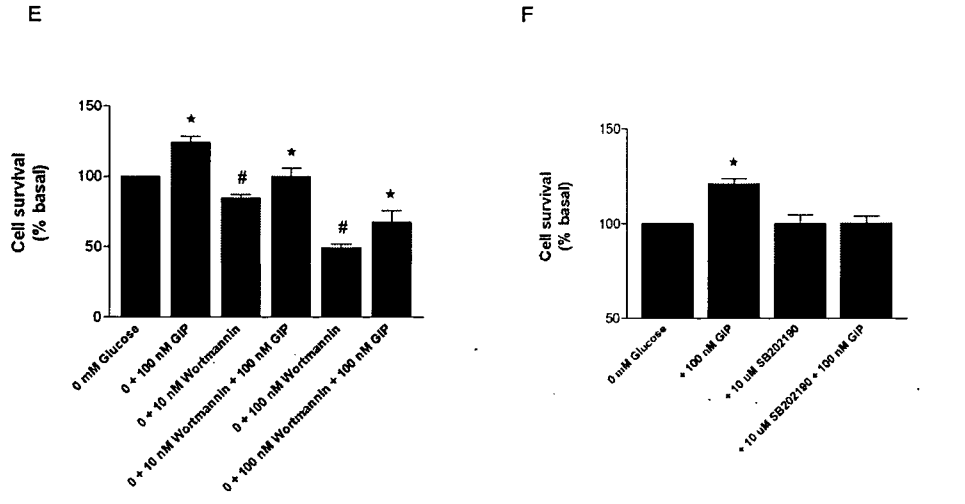


Figure 32: GIP promotion of INS-1 (832/13) cell survival during glucose deprivation involves p38 MAPK. Cells were serum and glucose starved for 48 h with or without the addition of GIP for the final 24 h. Final cell numbers were always less than cells plated, indicative of cell death. Protein kinase inhibitors were added to the medium 15 min prior to the final 24 h culture in the absence or presence of 100 nM GIP. The PKA inhibitor, H89, was unable to reverse GIP (A) or forskolin (B) mediated cell survival. The Mek1/2 inhibitors (PD98059 and U0126) both promoted cell survival with no further effect by GIP (C and D). Wortmannin had deleterious effects on cell survival (E), which were partially reversed by GIP; note the different Y-axis scale. Panel F represents the involvement of p38 MAP kinase, via specific inhibition with SB202190. Final cell numbers were quantified fluorometrically by CYQUANT™, and data represent means of 3-8 experiments done in triplicate, where * and # represent $p < 0.05$ vs. respective controls (Student's t-test).

4.2.7 GIP and cAMP inhibit caspase-3 activity in INS-1 cells

Since the initial discovery that a cysteine protease (CED-3) is involved in apoptosis in the nematode *Caenorhabditis* elegans, caspase activation has been identifiable with the induction of cellular apoptosis (Strasser et al. 2000). The name 'caspase' is derived from the finding that these cysteine proteases cleave after an Asp residue in their substrates (Shi 2002). There are now at least 14 distinct mammalian caspases that are generally divided into initiator caspases (caspases-2, -8, -9, -10) and effector caspases (caspases-3, -6, -7). All caspases are initially found in cells as zymogens, and must undergo proteolytic cleavage to be activated during apoptosis. While initiator caspases are autoactivated, effector caspases, such as caspase-3, are cleaved by initiator caspases resulting in their activation. Effector caspases are then responsible

for the broad proteolytic cleavage resulting in cell death. This includes destruction of structural components, regulatory proteins, inhibitors of deoxyribonuclease, and DNA repair enzymes such as poly (ADP-ribose) polymerase (PARP) (Reviewed by Shi 2002).

To establish whether the cell survival effects of GIP were due to anti-apoptotic actions of the polypeptide, activation of the effector caspase-3 and a screen for various caspases was conducted during glucose deprivation and wortmannin treatment. Figure 33A illustrates that 0 mM glucose promoted caspase-3 activity by 6 h (not by 2 h; data not shown, n=3), and that this effect was completely reversed by the concurrent addition of GIP or forskolin. Greater stimulation of caspase-3 activity was achieved by wortmannin treatment, and responses were also completely inhibited by concomitant GIP treatment ($p<0.05$, n=3; see caspase-3 data, Figure 36D). In further experiments it was shown that GIP treatment 24 h after initiation of caspase-3 activation by glucose deprivation was also able to reverse the activation (data not shown; $p<0.05$, n=3). The selective caspase-3 inhibitor, Ac-DEVD-CHO, and immunoblotting for caspase-3, were used to ensure that measured protease activity was in fact due to caspase-3 (Figure 33B and data not shown). In a recent study from our laboratory, evidence was obtained for a protective effect of the incretins on β-cells in streptozotocin (STZ)-induced diabetes in rats (Pospisilik et al. 2003). Therefore, we examined the ability of GIP to protect against STZ-induced β-cell death. When added 10 min prior to, and during, a 30 min STZ exposure, GIP and GLP-1 were both able to protect completely against the pro-apoptotic (caspase-3 activating) effects of STZ (Figure 33C).

In support of these data, we have begun to screen for various apoptotic proteins involved in GIP suppression of INS-1 cell death. While there was evidence for caspase-9 like activity in INS-1 cells that was completely reversed by GIP and elevated in the presence of wortmannin (Figure 34), no caspase-9 protein was detected. The fluorometric substrate, Ac-LEID-AFC, can also be acted upon by caspases-4 and –5, which may explain this discrepancy. Based on Kinexus KAPS screen data, we have also found that cleavage of pro- caspase-1 α and β, pro-caspase-5, and PARP were all reduced by GIP treatment (Figure 35). In this case, a reduction in pro-caspase protein levels can be interpreted as an increase in the active caspase (Figure 35).

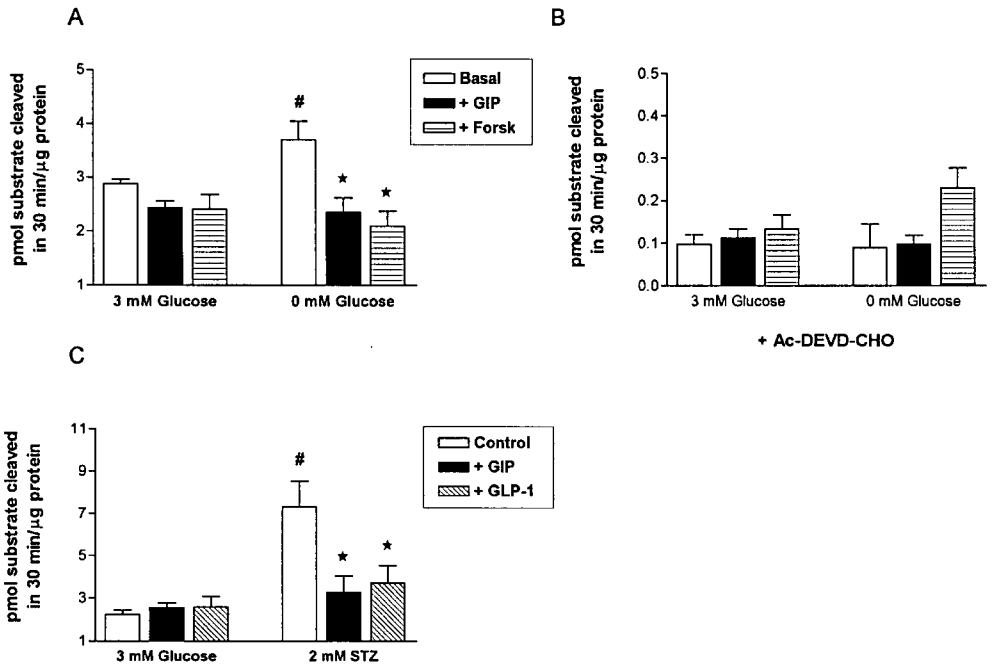


Figure 33: GIP and cAMP inhibit caspase–3 activation induced by 0 mM glucose (A, B) and STZ (C) in INS-1 (832/13) cells. Cells were serum starved before and during the experiment, and 100 nM GIP, 10 µM forskolin, or 100 nM GLP-1 were added for 6 h in the presence and absence of glucose (3 mM), or STZ to assess affects on caspase-3 activity. Caspase-3 activity was quantified using the substrate, Z-DEVD-AMC, over 30 min. Caspase-3 activity was corrected for total protein concentration using the BCA protein assay. Activity was ensured to be specific by using the caspase-3 inhibitor Ac-DEVD-CHO (B). All experiments are representative of n=3 (A, B) or n=5 (C), where * and # represent $p < 0.05$ vs. respective controls (ANOVA with Newman Keuls Multiple comparison post hoc test).

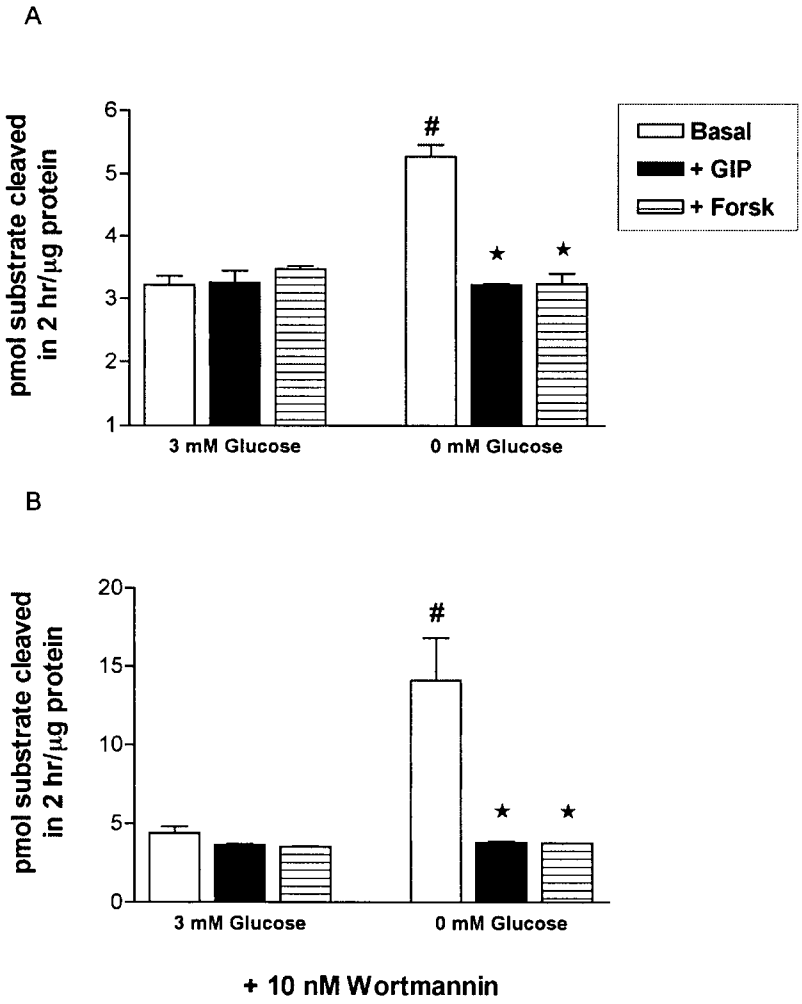


Figure 34: GIP and cAMP inhibit caspase–9 like activity induced by 0 mM glucose (A) and 0 mM glucose + Wortmannin (B) in INS-1 (832/13) cells. Cells were serum starved before and during the experiment, and 100 nM GIP, or 10 μM forskolin were added for 6 h in the presence and absence of glucose (3 mM), without (A) and with (B) 10 nM Wortmannin. Caspase-9 like activity was quantified using the substrate, Ac-LEID-AFC, over 2 h. Caspase activity was corrected for total protein concentration using the BCA protein assay. Experiments are representative of n=3, where * and # represent $p < 0.05$ vs. respective controls (ANOVA with Newman Keuls Multiple comparison post hoc test).

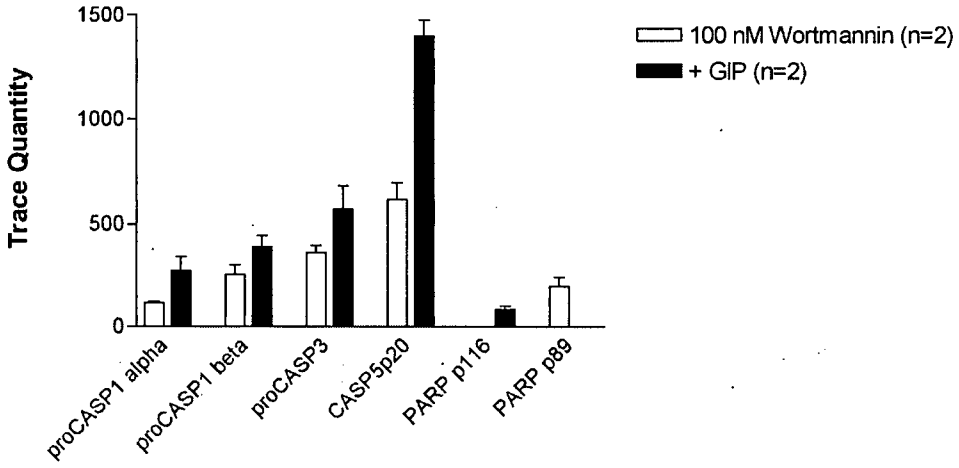


Figure 35: Pro-survival effects on GIP in wortmannin treated INS-1 (832/13) cells. Cells were serum starved before and during the experiment, and cells were stimulated to undergo apoptosis with the addition of 100 nM wortmannin for 6 h, in the absence or presence of 100 nM GIP. Trace quantities are based on densitometry of Western blots (n=2) detecting pro-caspase-1 α/β (proCASP1 alpha/beta), pro-caspase-3 (proCASP3), pro-caspase5 (CASP5p20), PARP p116, and the PARP cleavage product, PARP p89.

4.2.8 MAP kinases (Mek1/2-ERK1/2 and P38) regulate caspase-3 activity in INS-1 cells

A pharmacological approach was also taken to identify the GIP receptor mediated signaling pathway responsible for inhibition of caspase-3 activation, with the objective of correlating these findings to those for survival. In support of the cell survival data, there were no apparent roles for PKA (H89, 5 and 10 μM tested), Mek 1/2 (UO126), or PI3K (wortmannin) in GIP or cAMP inhibition of caspase-3 activation (Figure 36B, C, D). The effect of inhibitors was controlled for by quantifying ERK1/2 phosphorylation (data not shown, n=3). The adenylate cyclase activator, forskolin, was able to mimic GIP actions on caspase-3, however, these actions were also unaffected by the PKA inhibitor H89 (Figure 36A, 37). Thus, we propose that GIP is acting in a cAMP-dependent, but PKA-independent manner in regulating cell survival via caspase-3 inhibition. We have previously shown that GIP can signal via Rap1, the small GTPase upstream of B-Raf, and we therefore tested the effect of various Rap1 constructs (wild type, constitutively active G12V, dominant negative N17) on GIP inhibited caspase-3 activation.

There was no significant effect of any of these (data not shown; n=2). Further, in contrast to forskolin, the Epac-selective analogue, 8-CPT-OMe-cAMP was not capable of reversing caspase-3 activity (Figure 37).

We were somewhat surprised by the lack of affect of the Mek1/2 inhibitor on caspase-3 activation (Figure 38C), given the role for this kinase in regulating the ERK1/2 module and cell survival in other systems. Since the Mek1/2-ERK1/2 pathway is highly regulated by GIP (Kubota et al. 1997; Trumper et al. 2001; Ehses et al. 2002A), we wanted to assess the effect of long-term inhibition of this pathway on caspase-3 regulation by glucose deprivation and GIP. RNA interference (RNAi) studies were performed and found to have maximal effects on Mek1/2 protein levels on days 2 and 3 post transfection. Figure 38A depicts the level of protein knockdown achieved on day 3, with the caspase-3 activity measured on the same day (38B). The dsRNA oligonucleotide targeted at rat Mek1 also influenced Mek2 expression (Figure 38A). It is evident that even partial removal of Mek1/2 results in a parallel reduction in caspase-3 activity under all conditions (basal activity was decreased from 5.6 ± 0.4 to 2.6 ± 0.4 pmol substrate cleaved/30 min; and maximal activity was also decreased from 18.5 ± 3.5 to 6.4 ± 0.7 pmol substrate cleaved/30 min; Figure 38B). The overall elevated caspase-3 activity in these studies was due to the effects of cell transfection, which was also found to increase caspase-3 activity in the aforementioned Rap1 experiments. However, we do not believe this detracts from the interpretation of the results, given the similar profile of activity.

Although the fold caspase-3 activation by glucose deprivation is relatively similar (3.3 ± 0.6 fold for scramble and 2.5 ± .03 for Mek1/2 RNAi; n=3, $p>0.05$), the reversal of caspase-3 activity by GIP is slightly reduced by partial removal of Mek (97 ± 6 % for scramble and 68 ± 10 % for Mek1/2 RNAi; n=3, $p<0.05$). Thus, we believe that there is a modest role for Mek1/2 in the regulation of caspase-3 activity in INS-1 cells, although its exact contribution could not be quantified due to the inability to completely knockdown the Mek isoforms.

Since p38 MAP kinase was implicated in the above cell survival effects of GIP (Figure 32D), we next examined its role in INS-1 caspase-3 activity. Treatment of cells with SB202190 mimicked the effects of GIP and cAMP (Figure 39), indicating that GIP probably acts on caspase-3 activation by inhibiting stress-induced p38 MAP kinase signaling. It therefore appears that that p38 MAP kinase is the major short-term regulator of caspase-3 activity in INS-1 cells, whereas the kinases Mek1/2-ERK1/2 may play a more modest role and regulate long-term caspase-3 activity.

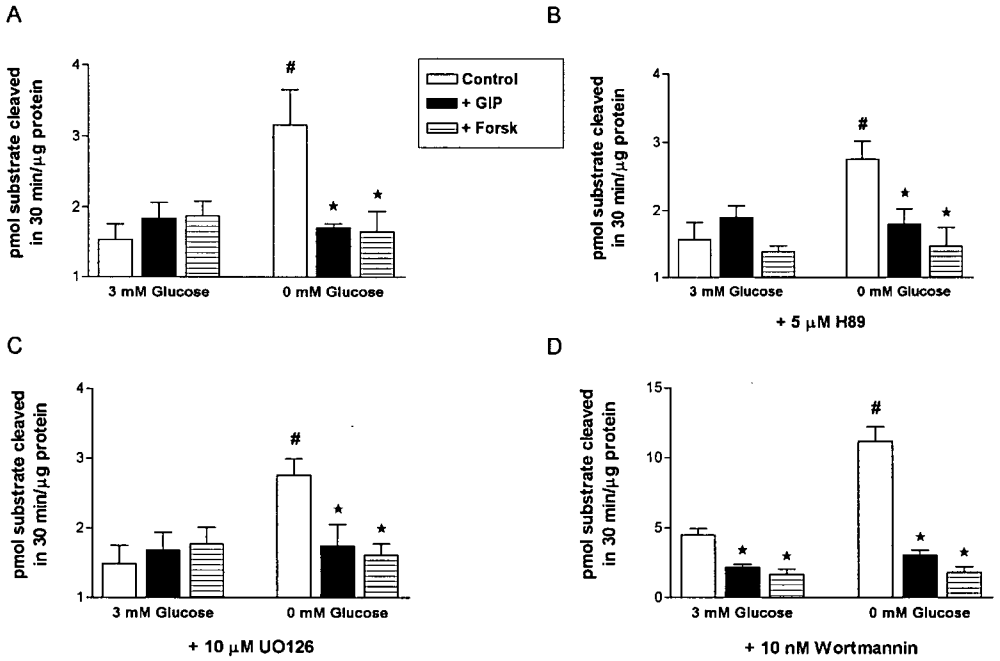


Figure 36: GIP inhibition of caspase-3 activation is independent of PKA, Mek1/2, and PI3K. Protein kinase inhibitors were added to the medium 15 min prior to and during the initiation of further caspase-3 activation by glucose deprivation. GIP was able to completely reverse caspase-3 activation by 0 mM glucose (A) independent of PKA (B), Mek1/2 (C), and PI3K (D). Caspase-3 activity was assessed fluorometrically after 30 min of substrate incubation and corrected for total protein. Data are representative of n=3-4 for all, where * and # represent $p < 0.05$ vs. respective controls (ANOVA with Newman Keuls Multiple comparison post hoc test).

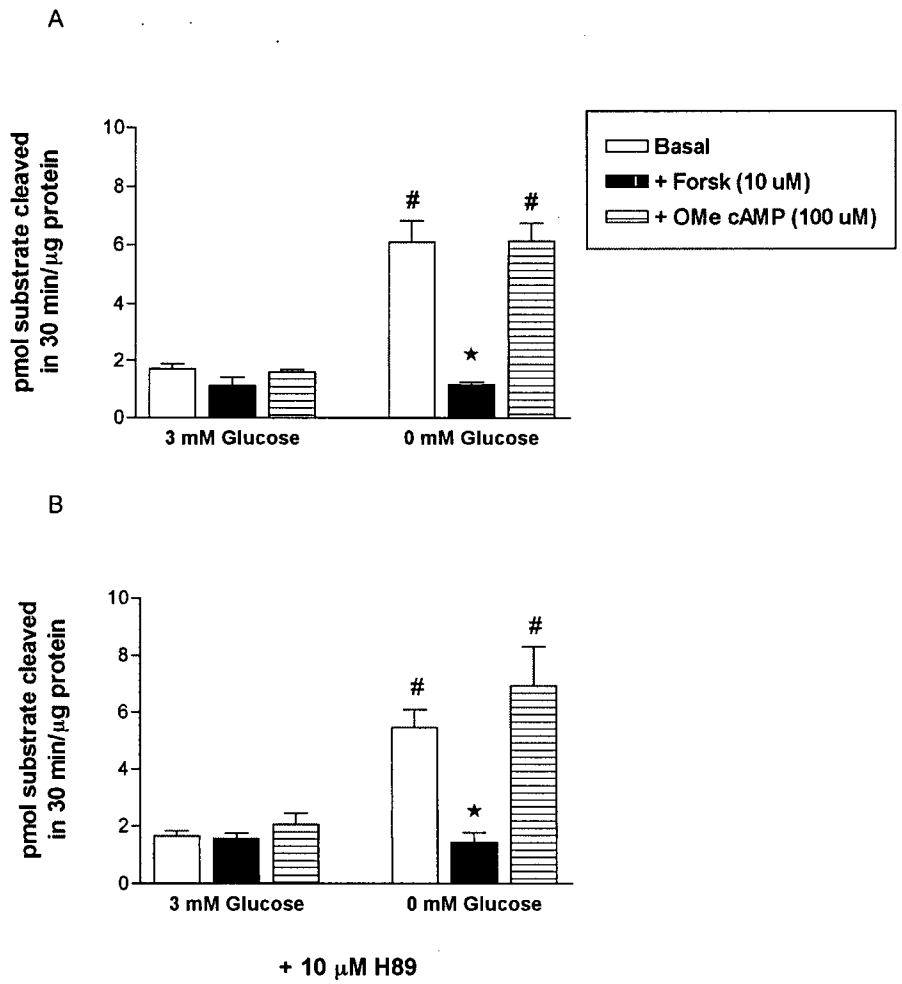
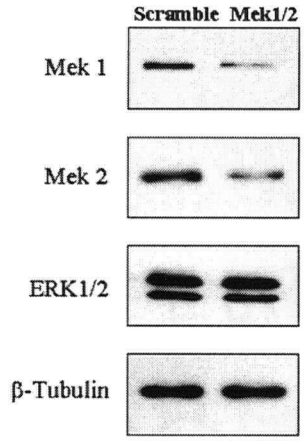


Figure 37: cAMP-mediated inhibition of caspase-3 activation is independent of Epac. The adenylyl cyclase activator, forskolin, and the Epac-selective cAMP analogue, CPT-2OMe-cAMP (Ome cAMP) were added during glucose deprivation for 6 h. In B, the PKA inhibitor H89 was added for 15 min prior to and during the entire experiment. Caspase-3 activity was assessed fluorometrically after 30 min of substrate incubation and corrected for total protein. Data are representative of n=4 (A) and n=3 (B), where * and # represent $p < 0.05$ vs. respective controls (ANOVA with Newman Keuls Multiple comparison post hoc test).

A



B

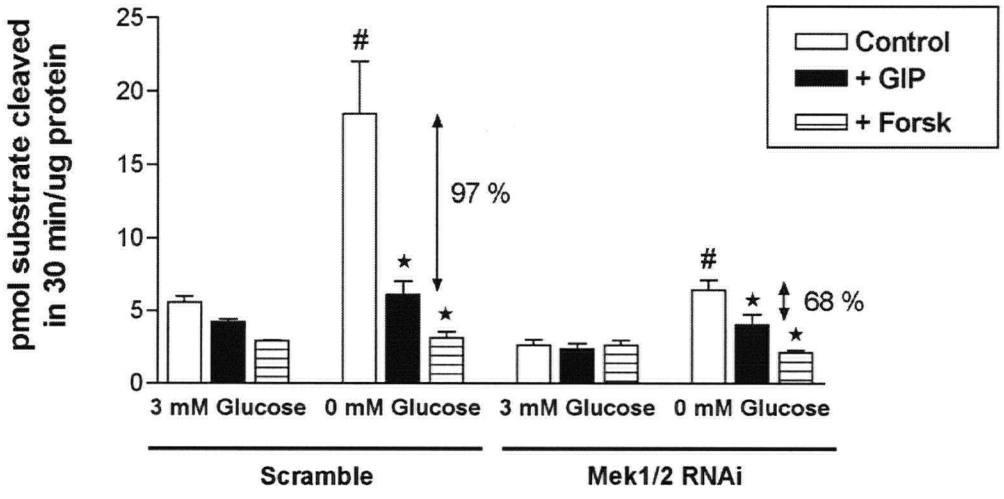


Figure 38: A role for Mek1/2 in the cellular regulation of INS-1 caspase-3 activity. dsRNA oligonucleotides were introduced into cells by Oligofectamine™ and protein knockdown was greatest on day 3 (A), with no effect on total ERK1/2 protein levels. Scramble represents the dsRNA negative control; see experimental procedures for details. Caspase-3 activity (B) was assessed on day 3 post transfection and elevated levels are due to cell trauma endured during transfections. Mek1/2 knockdown caused overall diminished caspase-3 activity with minimal effects on fold induction by glucose deprivation. GIP reversal of caspase-3 activity was significantly affected; 97 ± 6 % inhibition for scramble and 68 ± 10 % for Mek1/2 RNAi; n=3, $p<0.05$. Blots in (A) are representative and data in (B) are representative of n=3, where * and # represent $p < 0.05$ vs. respective controls (ANOVA with Newman-Keuls Multiple comparison post hoc test).

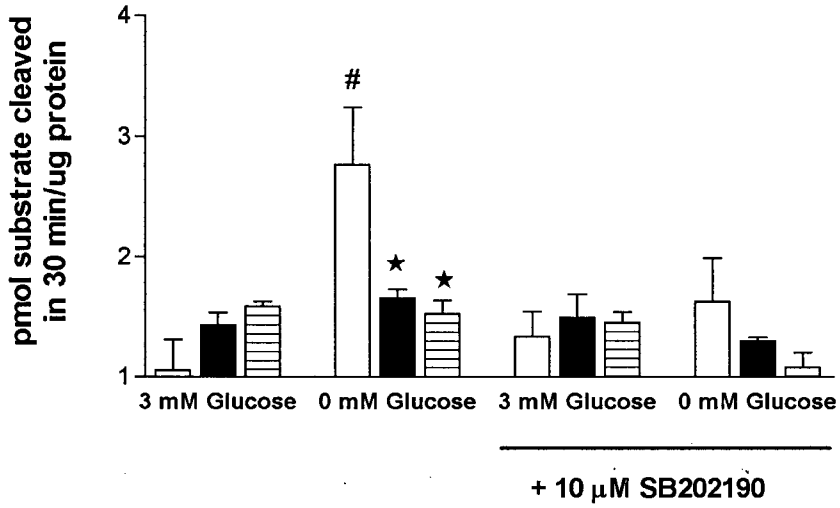


Figure 39: Caspase-3 activation by glucose deprivation is dependent on p38 MAPK activity. The protein kinase inhibitor, SB202190, was added to the medium 15 min prior to and during the initiation of further caspase activation by glucose deprivation. Caspase-3 activity was assessed fluorometrically after 30 min of substrate incubation and corrected for total protein. Data are representative of n=3, where * and # represent $p < 0.05$ vs. respective controls (ANOVA with Newman Keuls Multiple comparison post hoc test).

4.2.9 GIP regulation of p38 MAP kinase phosphorylation and dephosphorylation

In order to test the hypothesis that reversal of caspase-3 activation by GIP results from inhibition of stress-induced p38 MAP kinase activity, phosphorylation of the p38 MAP kinase module (MKK3/6, p38 MAP kinase, ATF-2) by glucose deprivation was studied. In contrast to its effects on ERK1/2, glucose withdrawal led to an activation of the p38 MAP kinase module as assessed by phospho-specific antibodies (Figure 40). Since caspase-3 activation was assessed at 6 h, we examined the phosphorylation of MKK3/6, p38 MAP kinase, and ATF-2 by incubation in 0 mM glucose over this time period. Activation was pronounced and exhibited slow kinetics, reaching a phosphorylation maximum for p38 MAP kinase at 4 h, followed by a decline. Thus, the 4 h time point was chosen to assess the effects of GIP on 0 mM glucose activation of p38 MAP kinase. As predicted, GIP and forskolin were both capable of reversing phosphorylation of p38 MAP kinase induced by either glucose deprivation or wortmannin treatment (Figure 41A

and B). The specificity for activation of p38 phosphorylation was assessed using the inhibitor SB202190 (Figure 41A). While total protein levels were assessed, changes in such long-term phosphorylation may be due to differences in total p38 MAP kinase protein. Examination of upstream MKK3/6 phosphorylation, however, revealed no consistent effects of GIP or forskolin at the 4 h time point. In fact, in the presence of wortmannin, there was no phosphorylation of MKK3/6 or ATF-2, in contrast to the marked phosphorylation of p38 MAPK (data not shown and Figure 41A). These results, therefore, suggest that GIP acting via cAMP is able to reverse caspase-3 activity by promoting long-term dephosphorylation of p38 MAP kinase (via a hereto-unidentified kinase/phosphatase; see Figure 43A).

Since the other incretin hormone, GLP-1, has been shown to activate p38 MAP kinase and thereby regulate cell growth (Buteau et al. 1999; Buteau et al. 2001), and in light of the results shown in Figure 3, we looked at the potential of GIP to activate the p38 MAP kinase module under 3 mM glucose conditions and short-term kinetics (Figure 42). GIP affected phosphorylation of the entire MKK3/6, p38 MAPK, ATF-2 module. Activation of MKK3/6 and p38 MAPK was transient, but significant, with an ensuing reduction in phosphorylation after 1 h that is in agreement with a trend towards dephosphorylation. However, downstream ATF-2 phosphorylation was stimulated by GIP treatment over the entire 2 h time course. This resembled the phosphorylation kinetics of ERK1/2 more than p38 MAPK, due to the sustained activation at 2 h Since Thr^{71} of ATF-2 has recently been identified as a phosphorylation target of ERK1/2 (Ouwens et al. 2002), this may explain these observations and also lend insight into the discrepant findings of ATF-2 phosphorylation noted at 4 h in the study above. Thus, in addition to reversing sustained stress-induced p38 activation by glucose deprivation or wortmannin treatment, GIP is also able to regulate p38 MAP kinase through rapid and transient phosphorylation events (see Figure 43). We propose therefore, that GIP dynamically regulates p38 MAP kinase, rapidly promoting phosphorylation of MKK3/6, p38, and ATF-2 under normal conditions, but promoting dephosphorylation of p38 during long-term stress conditions (no glucose or serum). These two regulatory events are proposed to regulate INS-1 cell growth and apoptosis respectively (see Figure 43).

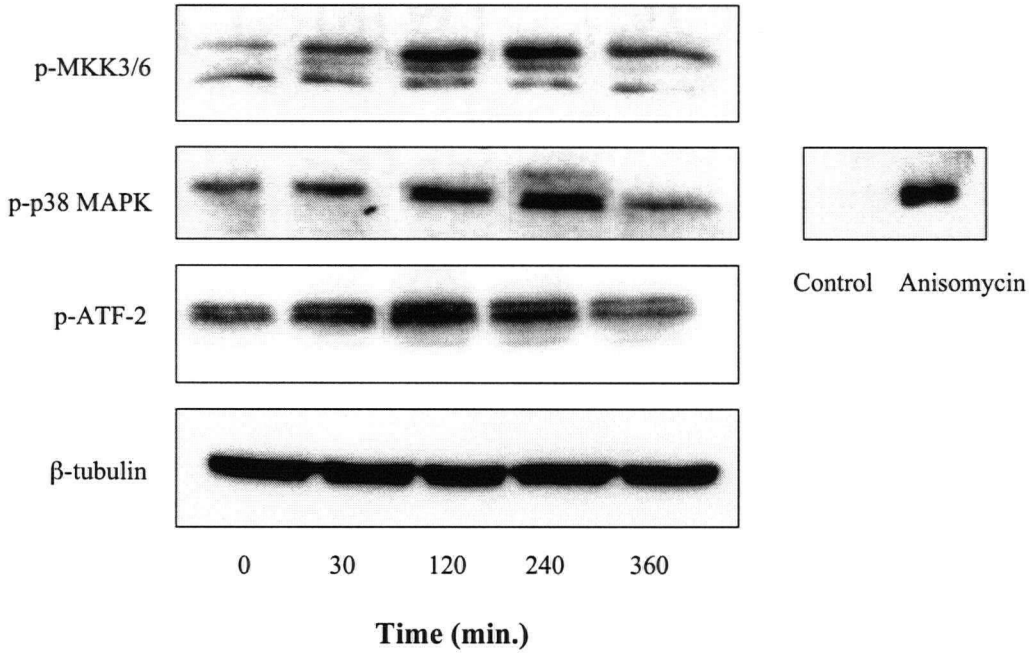
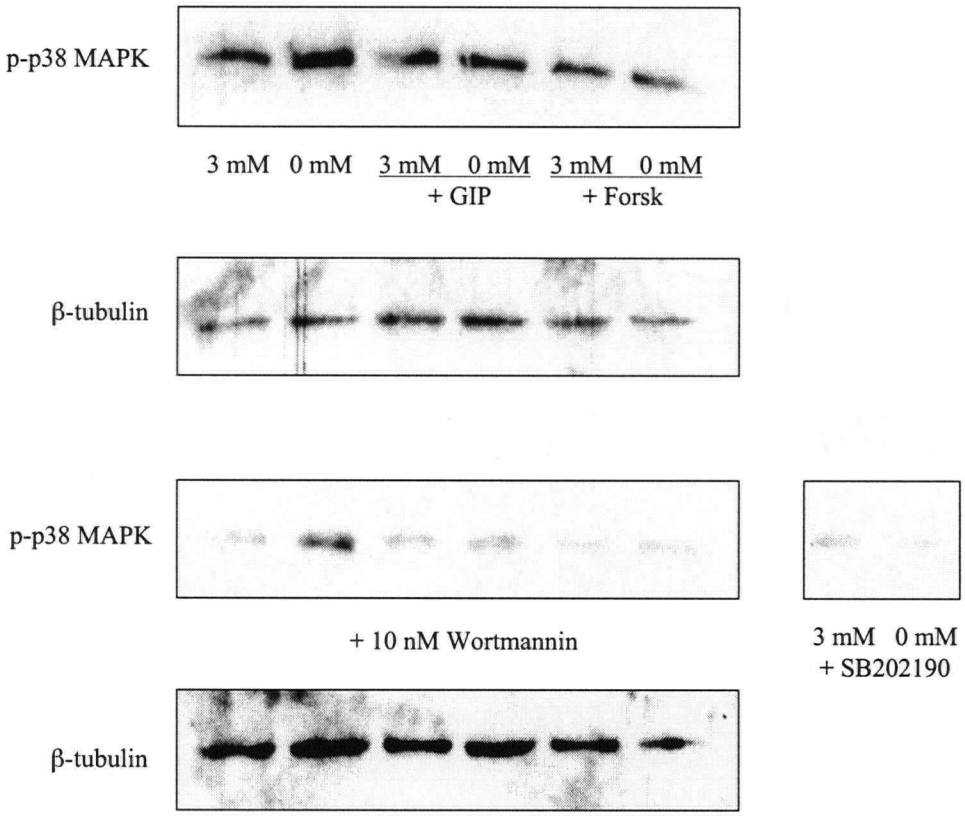


Figure 40: Glucose deprivation induces phosphorylation of MKK3/6, p38 MAPK, and ATF-2 in INS-1 (832/13) cells. Cells were plated in 6-well plates 2 days prior to experiments at 2 x 10^6 cells/well, and serum starved overnight before glucose deprivation. Fifty μg protein samples were separated by SDS-PAGE and membranes blotted with antibodies against Phospho T180, Y182-p38 MAPK (p-p38 MAPK), Phospho S189/207 MKK3/6 (p-MKK3/6), and Phospho T71 ATF2 (p-ATF2). The anisomycin blot shown served as a positive control for phospho-p38 MAPK. Data are representative blots of at least three independent experiments. The far right blot is a positive control depicting phosphorylation of p38 MAPK in cells treated with Anisomycin.

A


p-p38 MAPK
3 mM
0 mM
3 mM
0 mM
+ GIP
3 mM
0 mM
+ Forsk
β-tubulin
p-p38 MAPK
+ 10 nM Wortmannin
3 mM
0 mM
+ SB202190
β-tubulin

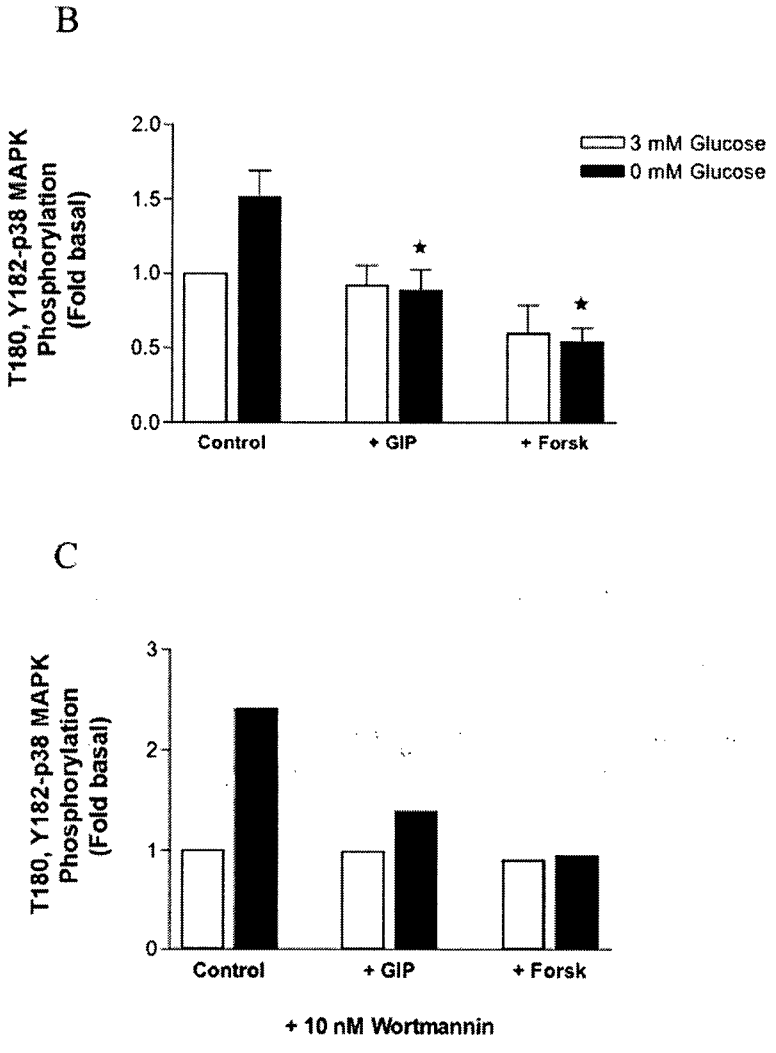
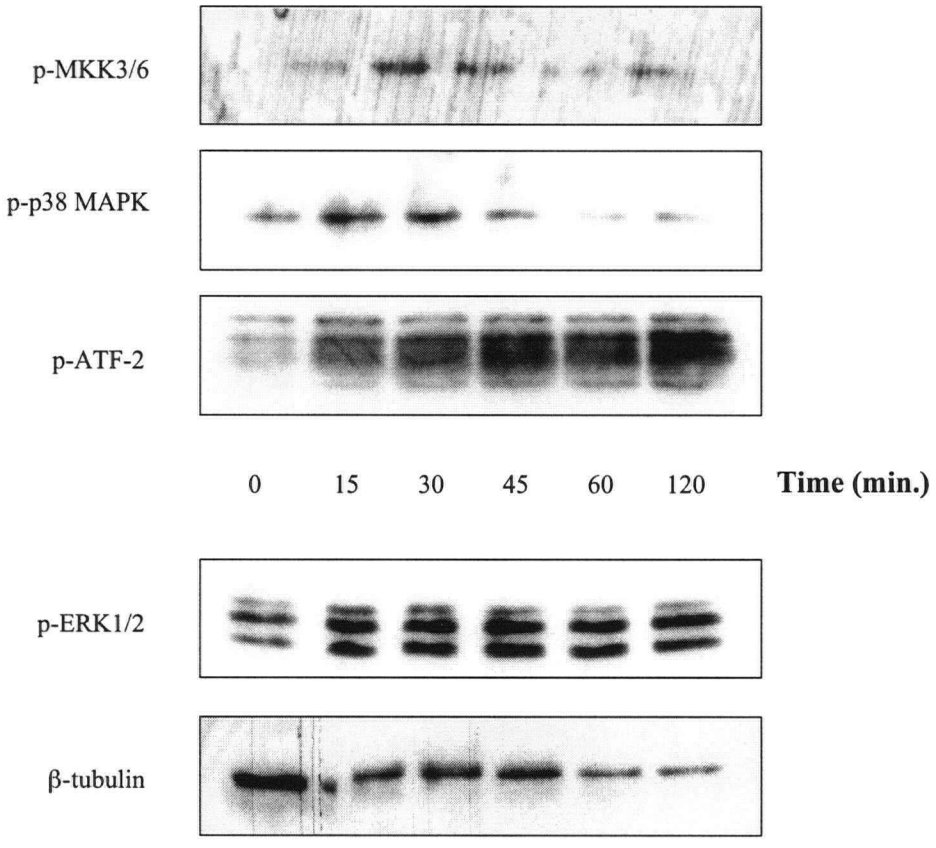


Figure 41: GIP and cAMP reverse long-term phosphorylation of p38 MAPK by glucose deprivation (A, B), or wortmannin treatment (A, C). Based on data from Figure 8, glucose deprivation was sustained for 4 h in the absence or presence of 100 nM GIP or 10 μM forskolin. GIP and forskolin were also able to reverse the activation of p38 MAPK in response to a more potent death stimulus, wortmannin (A, C). Fifty μg protein samples were separated by SDS-PAGE and membranes blotted with antibodies against Phospho T180, Y182-p38 MAPK (p-p38 MAPK). Data are representative blots of three (A, B) and two (+ wortmannin; A, C) independent experiments. Specificity of p38 MAPK phosphorylation was determined using the inhibitor SB202190 (A).

A


p-MKK3/6
p-p38 MAPK
p-ATF-2
0
15
30
45
60
120
Time (min.)
p-ERK1/2
β-tubulin

B

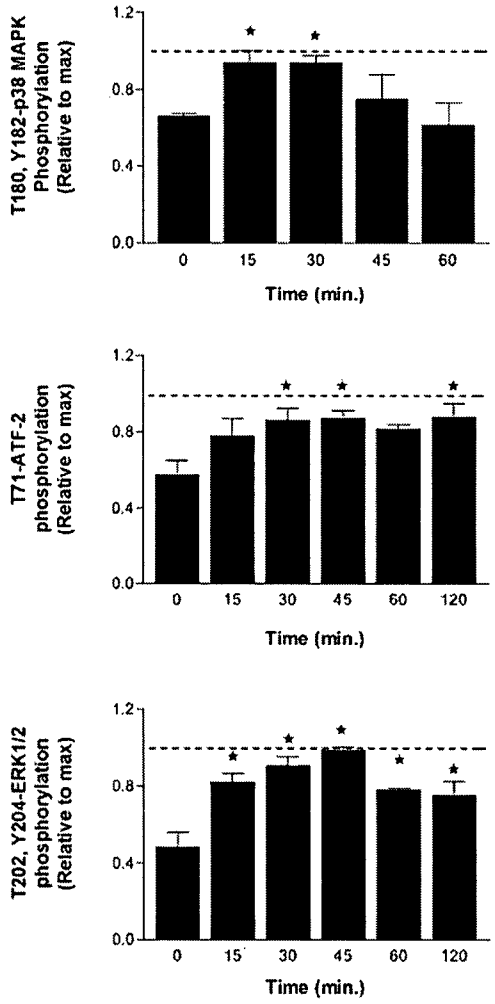


Figure 42: GIP regulates the phosphorylation of MKK3/6, p38 MAPK, and ATF-2 in INS-1 (832/13) cells. Cells were plated in 6-well plates 2 days prior to experiments at 2 x 10^6 cells/well, and serum starved overnight before experiments were conducted in 3 mM glucose RPMI + 0.1 % BSA. Fifty μg protein samples were separated by SDS-PAGE, and probing of the membranes was performed with Phospho T180, Y182-p38 MAPK (p-p38 MAPK), Phospho S189/207 MKK3/6 (p-MKK3/6), and Phospho T71 ATF2 (p-ATF2) obtained from Cell Signaling Technology, and Phospho T202, Y204-ERK1 (p-ERK 1/2), purchased from Santa Cruz Biotechnologies. Densitometry of blots in (A) are depicted in (B). All blots are representative of at least three independent experiments and quantification of phosphorylation is representative of n=3, where * represents $p < 0.05$ vs. respective controls (ANOVA with Dunnett's post hoc test).

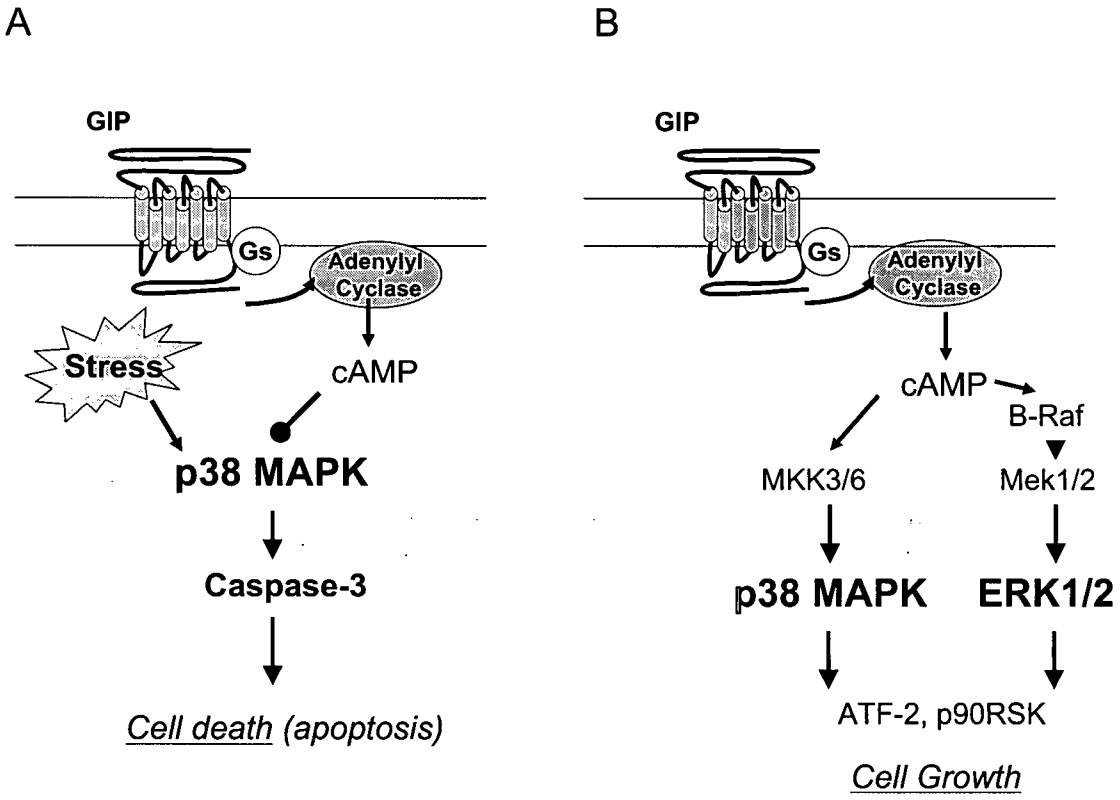


Figure 43: Proposed mechanism underlying GIP regulation of INS-1 cell fate. Under cellular stress induction (A), GIP is able to reverse caspase-3 activation by promoting long-term dephosphorylation of p38 MAP kinase. In contrast, under normal conditions (B), GIP rapidly promotes phosphorylation of the p38 MAP kinase and ERK1/2 module in INS-1 cells. This is thought to contribute to the growth promotive actions of GIP. Hypothesis based on data from the present and previous Chapter. See text for details.

4.3 DISCUSSION

4.3.1 GIP regulation of ERK 1/2 phosphorylation

Several members of the glucagon/PACAP superfamily of peptide hormones have been identified as regulators of MAPK modules. PACAP, glucagon, and the incretin GLP-1 have been shown to positively regulate ERK 1/2 in neuronal PC12 cells (Barrie et al. 1997), HEK cells (Jiang et al. 2001), and in beta and non-beta cells, respectively (Montrose-Rafizadeh et al. 1999; Buteau et al. 2001). Both PACAP and glucagon mediated cAMP elevations were found to regulate these protein-serine/threonine kinases. Until recently, however, the role of cAMP in ERK 1/2 regulation was controversial with some studies demonstrating positive effects, and others negative. A recent study on the glucagon receptor provided the first insight into how this superfamily of peptide hormones may regulate ERK 1/2 activity in a cAMP-PKA-dependent manner (Jiang et al. 2001). Data in the current Chapter demonstrates that GIP can activate the ERK 1/2 module via proximal cAMP-PKA-Rap1 activation, with a proposed role for B-Raf in the positive regulation of this module.

In attempting to delineate GIP receptor signaling pathways in CHO and β-cells, we have begun to map the expression of a wider range of protein kinases and phosphatases (Table 1-5). While various protein kinases have been identified in pancreatic β-cells, few have been identified in a single cell simultaneously (Jones and Persaud 1998). We confirmed the presence of four intact MAPK modules (Raf1/B→Mek1/2→ERK1/2, Cot/Tpl-2→Mek4/7→SAPK, PAKα→Mek6→ p38α, and ERK3) in rGIP-15, βTC-3, and INS-1 (832/13) cells (Table 1). We did not assess the levels of the p38 MAPK activator Mek3, because we could not identify a reliable antibody that was commercially available. However, another p38 MAPK activator Mek6 was clearly detected in the βTC-3, and INS-1 (832/13) cells. By assessing the relative expression levels of the identified kinases, it is possible to generate predictions on regulatory pathways that may be particularly important in various cell types, and select established cell lines that more closely resemble primary cells in the architecture of their signaling networks.

In most cell types, cAMP inhibits cell growth, and initial evidence detailing the molecular basis for this effect focused on cAMP inhibition of ERK 1/2 activity. This was mapped to occur at the level of Raf-1, via PKA mediated phosphorylation of S43 (Wu et al. 1993), S259 (Dhillon et al. 2002B), and S621 (Mischak et al. 1996), resulting in enzyme inhibition. However, recent studies have now highlighted a role for cAMP in positively

regulating ERK 1/2 activity in pituitary, ovarian, and neuronal cells. The basis for this interaction has been most extensively studied in the rat pheochromocytoma PC12 cell line. These studies have culminated in a model where cAMP activates ERK 1/2 when B-Raf is expressed in cells (Houslay and Kolch 2000). Unlike Raf-1, which undergoes complex protein interactions to regulate its activity, B-Raf is mainly regulated by small GTPase proteins (Kolch 2000). The key regulator is the small G-protein, Rap1, which is directly activated by elevated cAMP levels via PKA (Altschuler et al. 1995; Houslay and Kolch 2000). Recent work has demonstrated that Rap1 can be activated by both PKA-dependent and –independent mechanisms. Furthermore, data indicate that the amount of 14-3-3 binding protein associated with B-Raf may also explain the tissue specific effects of cAMP on B-Raf and ERK 1/2 activity (Qiu et al. 2000). The presence of B-Raf in our cell model supports the notion that cAMP mediated signaling via GIP can positively influence ERK1/2 activity.

We propose, therefore, that GIP stimulates ERK1/2 by influencing B-Raf activity in pancreatic β-cells. In support of this hypothesis, other GPCRs have also been shown to regulate ERK 1/2 activity via B-Raf and Rap1. For example, the adenosine receptor (A_{2A}), the prototypical β_2-adrenergic receptor, and the M_1 muscarinic receptor have all been found to stimulate ERK1/2 via Rap1 and B-Raf in CHO, HEK, and PC12 cells, respectively (Seidel et al. 1999; Schmitt and Stork 2000; Guo et al. 2001). These cells all express the Raf isoform B-Raf, and are therefore able to activate ERK1/2 via cAMP signaling. Since Rap1 is able to increase GIP-mediated ERK1/2 activity, it is likely that PKA-dependent activation of Rap1 is able to regulate B-Raf and thereby activate Mek1/2→ERK1/2→p90RSK.

Neuronal and endocrine cells share many similar phenotypic features. It may be expected therefore, that cAMP can positively influence ERK 1/2 activity and cellular fate in both cell types. Cyclic-AMP regulation of ERK 1/2 activity in CHO cells has been controversial, with studies showing both activating and inhibitory affects (Stevetson et al. 1993; Verheijen and Defize 1997; Seidel et al. 1999). A previous report investigating GIP signaling demonstrated an inhibitory affect of cAMP on ERK1/2 activity in CHO cells (Kubota et al. 1997). In the present study, however, we report that cAMP is able to positively influence the ERK1/2 module in both CHO (rGIP-15) and β-cells (INS-1, βTC-3, and BrinD11 cells). This similarity in cAMP actions in these cell lines, together with the similar expression level of protein kinases profiled in our cell model versus β-cell lines, supports our model system as a means for elucidating GIP receptor signaling.

It may seem counterintuitive for GIP to inhibit Raf-1 activity, while positively regulating downstream ERK 1/2 via Rap1 activation (Figure 26). However, similar results have been found for neuronal growth factor (NGF) and β_2-adrenergic receptor signaling (York et al. 1998; Schmitt and Stork 2000). The balance between Raf-1/B-Raf activation or inhibition represents a mechanism by which downstream kinetics of ERK 1/2 activity may be regulated, thereby modulating different physiological processes. For example, both NGF and the β_2-adrenergic receptor are able to activate Ras and ERK 1/2 rapidly and transiently via Raf-1 (York et al. 1998; Schmitt and Stork 2000). However, sustained (prolonged) activation of the ERK 1/2 module occurs through B-Raf in these systems. This attractive model may explain the molecular basis responsible for regulating differentiation (sustained ERK1/2 activation) versus proliferation (transient ERK1/2 activation) in PC12 cells. While we provide evidence for extremely rapid kinetics of ERK1/2 activation, there may be prolonged effects mediated by GIP via Raf-1 in β-cells, which still need to be elucidated. While GIP is able to regulate β-cell proliferation (Trumper et al. 2001; Ehses et al. 2002B), the second incretin hormone, GLP-1, has recently been identified as both a proliferation and differentiation factor in β-cells and their precursor ductal cells (Xu et al. 1999; Buteau et al. 2001; Hui et al. 2001; Tourrel et al. 2001). Thus, one could envision a similar control of cellular fate by the incretins at the level of the pancreas, as is the case for NGF and neurons.

The GIP stimulated cAMP-PKA-Rap1-ERK pathway identified here may be an important mechanism by which GIP regulates cellular proliferation/differentiation and/or gene transcription events in pancreatic β-cells. In elucidating this cascade, we have also identified β-cells as another example where cAMP can couple to activation of the ERK 1/2 module. Extrapancreatic GIP target tissues (e.g. adipose tissue or skeletal muscle), which express B-Raf, may also present themselves as systems where cAMP signaling positively influences ERK module activation. Interestingly, the antagonism of Raf-1 by GIP may represent an important balance by which GIP can regulate transient and/or sustained ERK 1/2 module activation. Together with recent data identifying GIP as a growth factor for β-cells, our findings support a role for GIP in regulating the molecular events responsible for such biological functions.

4.3.2 MAPK signaling and GIP-mediated survival actions

It has been suggested that the etiology of both type 1 and 2 diabetes mellitus involves a reduction in the mass of functional pancreatic β-cells. In order to maintain euglycemia, β-cell mass must be held relatively constant through a dynamic process that involves neogenesis and/or differentiation, proliferation, and apoptosis (Bonner-Weir 2000, 2001). Only recently, have the growth factors and hormones responsible for maintaining this equilibrium been identified, and they include glucose, insulin, prolactin, growth hormone, insulin-like growth factor (IGF), and most recently the incretin, GLP-1. From recent work (Trumper et al. 2002) and the current studies, it is evident that GIP stimulates β(INS−1)-cell proliferation, as well as promoting cell survival. The proliferative effects of GIP on INS-1 (832/13) β-cells were comparable to those obtained with two established growth factors for pancreatic β-cells, GH and GLP-1, while the cell survival effects may be a common property of the glucagon superfamily, since GLP-2, PACAP, and VIP have also been shown to exhibit this property (Vaudry et al. 2000; Yusta et al. 2000). While these data are limited to cell models, we have recently found a dysregulation of islet size in GIPR -/- mice (Pamir et al. 2003), in addition to a protective role for both GIP and GLP-1 in STZ-induced diabetic rats (Pospisilik et al. 2003), implying a physiological role for GIP in the regulation of cell fate.

A decrease in glucose transport has recently been shown to be an essential component of the execution pathway during cytokine- and growth factor-induced cell death (Moley and Mueckler 2000; Plas and Thompson 2002). Clearly, glucose depletion or withdrawal is sufficient to activate this apoptotic cascade on its own (Moley and Mueckler 2000; Pipeleers et al. 2001; Plas and Thompson 2002). Glucose deprivation is commonly studied in the context of cardiac and neuronal hypoxia/ischemia, however, in the current study we employed this treatment as a simple method of activating the apoptotic cascade and causing cell death. Our data support this notion of apoptotic cell death induced by glucose deprivation in beta cells; cell numbers were decreased by serum-free 0 mM glucose media (Figure 31C), caspase-3 and caspase–9 like activity were activated (Figure 33), and the stress kinase module, p38 MAP kinase, was activated (MKK3/6 → p38 MAPK → ATF-2) (Figure 40). Similar activation of SAPKs and p38 MAP kinase occur after withdrawal of NGF or nutrients from PC12 cells or neuronal cultures (Xia et al. 1995).

GIP signals via cAMP in beta cells and non-beta cells, and to date all physiological actions of GIP have been shown to be dependent on this pathway (Amiranoff et al. 1984; Pederson 1994; Wheeler et al. 1995; McIntosh et al. 1999; Ehses et al. 2001, 2002A). Despite the contrasting action of cAMP in numerous cell types, the studies described in this Chapter illustrate that cAMP is able to positively regulate the Raf → ERK1/2 → p90 RSK pathway in INS-1 cells (Ehses et al. 2002A). Similar to neuronal cAMP actions, the regulation of the ERK1/2 module by cAMP in beta cells seems to underlie its ability to regulate cell fate. Interestingly, the mitogenic actions of cAMP (which are cell-type specific) often correlate well with its survival actions (Cass et al. 1999; Mei et al. 2002). Our present data support this notion, insomuch as cAMP (forskolin stimulation) is also able to promote cell survival in beta cells (Figure 32) by inhibiting caspase-3/9 activation (Figure 33) and reversing prolonged p38 MAPK activation (Figure 40). The secretin/glucagon peptide family members, PACAP and GLP-2, are also both able to inhibit activation of caspase-3 via a postulated cAMP-dependent pathway (Vaudry et al. 2000; Yusta et al. 2000, 2002), implying a common signaling property of these class II GPCRs.

Our data also suggest that the survival actions of GIP are partially PI3K independent in INS-1 cells, since wortmannin was unable to affect the influence of GIP on cell survival, caspase activity (capase-1 α/β, caspase-3, caspase-5, caspase-9 "like") or p38 MAPK phosphorylation (Figure 32, 34, 35, 36, 41). These findings concur with GLP-2 studies (in the case of reversing cycloheximide-induced cell death), where GLP-2 actions were also shown to be PI3K- and Mek1/2-independent, while being mimicked by cAMP (Yusta et al. 2000). A recent study, also identified a PKA- and PI3K-independent mechanism for GIP mediated cell survival (Trumper et al. 2002). The present findings extend these studies, however, by demonstrating that GIP is able to regulate cell fate through regulation of stress-induced p38 MAPK activity. Cyclic AMP has also been reported to directly regulate both PKB and GSK-3β, independently of PI3K activity (Filippa et al. 1999; Li et al. 2000). Interestingly, in recent studies of GLP-2 actions it was suggested that cAMP can act on GSK-3 downstream of PKB (Yusta et al. 2002). Since both PKB and GSK-3β have also been shown to be regulated by GIP in INS-1 cells (Trumper et al. 2001), a common property of the secretin/glucagon receptor family may be to promote cell survival via cAMP-dependent regulation of PKB and GSK-3β. Nevertheless, the current study indicates that regulation of p38 MAPK is central to the anti-apoptotic actions of GIP and cAMP in INS-1 cells.

It is generally accepted that the balance between ERK and p38/JNK pathways determines whether an extracellular stimulus promotes (beta) cell growth or acts in a detrimental manner (apoptotic) (Mandrup-Pouslen 2001; Matsuzawa and Ichijo 2001). Prolonged p38 MAP kinase activation targets cells to apoptosis, whereas selective activation of ERK generally prevents apoptosis and ensures cell survival. Thus, GIP presents itself as a prime candidate for the enhancement of cell viability by inhibiting long term p38 MAP kinase activation, while concurrently activating ERK1/2 rapidly and for a sustained period (Figures 41 and 42) (Trumper et al. 2001). Furthermore, caspase-3 activity is functionally regulated by both these kinases in β-cells; GIP regulates caspase-3 activity primarily via actions involving p38 MAPK (Figures 39 and 41), but also through Mek1/2 kinases (ERK1/2 activators; Figure 38). The effects of GIP on ERK1/2, however, are insufficient to account for the survival and caspase-3 inhibition facilitated by GIP in β-cells (based on inhibition with PD98059 and U0126, and RNAi studies). This contrasts with the recent proposal that the ERK1/2 pathway is central to the anti-apoptotic actions of GIP (Trumper et al. 2002). However, these studies were conducted in elevated glucose conditions (7.5 mM), making it impossible to distinguish actions of GIP from the autocrine insulin actions, which were absent in the present study.

Interestingly, there are now reports that rapid, transient activation of p38 and/or JNK are correlated with cellular proliferation, whereas, prolonged activation of these stress pathways results in cellular execution (Chen et al. 1996; Roulston et al. 1998; Matsuzawa and Ichijo 2001). In neuronal cells, nerve growth factor (NGF) is able to transiently activate p38 MAPK and thereby regulate neuronal differentiation in PC12 cells (Morooka and Nishida 1998; Zentrich et al. 2002). Furthermore, transient and persistent activations of ERK1/2 also led to different cell fates, since transient activation of ERK by EGF stimulates cell growth in PC12 cells, while sustained activation is also implicated in neuronal differentiation by NGF (Stork and Schmitt 2002). Our present study, in addition to previous work, illustrates that GIP is able to activate MKK3/6 → p38 MAP kinase transiently while inhibiting its long-term activation by stress, and concurrently activating ERK1/2 (and ATF-2) both rapidly and persistently. Thus, based partly on GLP-1 studies (Buteau et al. 1999), we propose that GIP is able to regulate cell survival via rapid/transient activation of p38 MAP kinase (proliferative stimulus) and long-term inhibition in response to stress (anti-apoptotic actions; see schematic Figure 43).

Although much research is now being conducted on mapping the signal transduction networks involved in immune-mediated beta cell apoptosis (Eizirik and Mandrup-Poulsen 2001; Mandrup-Pouslen 2001), there is a paucity of information regarding pro-survival hormones, and

their regulation of these networks. In this Chapter, insight is provided into the role of MKK3/6, p38 MAPK, ATF-2, and Mek1/2 in the survival actions of GIP on INS-1 (832/13) cells. GIP is able to reverse caspase activation in a cAMP-dependent manner through the dynamic regulation of p38 MAPK phosphorylation; furthermore, we propose that GIP is able to regulate both cell growth and cell death via dynamic control of the p38 MAPK module. A modest role for the Mek1/2 (and hence ERK1/2) MAPK pathway in the regulation of caspase-3 activity by GIP was also identified, suggesting that the regulation of β-cell fate by GIP includes the dynamic regulation (transient vs. sustained phosphorylation) of both p38 and ERK1/2 MAPK modules. The regulation of stress kinase pathways by GIP is intriguing since they are also utilized in immune-mediated β-cell attack. Our study therefore further supports the notion that GIP may be therapeutically useful for the modulation of β-cell growth and survival and suggests that an additional contributing factor to type 2 diabetes, where GIP actions are blunted (Lynn et al. 2001, 2003), may be a lack of GIP-mediated proliferative/survival signals at the level of the β-cell.

CHAPTER 5: REGULATION OF THE RAT INSULIN PROMOTER BY GIP

5.1 BACKGROUND

GIP is a major regulator of pancreatic β-cell function (Pederson 1994; Miyawaki et al. 1999), exerting its effects through binding to its G-protein coupled receptor, resulting in an elevation of intracellular cAMP, and the promotion of lipid (Ehses et al. 2001) and protein kinase signaling (Kubota et al. 1997; Trumper et al. 2001, 2002; Ehses et al. 2002A). To date, all functional effects of GIP can be linked to proximal cAMP signaling, including the regulation of insulin exocytosis, and the promotion of β-cell growth and survival (Ehses et al. 2002; Ehses et al. 2002). However, despite evidence supporting a role for GIP in the promotion of insulin gene transcription and protein expression (Fehmann and Göke 1995; Wang et al. 1996), the intracellular mechanisms coupling these physiological actions have not been elucidated.

In addition to *in vitro* studies illustrating that GIP is able to upregulate intracellular insulin stores in the β-cell, we have recently shown that insulin gene expression in the GIPR knockout mouse (GIPR -/-) is significantly reduced (Pamir et al. 2003). This is despite increased compensatory actions of the second incretin hormone, glucagon like peptide-1 (GLP-1), the β-cell actions of which parallel those of GIP. Therefore, the glycaemic control exhibited by GIP is clearly dependent on its long-term regulation of pancreatic insulin content.

The extreme importance of the CRE element in the rat insulin 1 promoter (RIP1) was recently demonstrated in INS-1 cells (Chepurny et al. 2002), however, its regulation by proximal signals still remains enigmatic. The GLP-1 receptor is thought to be coupled to the regulation of the RIP1 via a CREB family transcription factor (Chepurny et al. 2002), however the regulation of CREB itself has not been studied. CREB is one of the most diverse integrators of signal transduction. The paradigm of cAMP/PKA mediated phospho-regulation of serine 133 (S133) has now been expanded to include other protein kinases which can phosphorylate this residue, including p90 RSK, PKC, Akt/PKB, MSK-1 (mitogen and stress activated kinase-1), and MAPKAP-2 (RSK-2; MAPKAP = MAPK activated protein) (Shaywitz and Greenberg 1999; Mayr and Montminy 2001). All of these kinases are potential targets of GPCR activation and the majority have been shown to be involved in Family B GPCR signaling.

The studies described in the current Chapter sought to further elucidate the intracellular mechanisms coupling the GIP receptor to CREB phosphorylation and to regulation of the rat

insulin promoter in β-(INS-1) cells. We hypothesized that GIP signals via cAMP/PKA→CREB to regulate insulin gene transcription.

5.2 RESULTS

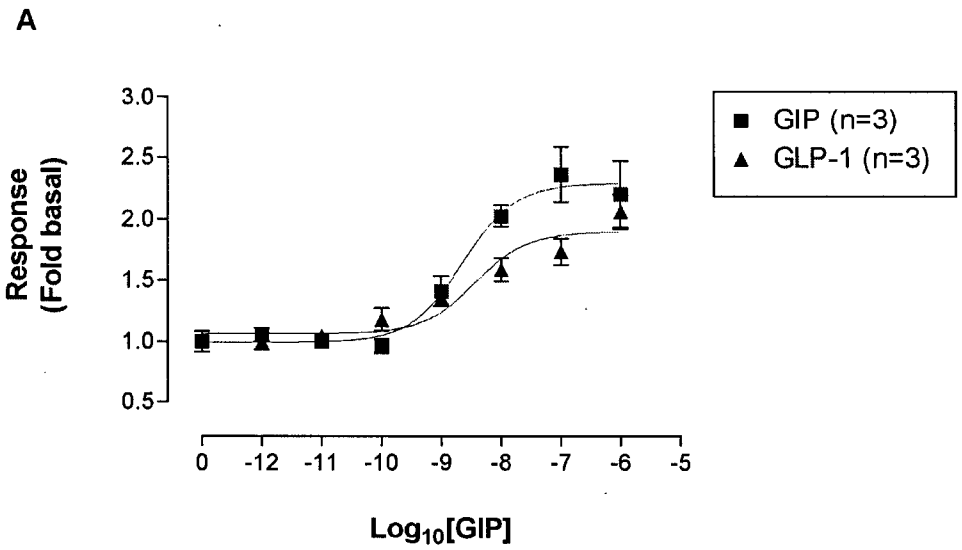
5.2.1 GIP regulates RIP1 via cAMP/PKA

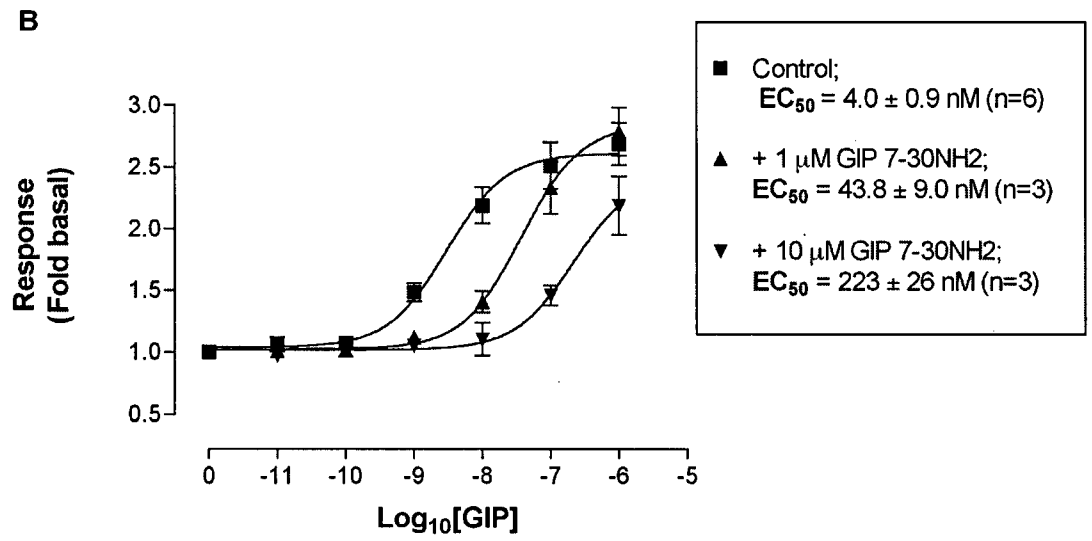
Glucose was found to regulate both RIP1 and RIP2 expressed in INS-1 cells over a 5-22 mM range of glucose (24 h stimulation; see Section 2.14 for details), however, GIP was found to only potentiate RIP1 activity with no effect on RIP2 (Figure 44 and data not shown, n=3). Thus, subsequent experiments focused on GIP receptor regulation of RIP1.

Examination of GIP induced RIP1 activity confirmed earlier findings (Lu et al. 1993A; Fehmann and Göke 1995), and demonstrated that GIP and GLP-1 stimulated RIP1 activity with similar affinity and efficacy over 4 h (EC_{50}= 3.2 ± 1.2 nM vs. 2.7 ± 0.6 nM, n=3; Figure 44). This response was ensured to be due to GIP receptor activation by using the competitive antagonist, $GIP_{7\text{-}30NH2}$, and the biologically inactive fragment $GIP_{3\text{-}42}$ (Figure 44). Preincubation with and concomitant addition of 1 μM and 10 μM $GIP_{7\text{-}30NH2}$ resulted in a significant rightward shift in the EC_{50} of 10 and 50-fold respectively (ANOVA with Dunnett's post hoc test, $p<0.05$). Further, $GIP_{3\text{-}42}$ had no effect on RIP1 activity in INS-1 cells.

Studies were initiated to characterize the effector signaling and protein kinases involved in this response. Due to the importance of cAMP in GIP receptor signaling, we hypothesized that cAMP/PKA would be involved in regulating RIP1 via CREB. However, since ERK1/2 and p38 MAPK signaling systems have been implicated in regulating CREB phosphorylation and we have recently demonstrated a role for GIP in regulating MAPK modules (Chapter 4) (Ehses et al. 2002A; Ehses et al. 2002B), we also investigated a possible role for these kinases in regulating GIP-mediated RIP1 activity. The PKA inhibitor, H89, was able to blunt GIP and forskolin-stimulated RIP1 activity significantly, implicating cAMP/PKA signaling in the mediation of these effects (Figure 45). Despite effects on basal RIP1 activity, U0126 was without effect on GIP reponsiveness and these results were confirmed by RNAi knockdown of Mek1/2, the upstream regulator of ERK1/2 (Figure 45 and 46). Inhibition of p38 MAPK enhanced the effects of GIP on RIP1 (Figure 45). These findings are in agreement with previous studies on GLP-1 (Kemp and Habener 2001), and the effects of stress on insulin gene expression (Kaneto et al.

2002). Further experiments investigating a role for arachidonic acid in regulating RIP1 activity found no effect by this fatty acid (data not shown; n=3). Thus, proximal cAMP/PKA signaling is integral to the regulation of RIP1 activity by GIP.





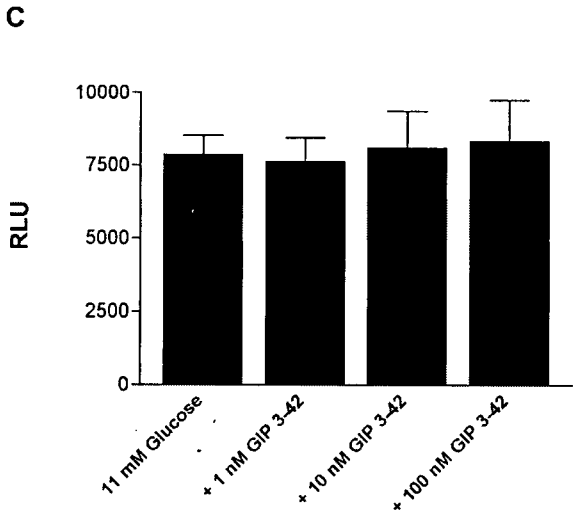


Figure 44: The GIP receptor stimulates rat insulin 1 promoter (RIP1) activity in INS-1 β-cells. INS-1 cells were transfected with the -410 RIP1 promoter reporter construct and stimulated for 4 h in 11 mM glucose RPMI media in A-C. In B, cells were preincubated with $GIP_{7\text{-}30NH2}$ for 15 min prior to and during GIP stimulation resulting in a significant increase in EC_{50} (ANOVA with Dunnett's post hoc, $p<0.05$). Addition of the biologically inactive fragment $GIP_{3\text{-}42}$ had no effect on RIP1 activity (C; n=3). Stimulation was terminated by the addition of Steady-Glo luciferase assay buffer and luciferase activity was quantified using a Turner Design (TD 20/20) luminometer.

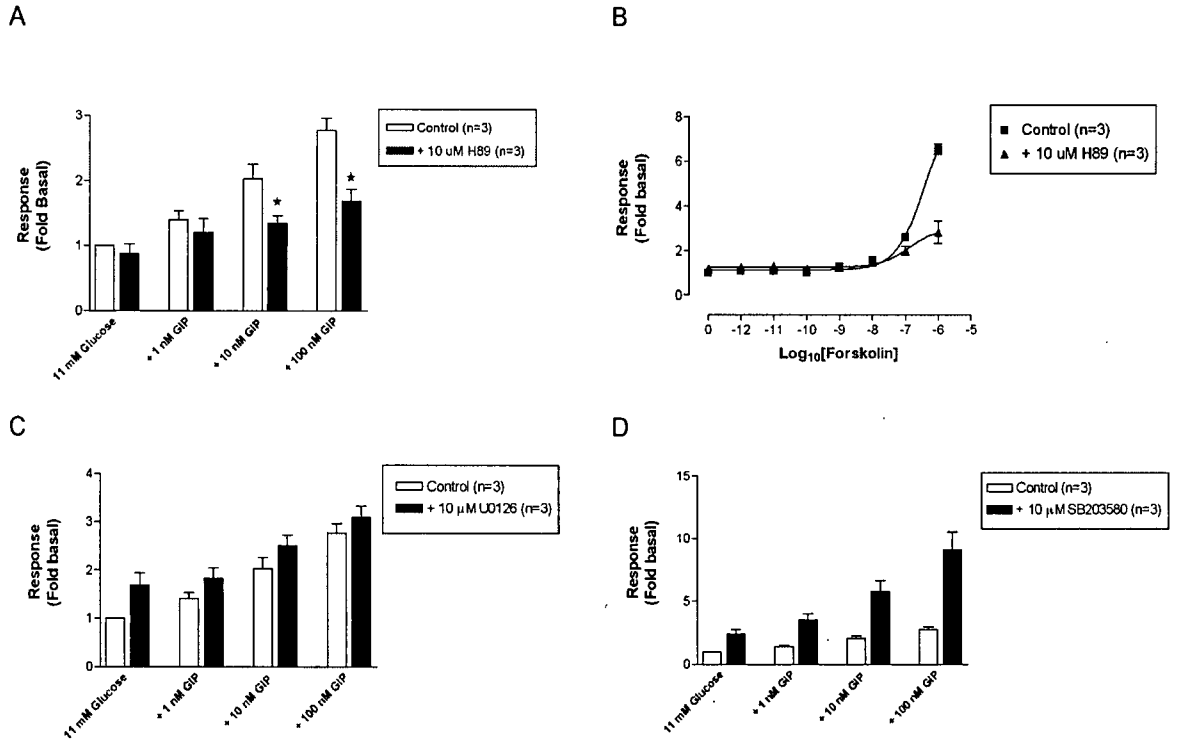


Figure 45: Evidence that GIP signals via cAMP/PKA to regulate RIP1 activity in INS-1 β-cells. INS-1 cells were transfected with the -410 RIP1 promoter reporter construct and preincubated with the indicated inhibitors for 15 min prior to and during stimulation with GIP or forskolin. GIP and forskolin stimulated RIP1 via activation of PKA (A, B), but GIP actions were not affected by inhibition of Mek1/2 (C), and were potentiated by inhibition of p38 MAPK (D). All data are representative of n=3, for experiments done in triplicate, where * represents $p<0.05$, as tested by Student's t-test.

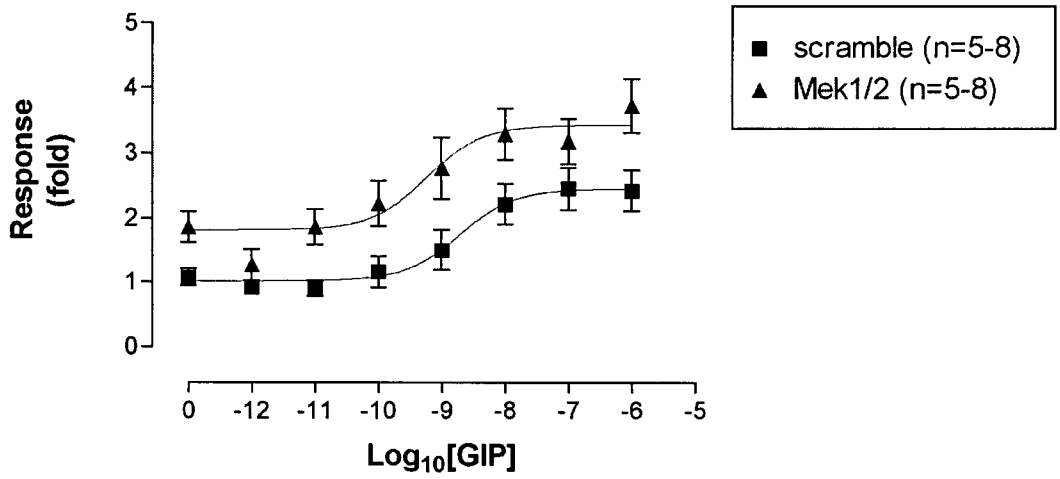


Figure 46: Effect of RNAi knockdown of Mek1/2 on GIP-stimulated RIP1 activity in INS-1 β cells. dsRNA oligonucleotides were introduced into cells (day 0) by Oligofectamine™ and protein knockdown was greatest on day 3 (see Figure 38), with no effect on total ERK1/2 protein levels. Scramble represents the dsRNA negative control; see experimental procedures for details (Section 2.13). INS-1 cells were transfected with the -410 RIP1 promoter reporter construct 24 h after dsRNA transfection (day 1), plated on day 2, and the experiment was conducted on day 3. As with U0126 treatment (Figure 45), knockdown resulted in elevated basal RIP1 activtiy with no effect on EC_{50}, 2.2 ± 1.1 for scramble and 3.4 ± 2.9 for Mek1/2 (n=5).

5.2.2 GIP activates RIP1 via a CREB family transcription factor

Based on the importance of the CRE element in mediating GLP-1 effects on the rat insulin promoter (Skoglund et al. 2000; Chepurny et al. 2002) and the role of cAMP/PKA in regulating RIP1, we investigated the effect of various CREB constructs on the ability of GIP to stimulate RIP1 activity in INS-1 cells. The dominant negative A-CREB is a mutant in which the basic residues within the bZIP domain have been mutated to acidic residues (Vinson et al. 2002). This mutant is thought to heterodimerize with wild type CREB and thereby inhibit its DNA binding. However, due to the high homology of CREB family members, A-CREB does not differentiate between CREB, ATF-1, or CREM (Shaywitz and Greenberg 1999). A-CREB was able to almost ablate the ability of GIP to drive RIP1 activity over the entire concentration-response profile (Figure 47). The M1-CREB mutant contains a S133A mutation rendering CREB non-phosphorylatable on S133; M1-CREB occupies the CRE without allowing wild type CREB to bind. In support of findings with GLP-1, the M1-CREB construct was unable to interfere with GIP responsiveness. It therefore came as no surprise that the gain-of-function mutant Thy143Phe or F/Y CREB (Du et al. 2000) also had no effect on GIP signaling to the RIP1 (Figure 47). Taken together with the effects of these constructs on the ability of forskolin to regulate the rat insulin promoter (Figure 47), it appears that GIP promotes RIP1 activity via a CREB family transcription factor, independent of Serine 133 CREB phospho-regulation.

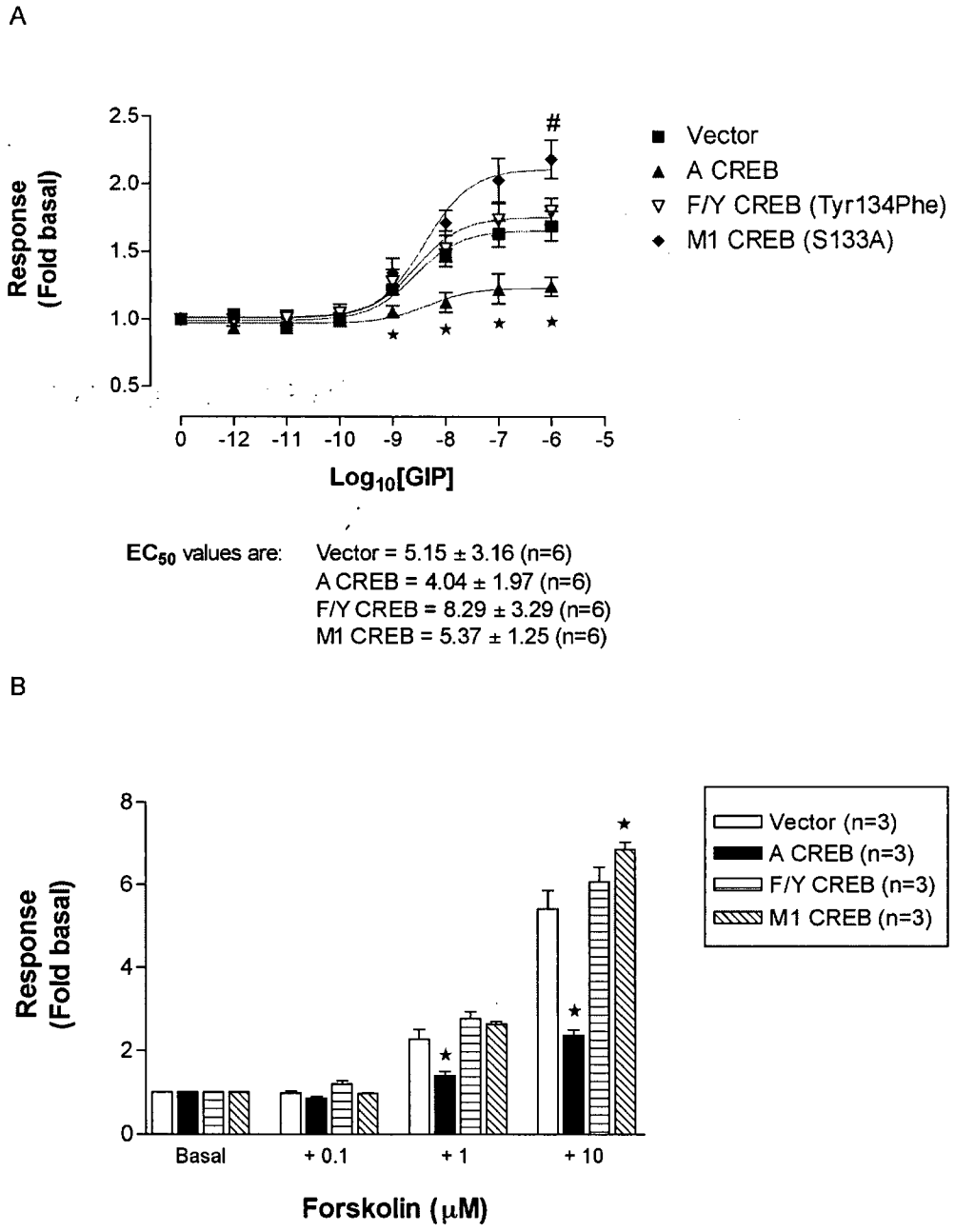


Figure 47: GIP stimulates RIP1 activity via a CREB family transcription factor in INS-1 β-cells. INS-1 cells were cotransfected with the -410 RIP1 promoter reporter and various CREB constructs. Cells were stimulated for 4 h with GIP (A) or forskolin (B) in 11 mM glucose RPMI media containing 0.1 % BSA, and cells were lysed with Steady-glo luciferase assay buffer prior to quantification of luciferase activity. Statisical significance was tested with ANOVA and Dunnett's post hoc test, where * or # represents $p<0.05$ vs. vector control. There was no significant difference between EC_{50} values ($p>0.05$).

5.2.3 GIP stimulates CREB/CREM/ATF-1 phosphorylation

Relatively few studies have investigated the phospho-regulation of CREB/CREM/ATF-1 in β-cells. Due to the importance of a CREB family transcription factor in mediating the effects of GIP on the rat insulin promoter (Figure 47), we investigated the phospho-regulation of CREB/CREM/ATF-1 by GIP in INS-1 β-cells. The monoclonal antibody we used has previously been shown to detect all three CREB family members in INS-1 cells (Kemp and Habener 2002). Figure 48 demonstrates that activation of the GIP receptor results in transient phosphorylation of S133 CREB, S117 CREM, and S63 ATF-1 with maximum phosphorylation occurring by 15 min and a return to basal by 60 min. In fact, we detected phosphorylation of all three bZIP family members as early as 5 and 10 min (data not shown, n=2). The kinetics of these phosphorylation events are consistent with transcriptional events mediated via CREB (Mayr and Montminy 2001). Glucose (11 mM) was without effects on CREB/CREM/ATF-1 phosphorylation despite overnight incubation in 3 mM glucose prior to stimulation. The concentration dependence of this response was thus investigated at 15 min in the presence of 11 mM glucose (Figure 49). GIP was able to phospho-regulate all three CREB family transcription factors, with significant increases in the picomolar range (CREB and ATF-1) and nanomolar range (CREM; $p<0.05$, n=3), and maximal responses of 3 (pCREB and pATF-1) to 5-fold (pCREM).

Recently, phospho-regulation of Serine 142/143 was implicated in neuronal Ca^{2+}-mediated transcriptional effects via CREB (Kornhauser et al. 2002). Since GIP has been shown to increase influx of Ca^{2+} in islets and β-cells (Wahl et al. 1992; Lu et al. 1993B; Wheeler et al. 1995), we investigated the ability of GIP to regulate these phospho-sites. Despite evidence for basal phosphorylation of S142/143 in INS-1 cells, we found no effects due to cell depolarization or GIP treatment on these sites (data not shown and Figure 49). However, INS-1 cell depolarization (with high K^+) was able to increase phosphorylation of S133 CREB/S117 CREM/S63 ATF-1 (data not shown). The phospho-regulation of S142/143 has also not been reproduced by other groups (M. Montminy, personal communication).

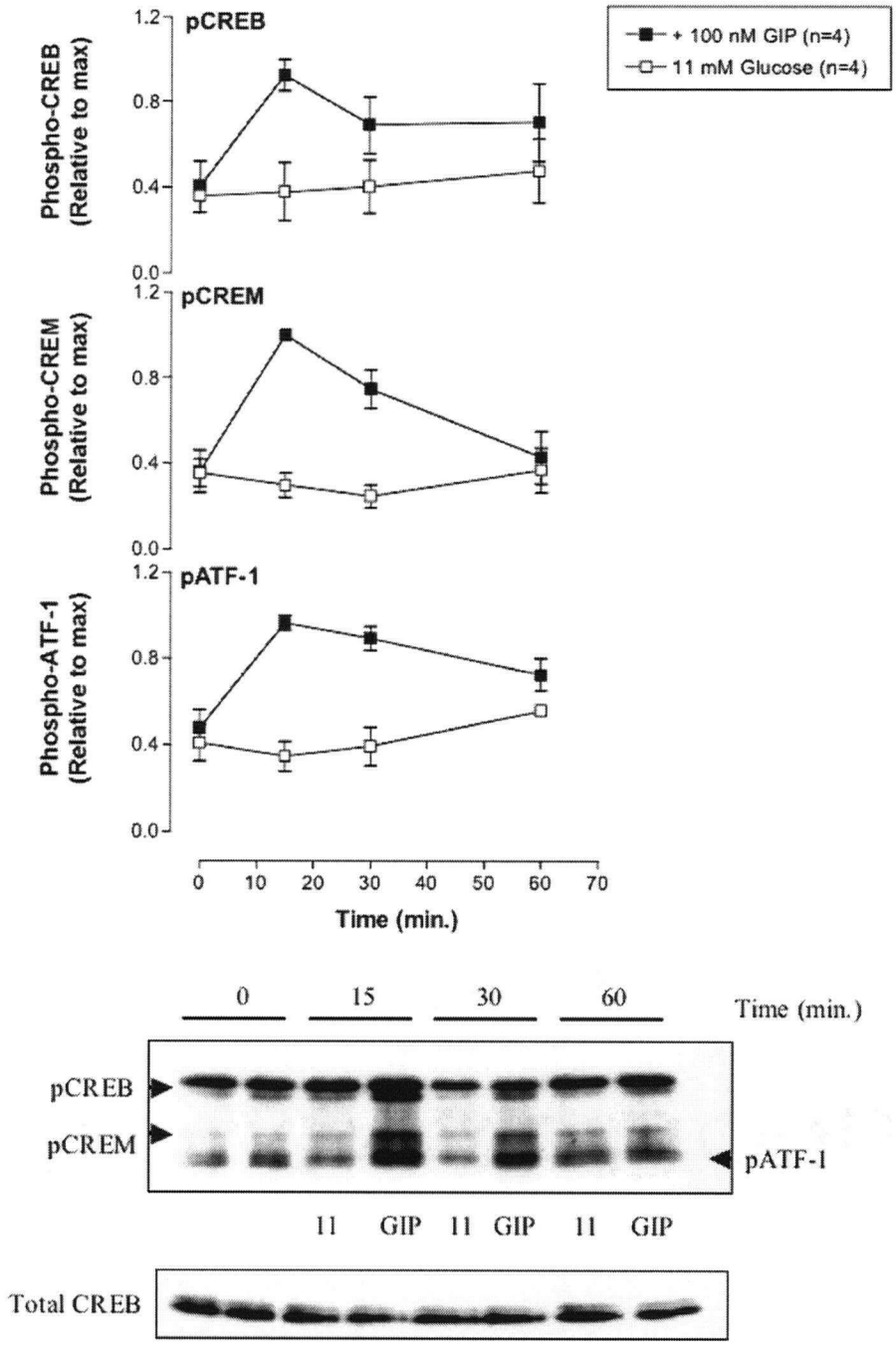


Figure 48: Temporal profile of GIP stimulated phosphorylation of S133 CREB/S117 CREM/S63 ATF-1 in INS-1 β-cells. INS-1 cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight and stimulated for the indicated time points with 11 mM glucose or glucose + 100 nM GIP. Thirty-μg protein samples were separated by SDS-PAGE and membranes were blotted with a phospho-CREB (pCREB) antibody, which also recognizes phospho-CREM and –ATF-1 (pCREM, pATF-1), and an antibody recognizing total CREB. Blots were quantified using densitometric analysis and are representative of n=4.

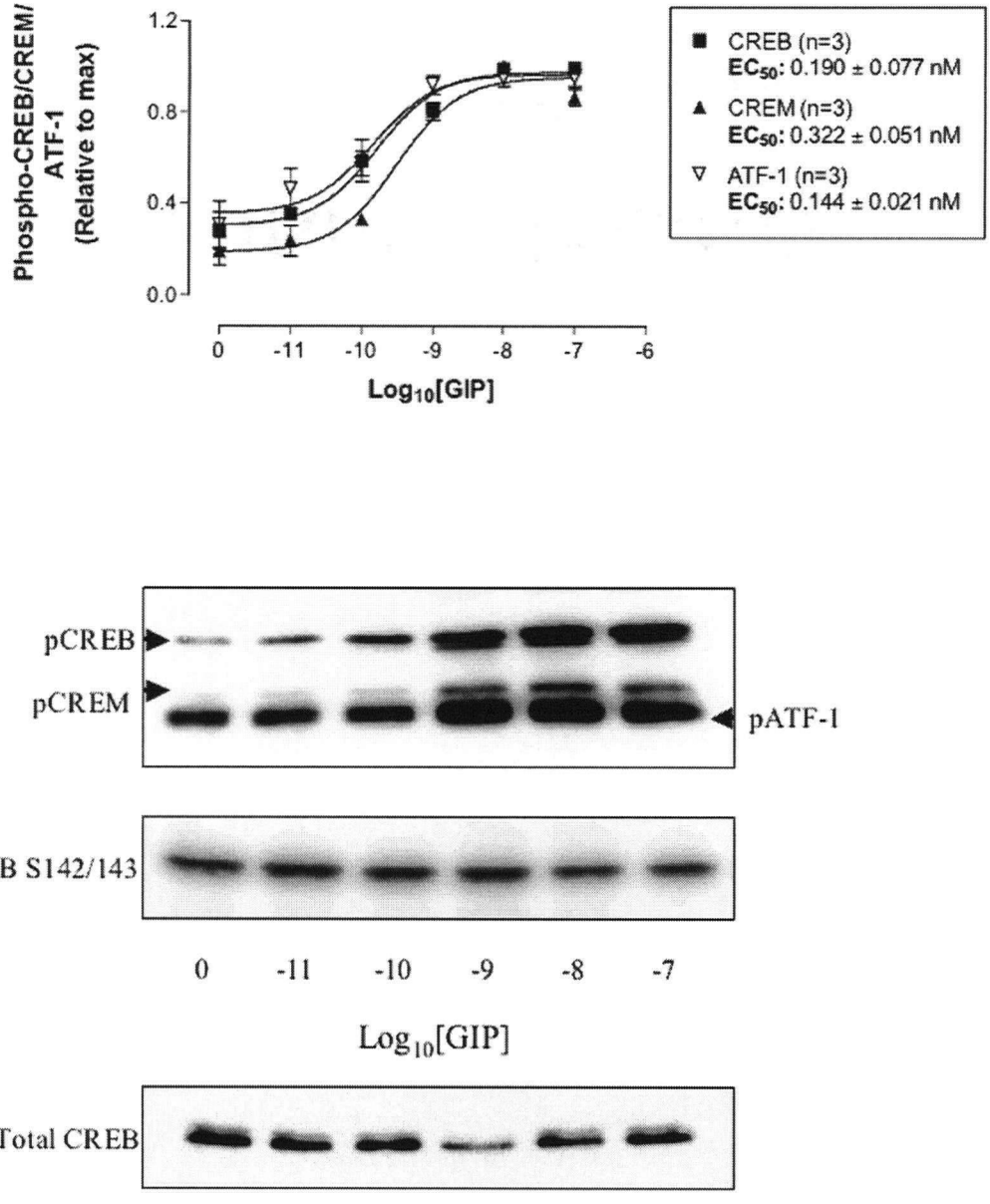


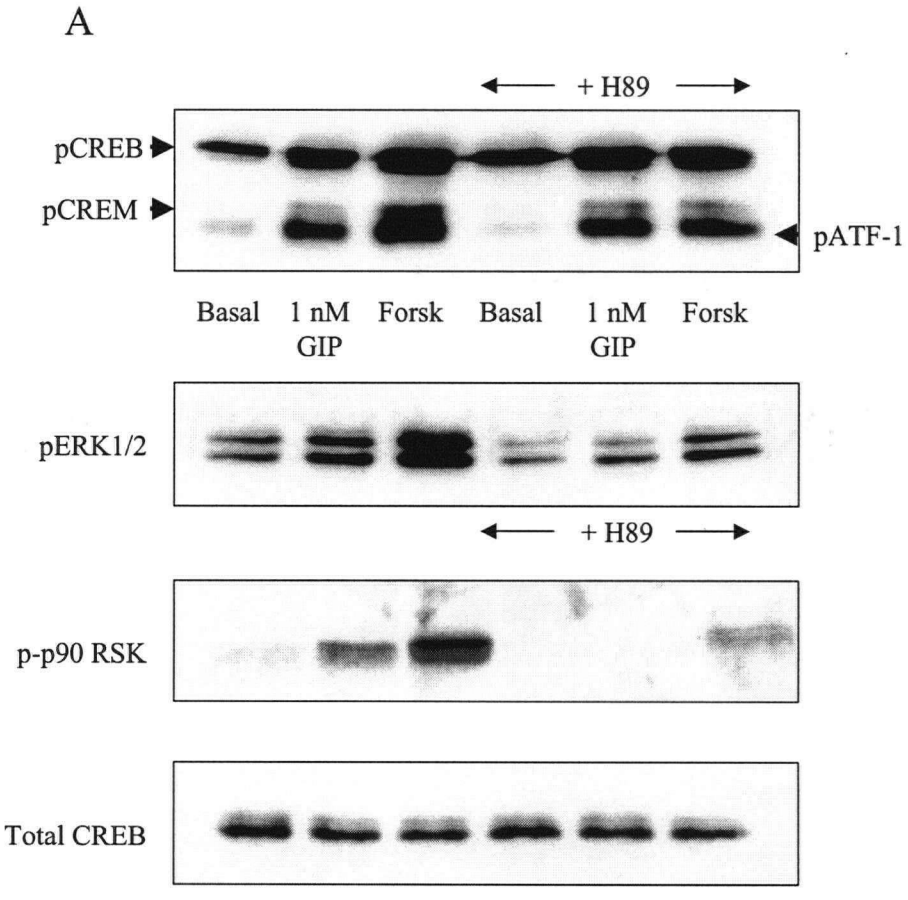
Figure 49: GIP stimulates phosphorylation of S133 CREB/S117 CREM/S63 ATF-1 in a concentration-dependent manner in INS-1 β-cells. INS-1 cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight and stimulated for 15 min with 11 mM glucose (Point "0") or glucose and the indicated GIP concentration. Thirty-µg protein samples were separated by SDS-PAGE and membranes were blotted with a phospho-CREB (pCREB) antibody, which recognizes phospho-CREM and –ATF-1 (pCREM, pATF-1), a phospho S142/143-CREB antibody, and an antibody recognizing total CREB. GIP significantly increased phosphorylation of CREB and ATF-1 in the picomolar range, and CREM in the nanomolar range (ANOVA with Dunnett's post hoc, $p<0.05$). Blots were quantified using densitometric analysis and are representative of n=3.

5.2.4 GIP stimulated phosphorylation of CREB/CREM/ATF-1 is PKA, Mek-ERK1/2-p90RSK, and p38 MAPK independent

Given the importance of cAMP/PKA and CREB/CREM/ATF-1 in mediating insulin gene promoter effects by GIP, we sought to determine which kinase was responsible for S133/S117/S63 phosphorylation of these bZIP family members. Further, given that S133 phospho-regulation is dispensable for GIP-mediated promoter effects (Figure 47), it was hypothesized that S133 CREB regulation may be via a PKA-independent mechanism. Figure 50 demonstrates that the PKA inhibitor H89 was unable to reverse GIP or forskolin stimulated phospho-CREB/CREM/ATF-1. This concentration of H89 has previously been reported to inhibit CREB/ATF-1 phosphorylation in INS-1 cells stimulated with IGF-1 (Liu et al. 2002). These PKA-independent effects on CREB were in contrast to GIP-mediated, PKA-dependent actions on the ERK1/2 module (ERK1/2→p90RSK; Figure 50), confirming previous data in CHO-K1 cells expressing the GIP receptor (Ehses et al. 2002A). The notion that the phospho-regulation of CREB/CREM/ATF-1 was also independent of the ERK1/2 module in β-cells was confirmed using the Mek1/2 inhibitor U0126 (Figure 51). Phospho-ERK1/2 and phospho-p90RSK activation by GIP and forskolin were completely ablated in the presence of U0126, without any effect on phospho-CREB/CREM/ATF-1. Furthermore, the p38 MAPK inhibitor SB203580 was also without effect on GIP-stimulated phosphorylation of the CREB family members (Figure 51), while having dramatic effects on insulin gene promoter activity (Figure 43). Therefore, we provide evidence that GIP and cAMP mediated phosphorylation of CREB/CREM/ATF-1 are independent of PKA, p38 MAPK, and the Mek1/2→ERK1/2→p90RSK module. Furthermore, these data lend support to the notion that GIP regulation of the rat insulin gene promoter is independent of S133 CREB, and suggest an independence of S117 CREM and S63 ATF-1 as well.

There has been recent interest in cAMP-dependent, PKA-independent actions that involve the stimulation of cAMP-GEFs or Epac 1 and 2 and the subsequent regulation of the small GTPases Rap1 and Rap2 (Enserink et al. 2002; Stork and Schmitt 2002). In fact, we have provided evidence that GIP may signal via Rap1 to regulate the ERK1/2 module in CHO-K1 cells (Chapter 4) (Ehses et al. 2002A), and the GLP-1 receptor has recently been shown to be coupled to CICR via Epac regulation in INS-1 cells (Kang et al. 2003). Due to the apparent PKA-independent effects found in the current study, we investigated the Epac-selective cAMP analogue, 8CPT-2Me-cAMP, for its effects on CREB/CREM/ATF-1 and ERK1/2

phosphorylation. Stimulatory effects of the PKA-selective analogue, 8CPT-cAMP, were observed which were partially reversed by H89 (Figure 52), but there was no phospho-regulation induced by 8CPT-2Me-cAMP of these kinases. We also eliminated any role for Epac-selective events in regulating the rat insulin promoter (Figure 52). 8CPT-cAMP effects on the phosphorylation of CREB/CREM/ATF-1 were not reversed by H89, while RIP1 activity was significantly reduced. This further supports our hypothesis that the regulation of S133 CREB is not correlated with GIP mediated rat insulin promoter actions, although they are dependent on a CREB family transcription factor.



B

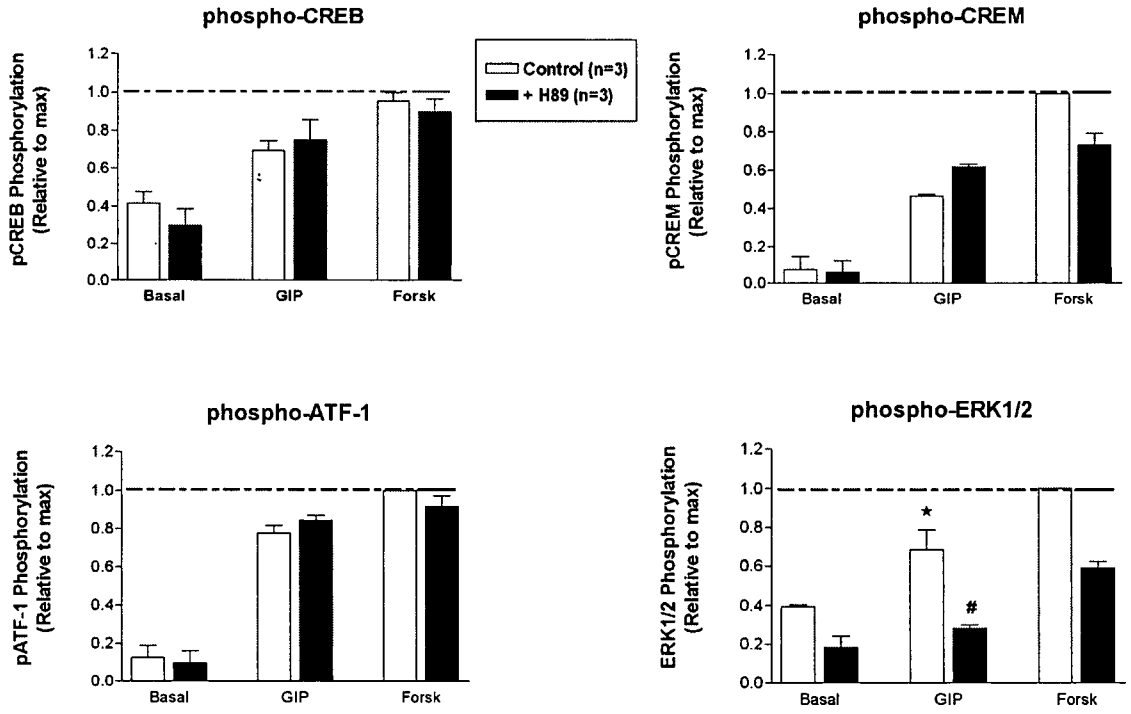


Figure 50: Evidence for cAMP-dependence and PKA-independence in GIP stimulated phosphorylation of S133 CREB/S117 CREM/S63 ATF-1 in INS-1 β-cells. INS-1 cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight and stimulated for 15 min with 11 mM glucose (Basal) or glucose and 10 nM GIP. H89 was added to cells during 15 min preincubation as well as during stimulation. 10 μM H89 did not blunt phospho-CREB/CREM/ATF-1, but did inhibit GIP-stimulated phospho-ERK1/2 (A, B) and phospho-p90RSK (A). Significance was tested using ANOVA with Tukey post hoc, where * represents $p<0.05$ vs Basal and # represents $p<0.05$ vs. Control. Blots were quantified using densitometric analysis and are representative of n=3.

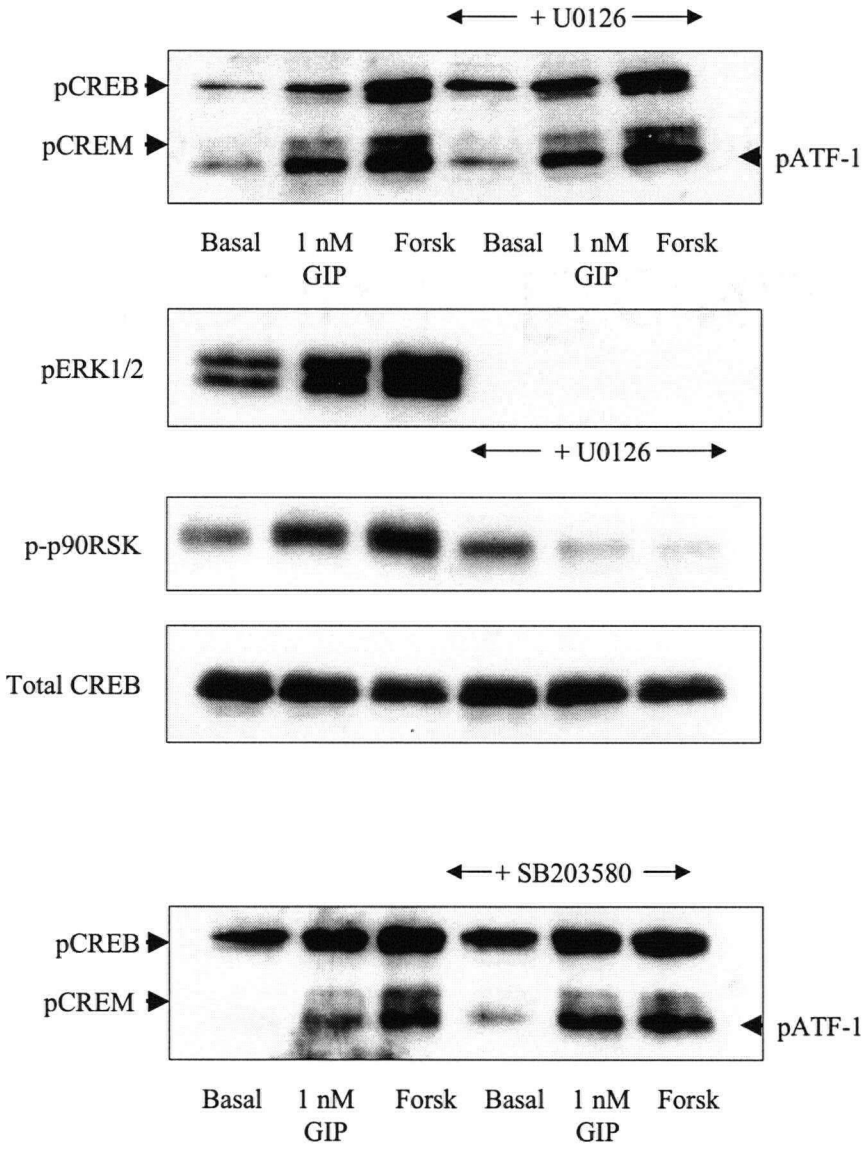
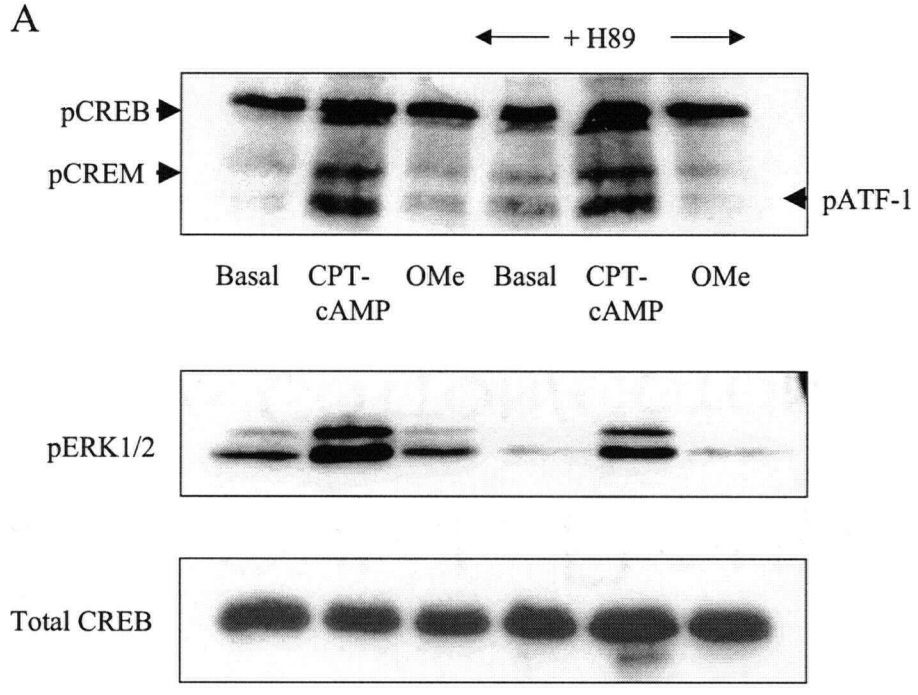
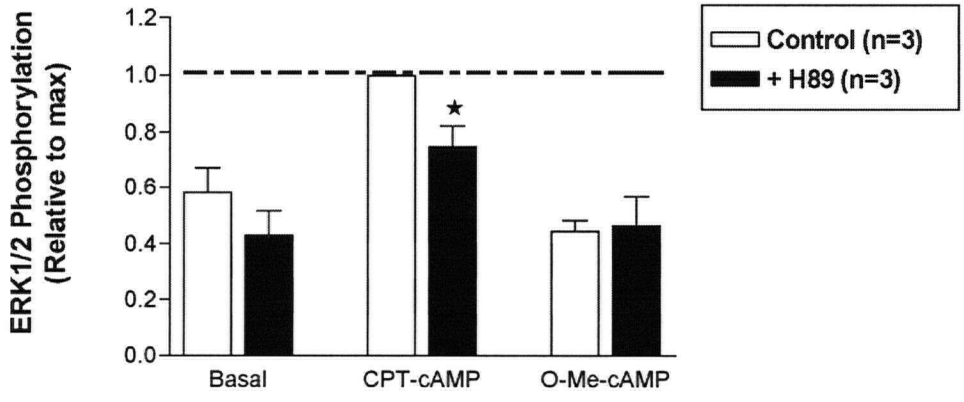


Figure 51: Evidence for Mek/ERK- and p38 MAPK-independence in GIP stimulated phosphorylation of S133 CREB/S117 CREM/S63 ATF-1 in INS-1 β-cells. INS-1 cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight and stimulated for 15 min with 11 mM glucose (Basal) or glucose and 10 nM GIP. Inhibitors (10 μM U0126 or 10 μM SB203580) were present during 15 min preincubation and during stimulation. 10 μM U0126 did not blunt phospho-CREB/CREM/ATF-1, but did inhibit GIP-stimulated phospho-ERK1/2 (A, B) and phospho-p90RSK (A). Significance was tested with ANOVA with Tukey post hoc, where * represents $p<0.05$ vs Basal and # represents $p<0.05$ vs. Control. Blots are representative of n=3.

A


+ H89
pCREB
pCREM
pATF-1
Basal
CPT-cAMP
OMe
Basal
CPT-cAMP
OMe
pERK1/2
Total CREB

B


ERK1/2 Phosphorylation (Relative to max)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Basal
CPT-cAMP
O-Me-cAMP
Control (n=3)
+ H89 (n=3)

C

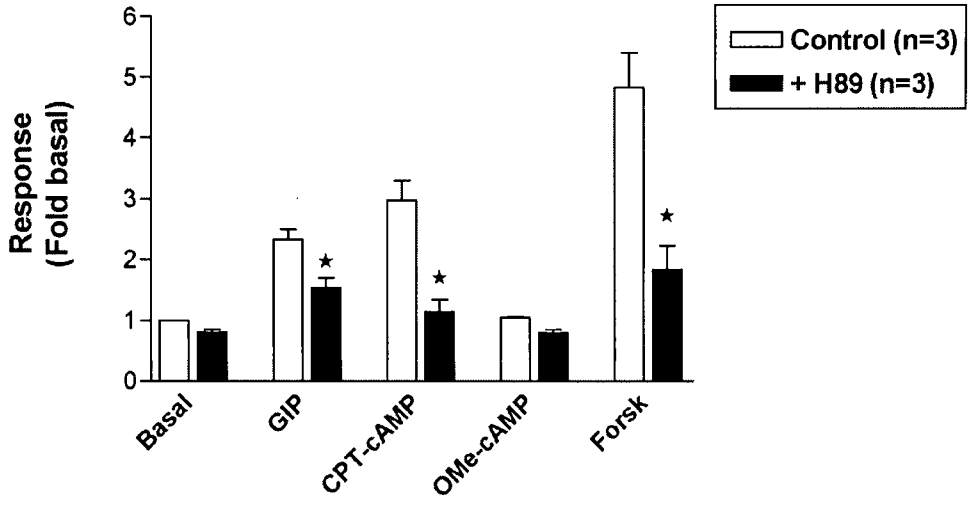


Figure 52: cAMP-mediated phosphorylation of S133 CREB/S117 CREM/S63 ATF-1 (A) and cAMP-stimulated RIP1 activity (C) is Epac-independent in INS-1 β-cells. INS-1 cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight and stimulated for 15 min with 11 mM glucose (Basal) or glucose and agonist (100 μM 8CPT-cAMP and 100 μM OMe-cAMP; Panel A). In B, the representative pERK1/2 blot in A is quantified (n=3). In C, INS-1 cells transfected with the –410 RIP1 reporter construct were stimulated with agonists for 4 h (10 μM forskolin). In all experiments 10 μM H89 was present during 15 min preincubation and during stimulation. Significance was tested with Student's t-test, where * represents $p<0.05$ vs Control.

5.3 DISCUSSION

Insulin stores are hormonally regulated at numerous levels, including gene trancription, translation, and post-translational events involved in exocytosis. The promoter region of the rat insulin 1 gene has been extensively studied and shown to contain numerous cis-elements integral to transcription factor mediated regulation of gene expression (Ohneda et al. 2000; Melloul et al. 2002). It has been known for some time that cAMP elevating agents such as the incretins, GIP and GLP-1, are able to regulate insulin gene expression (Nielsen et al. 1985; Drucker et al. 1987; Fehmann and Göke 1995). In addition to promoter activation, there have been documented effects on insulin mRNA stability (Welsh et al. 1985; Wang et al. 1995) and protein levels (Wang et al. 1996). Despite recent advances examining the regulation of the rat insulin promoter by GLP-1 (Skoglund et al. 2000; Chepurny et al. 2002; Lawrence et al. 2002), little has been elucidated with respect to GIP receptor activation. In the current chapter we highlight a role for a CREB family transcription factor in regulating GIP-mediated insulin promoter activity. Additionally, we shed light on the phospho-regulation of CREB/CREM/ATF-1 transcription factors by GIP and hypothesize that this regulation is independent of effects on the insulin gene.

The importance of the CRE element in regulating insulin gene expression has been recognized for two decades (Philippe and Missotten 1990). In contrast to the human insulin gene promoter, the rat CRE drives expression of the insulin gene. CREB is a member of the basic leucine zipper (bZIP) family of transcription factors responsible for binding to CRE sequences. The paradigm for CREB mediated gene activation includes cAMP/PKA phosphorylation of Serine 133, dimerization, and recruitment of the transcriptional coactivator, CREB binding protein (CBP) and its paralogue p300 (Mayr and Montminy 2001). In the present study we provide evidence that Serine 133 phosphorylation is dispensable for GIP and cAMP-mediated rat insulin gene activity in INS-1 β-cells. However, GIP-mediated effects on the rat insulin promoter are dependent on a CREB family transcription factor as demonstrated by the inhibitory effects of A-CREB (Figure 47). This is in support of earlier work on GLP-1-mediated RIP1 activation (Skoglund et al. 2000; Chepurny et al. 2002). Interestingly, the RIP1 contains a CRE that deviates from the normal palindromic CRE by a single C/A nucleotide substitution (5'-TGACGTCC-3' vs. 5'-TGACGTCA-3' respectively), implying that the bZIP responsible for binding the CRE may be an isoform of CREB, but not necessarily CREB itself. Our findings are in support of this hypothesis as well.

A recent study implicated cAMP/PKA signaling in GIP receptor mediated activation of CREB gene transcription (Trumper et al. 2001). However, these studies were carried out with transactivation reporter constructs where a Gal4-CREB fusion protein stimulates a Gal4 driven luciferase reporter construct. The ability of GIP to drive transcription was in this case PKA-dependent, as was the case with RIP1 in the current study. We have extended these findings to include analysis of phospho-regulation of CREB/CREM/ATF-1.

Phospho-regulation of CREB/CREM/ATF-1 in β-cells has not been extensively characterized. We investigated phosphorylation as a means to biochemically correlate/or discriminate these events from rat insulin promoter activity. Data in the present Chapter point towards a divergence between phospho S113 CREB/S117 CREM/S63 ATF-1 and regulation of the rat insulin promoter. Firstly, the M1-CREB mutant had no effect on GIP-mediated (or cAMP-mediated) RIP1 activity, while A-CREB almost ablated the response (Figure 47). This implies that the involvement of CREB in mediating these transcriptional events is not dependent on Serine 133 phosphorylation, and thus it is unlikely that CBP plays a role either. This is further supported by our phosphorylation data, since PKA (H89) and p38 MAPK inhibition (SB203580) had dramatic effects on gene promoter activity that were separate from any effects on GIP-mediated phosphorylation of CREB/CREM/ATF-1. It has previously been demonstrated in T-cells, that PKA inhibition blocked CREB-mediated transcription, without effect on Serine 133 phosphorylation (Brindle et al. 1995). These studies have led to the hypothesis that there is a second PKA-dependent event required for CREB-mediated transcriptional effects. While we cannot exclude the possibility that PKA is regulating a CREB family transcription factor in a manner independent of S113 CREB/S117 CREM/S63 ATF-1 to influence rat insulin promoter activity, we are intrigued by the possibility that these phospho-events may be crucial in the regulation of other physiological events.

In studying GIP receptor signal transduction in β-cell models we have begun to appreciate the similar phenotypic features of neuronal and endocrine cells at the level of intracellular signal transduction. The ability of cAMP signaling to influence the ERK1/2 module is conserved in neuronal and INS-1 cells, in addition to its role in promoting cell growth and regulating survival (Chapter 4 and (Ehses et al. 2002A; Stork and Schmitt 2002)). A role for CREB in regulating neuronal survival has received much recent interest (Walton and Dragunow 2000). There are also recent data demonstrating a role for CREB in pancreatic β-cell survival (M. Montminy, personal communication). We have preliminary evidence that CREB/CREM/ATF-1 are phosphoregulated by cellular stress in INS-1 β-cells (data not shown).

Owing to the recent elucidation of survival actions mediated by GIP (Ehses et al. 2002B; Trumper et al. 2002), it is tempting to speculate that GIP receptor stimulated phospho-regulation of CREB/CREM/ATF-1 may contribute to these actions. In fact, the diverse nature of CREB regulated genes lends itself to the hypothesis that S133-independent events may be crucial for insulin gene activity, while S133-dependent events may be integral to other physiological processes, such as the regulation of cell fate. Thus, we hypothesize that GIP receptor stimulated phosphorylation of CREB/CREM/ATF-1 does not play a role in regulating insulin promoter activity in INS-1 cells, but may be crucial to the regulation of survival or cell fate.

Our previous work implied a role for Rap1 and cAMP/PKA in regulating GIP receptor-mediated activation of the ERK1/2 (Raf→ERK1/2→p90RSK) module in CHO-K1 cells (Ehses et al. 2002A). The studies described in the current Chapter have extended some of this work to shed light on the regulation of the ERK1/2 module by GIP receptor activation in INS-1 β-cells. Our data supports the notion that the GIP receptor is proximally coupled to ERK1/2 and p90RSK phosphorylation by cAMP/PKA (Figure 50). Recent evidence demonstrates that the GLP-1 receptor is also coupled to ERK1/2 via PKA in MIN6 β-cells (Gomez et al. 2002). However, the Epac-selective analogue 8CPT-2Me-cAMP (100 μM) was unable to phosphorylate ERK1/2 (Figure 52) despite having effects on CICR at this concentration in INS-1 cells (Kang et al. 2003). This supports the recent hypothesis of Ensirink and colleagues, that cAMP activation of ERK1/2 and Rap1 may be independent processes (Enserink et al. 2002), and we speculate that cAMP may regulate the ERK1/2 module independent of Rap1 in INS-1 β-cells. It will be intriguing to elucidate the exact coupling mechanism between cAMP/PKA and the ERK1/2 module in β-cells. One can envision that this process may be dependent on multiple inputs such as Ca^{2+} influx, EGF receptor transactivation, or other small GTPases.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

There has been an overwhelming amount of work dedicated to the incretin GLP-1 in the last decade, given the therapeutic potential of the hormone and its receptor agonist, exendin-4, in treating type 2 diabetics. However, interest in GIP has also begun to mount given the therapeutic potential of long-acting GIP analogues (Hinke et al. 2002) and DPIV inhibitors (Pospisilik et al. 2002) in type 2 diabetes. Furthermore, recent studies (Trumper et al. 2001, 2002) and results presented in this thesis also implicate GIP in the regulation of β-cell fate (growth and survival). Thus, the potential of both GIP and GLP-1 for use in the therapeutic intervention of type 1 diabetes is also becoming a realistic possibility.

The intracellular signaling cascades stimulated by Family B GPCRs have been largely neglected, and there is a paucity of rigorous scientific analysis with regards to the molecular mechanisms coupling receptor effectors to physiological actions. Given the expansion of the signal transduction field in the last 20 yrs, effectors coupled to the GIP receptor clearly need to be identified. It is hoped that this thesis has provided some novel insights into the coupling of the GIP receptor to intracellular signaling of the pancreatic β-cell and thereby elucidated novel elements involved in GIP-stimulated insulin secretion, β-cell growth and survival, and insulin gene promoter activity. Results from this thesis can be characterized as follows:

a. GIP stimulates arachidonic acid release from βTC-3 cells via a Ca^{2+}-independent PLA_2, which is functionally coupled to GIP-potentiated insulin secretion.
b. GIP can stimulate insulin secretion from βTC-3 cells in a manner independent of the K^+_{ATP} channel via cAMP/PKA.
c. GIP-potentiated insulin secretion from βTC-3 cells is dependent on Krebs cycle flux under normal conditions, however is nutrient-independent under depolarizing conditions. Thus, the glucose-dependence of GIP-potentiated insulin secretion can be bypassed in βTC-3 cells by membrane depolarization.
d. GIP regulates the entire Raf→Mek1/2→ERK1/2→p90RSK module in CHO-K1 cells and INS-1 β-cells via a cAMP/PKA-mediated pathway.
e. GIP promotes INS-1 β-cell growth and survival.
f. GIP promotes INS-1 β-cell survival via cAMP-mediated inhibition of long-term p38 MAPK phosphorylation and caspase-3 activation.

g. GIP regulation of MAPK signaling (ERK1/2 and p38 MAPK) is intregral to its ability to regulate INS-1 β-cell fate.
h. GIP stimulates the rat insulin 1 promoter via cAMP/PKA and a CREB family transcription factor in INS-1 β-cells.
i. GIP phospho-regulation of CREB/CREM/ATF-1 is via a cAMP-dependent, PKA-independent mechanism in INS-1 β-cells, and is independent of GIP-stimulated RIP1 effects.

During the course of this thesis research, the proposed role of cAMP/PKA in mediating $G\alpha_s$ signals has become much more diverse than originally thought. There is now evidence that the incretins may stimulate insulin secretion in a cAMP-dependent, PKA-independent manner (Kashima et al. 2001), which may involve the regulation of CICR via Epac (or cAMPGEF) (Kang et al. 2003). Results presented in this thesis further highlight the potential for cAMP actions independent of PKA in β-cell lines. Results have been presented which implicate the free fatty acid, arachidonic acid (AA), in GIP receptor mediated effects on insulin secretion in βTC-3 β-cells (Chapter 3). The actions of GIP on AA release were paralleled by forskolin and not inhibited by the PKA inhibitor, H89. Furthermore, a similar conclusion was drawn from experiments investigating cell survival and caspase-3 inhibition (Chapter 4), in addition to phospho-regulation of CREB/CREM/ATF-1 (Chapter 5). It will be interesting to tease out those cAMP-dependent mechanisms which are PKA-dependent from the PKA-independent ones, with an intriquing potential for therapeutic intervention in regulating independent downstream biological effects.

Given the recent evidence that GLP-1 regulates CICR in a manner independent from PKA (Kang et al. 2003), and that both GIP and GLP-1 can stimulate insulin secretion independently of PKA (Kashima et al. 2001), it is tempting to speculate that AA signaling may be implicated in these events. Indeed AA has been coupled to fluxes in intracellular Ca^{2+} levels in pancreatic β-cells, and both GIP and GLP-1 are able to regulate intracellular concentrations of this ion (Lu et al. 1993B). The fact that arachidonic acid makes up the major fatty acyl constituent of phosphatidylserine phospholipids in the β-cell, lends support to its role as a second messenger. However, whether its role is as an intracellular effector or extracellular signal remain to be elucidated. Regardless, the role of PLA_2 in regulating insulin secretion in β-cells continues to be investigated by other groups (Ma et al. 2001, 2002). A recent finding by Itoh and

colleagues resulted in the identification of a novel GPCR for fatty acids in the pancreatic β-cell (Itoh et al. 2003). Data revealed that this receptor, GPR40, binds to numerous fatty acids, including arachidonic acid, and that its activation is coupled to fluxes in intracellular Ca^{2+} and insulin secretion in MIN6 β-cells. Thus, it is tempting to speculate that GIP receptor stimulation of AA release across the phospholipid bilayer may result in autocrine actions of the fatty acid to fine tune insulin secretion.

Elucidation of the glucose-dependence of GIP actions on insulin secretion in βTC-3 cells was an unexpected and intriguing discovery. Despite conflicting data from isolated mouse islets (Yajima et al. 1999), GIP and cAMP actions on insulin secretion were solely dependent on membrane depolarization with high K^+ in βTC-3 cells. Thus, the glucose-dependence of GIP in βTC-3 cells is based on the ability of the nutrient to cause cellular depolarization via the K_{ATP} channel (since glucose has no K_{ATP} channel-independent effects in these cells). Further, the actions of GIP and cAMP were shown to be not solely reliant on the K_{ATP} channel. However, the actions of arachidonic acid, despite being independent of the K_{ATP} channel, were dependent on a Krebs cycle intermediate. The unexpected nature of these data begs for reassessment in a more physiological model. The notion of glucose-dependence in pancreatic β-cells remains poorly understood on a molecular level, and an extension of these studies is warranted.

Data presented in this thesis have begun to highlight the regulation of the ERK1/2 module in pancreatic β-cells for the first time. While initial work in CHO-K1 cells implicated the small GTPase, Rap1, as the upstream kinase regulating positive input into the module (Chapter 4), parallel studies in INS-1 cells revealed that actions on ERK1/2 are likely not via Epac (Chapter 5). These data are suggestive of a cAMP/PKA dependent pathway that may be independent of Epac or Rap1, in the regulation of Mek1/2-ERK1/2-p90RSK. Indeed GLP-1 receptor coupling to ERK1/2 has been shown to be independent of Ras in MIN6 cells (Gomez et al. 2002). Thus, it remains unknown which upstream kinase regulates the ERK1/2 module in pancreatic β-cells, although Ca^{2+} influx is integral to these events (data not shown and Gomez et al. 2002). Furthermore, studies are also needed to characterize the potential role for receptor transactivation and desensitization/internalization in regulating GIP-stimulation of MAPK signaling events.

The physiological relevance of ERK1/2 phosphorylation in the pancreatic β-cell remains relatively unexplored, and the physiological role of GIP stimulation of the ERK1/2 module can only be extrapolated from other publications. Recent studies have implicated Mek1/2-ERK1/2 in

the mitogenic effects of GIP and GLP-1 on INS-1 β-cells (Trumper et al. 2002; Briaud et al. 2003). Based on the extensive literature available on ERK1/2, it can be postulated that this kinase module plays an integral role in regulating β-cell fate. It will be interesting to relate the activation of ERK1/2 to the regulation of cell cyle proteins by GIP. Further, results presented here also implicate Mek1/2-ERK1/2 in the regulation of caspase-3 activity and hence cell survival (Chapter 4). Indeed, these kinases are activated by cytokine-induced pancreatic β-cell death (Eizirik and Mandrup-Poulsen 2001). Our results are in agreement with a role for Mek1/2-ERK1/2 in promoting β-cell caspase-3 activity under long-term activation (such as during cytokine insult). These findings are counter-intuitive given the aforementioned role for these kinases in the promotion of cell proliferation. Thus, in addition to the duality in signaling implicated in the p38 MAPK module in this thesis (Chapter 4), it is also apparent that Mek1/2-ERK1/2 may regulate cell fate dynamically. Therefore, in assessing the role of MAPKs in mediating biological actions, it is essential to examine biochemically the temporal regulation of these kinases and evaluate gene products affected by this temporal regulation.

Finally, the regulation of phospho-S133 CREB/S117 CREM/S63 ATF-1 represents another level of β-cell signal regulation by GIP, independent of MAPK module input. Discovery of the upstream kinase regulating this event in the β-cell will be a novel finding with respect to β-cell signal transduction. The duality of signaling highlighted in the case of MAPKs may also apply to CREB signal transduction. GIP is able to stimulate phosphorylation of CREB, while we also have preliminary evidence that this kinase is stress regulated (data not shown). How these two processes are correlated will be interesting to unravel. Further, the inability of H89 to have effects on caspase-3 activity and CREB/CREM/ATF-1 phosphorylation may represent a link between these two processes. Thus, a study aimed at the possibility that CREB may be involved in RIP1 effects in addition to postulated survival effects represents a formidable endeavor with an intriguing outcome.

In conclusion, the aim of this thesis was to characterize the intricate network of GIP receptor signaling pathways underlying β-cell processes such as insulin secretion, β-cell growth and survival, and insulin gene promoter activity. While it is hoped that much insight has been provided, it is evident these studies have all been performed in β-cell model systems. Thus, research on the islet or purified β-cell level is warranted to support the conclusions herein. Ultimately, it is hoped that the basic science knowledge gained in this thesis will one day be applied to the treatment of type 1 and 2 diabetes mellitus.

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