

Glucose-Dependent Insulinotropic Polypeptide Stimulation of Lipolysis in Differentiated 3T3-L1 Cells: Wortmannin-Sensitive Inhibition by Insulin*

CHRISTOPHER H. S. McINTOSH, IRENE BREMSAK, FRANCIS C. LYNN,
RUTH GILL, SIMON A. HINKE, RICHARD GELLING, CUILAN NIAN,
GARY McKNIGHT, STEPHEN JASPERS, AND RAYMOND A. PEDERSON

Department of Physiology (C.H.S.M., I.B., F.C.L., R.G., S.A.H., R.G., C.N., R.A.P.), University of British Columbia, Vancouver, British Columbia, V6T1Z3, Canada; and Zymogenetics, Inc. (G.M., S.J.), Seattle, Washington

ABSTRACT

GIP is an important insulinotropic hormone (incretin) that has also been implicated in fat metabolism. There is controversy regarding the actions of GIP on adipocytes. In the current study, the existence of GIP receptors and effects of GIP on lipolysis were studied in differentiated 3T3-L1 cells. GIP receptor messenger RNA was detected by RT-PCR and RNase protection assay. Receptors were detected in binding studies (IC_{50} 26.7 ± 0.7 nM). GIP stimulated glycerol release with an EC_{50} of 3.28 ± 0.63 nM. GIP (10^{-9} – 10^{-7} M) + IBMX increased cAMP production by 1180–2246%. The adenylyl cyclase inhibitor MDL 12330A (10^{-4} M) inhibited GIP-induced glycerol production by >90%,

and reduced cAMP responses to basal. Preincubation of 3T3-L1 cells with insulin inhibited glycerol responses to GIP, and the inhibitory effect of insulin was blocked by the phosphatidylinositol 3'-kinase inhibitor, wortmannin. It is concluded that GIP stimulates glycerol release in 3T3-L1 cells primarily via stimulation of cAMP production, and that insulin antagonizes GIP-induced lipolysis in a wortmannin-sensitive fashion. It is suggested that effects of GIP on fat metabolism *in vivo* may depend upon the circulating insulin level, and that meal-released GIP may elevate circulating fatty acids, thus optimizing pancreatic β -cell responsiveness to stimulation by glucose and GIP. (*Endocrinology* 140: 398–404, 1999)

GLUCOSE-DEPENDENT insulinotropic polypeptide (gastric inhibitory polypeptide; GIP) is released from the small intestine in response to a meal and stimulates insulin secretion in the presence of elevated circulating glucose (1, 2). This incretin action is shared by peptide products of the proglucagon gene, glucagon-like peptide-1 (GLP-1(7–36amide)) and GLP-1(7–37) (3), and there is currently considerable interest in the potential of using incretin analogs for the treatment of noninsulin-dependent diabetes mellitus (NIDDM) (4). In addition to its effect on insulin secretion, GIP has also been implicated in the regulation of fat metabolism, an action that could also be of paramount importance in the normal regulation of insulin secretion (5).

Ingestion of triglycerides increases circulating GIP by a pathway that involves both metabolism (6, 7) and absorption of fatty acids (8, 9). Several lines of evidence support a role for GIP in the subsequent disposal of circulating triglycerides (10). Administration of GIP promoted chylomicron-associated triglyceride clearance from blood in dogs (11), and reduced peak plasma triglyceride levels during an intraduodenal fat infusion in rats (12). GIP has also been shown to stimulate lipoprotein lipase activity in cultured preadipocytes (13), an effect that would result in

increased cellular uptake of triglycerides. There is less known regarding the direct effects of GIP on adipocyte lipid metabolism, but it has been reported to enhance incorporation of both fatty acids (10) and glucose (14) into lipids, and to inhibit glucagon-stimulated lipolysis and cAMP production (15, 16). These studies have been interpreted as being consistent with an overall anabolic effect of GIP in adipose tissue. However, in the pancreatic islet, GIP exerts its major effects via stimulation of adenylyl cyclase (17, 18). Because hormonal stimulation of release of glycerol and fatty acids from adipocytes is generally associated with increased levels of cAMP and stimulation of hormone-sensitive lipase activity (19), it would be anticipated that GIP would exhibit lipolytic, rather than lipogenic, effects.

In the current study, the presence of GIP receptors and the effect of GIP on lipolysis were studied in differentiated 3T3-L1 cells (adipocytes), a cell line which has been shown to exhibit many of the characteristics of normal adipocytes, including responsiveness to insulin and lipolytic hormones (20). GIP receptors were detected in these cells, and GIP acted as a lipolytic hormone primarily via stimulation of cAMP production. In addition, insulin was found to be capable of antagonizing GIP-induced lipolysis in a wortmannin-sensitive fashion. It is suggested that GIP-induced elevations in circulating FFA are involved in the optimization of pancreatic β -cell responsiveness to the synergistic stimulatory actions of glucose and GIP. The response of the adipocyte to GIP may depend upon the prevailing circulating insulin level.

Received July 10, 1998.

Address all correspondence and requests for reprints to: Christopher McIntosh, Ph.D., Department of Physiology, Faculty of Medicine, University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia, Canada, V6T 1Z3. E-mail: mcintosh@unixg.ubc.ca.

* This work was supported by grants from Zymogenetics Inc. (Seattle, WA), the Medical Research Council of Canada (5-90007-RAP/CHSM) and the Canadian Diabetes Association (CHSM/RAP).

Materials and Methods

Culture of 3T3-L1 cells

3T3-L1 cells (American Type Culture Collection; ATCC) were cultured in DMEM containing high glucose and supplemented with 5% newborn calf serum plus penicillin/streptomycin (standard medium) in 12 or 24 well Corning or Falcon culture plates. Cells were induced to differentiate into the adipocyte phenotype by a modification of the method described by Rentsch and Chiesi (21). Two days after cells were confluent, medium was supplemented with dexamethasone (0.6 μ M), 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM) and insulin (16 μ M) for 72 h, after which cells were cultured in DMEM high glucose medium + 10% FCS. Differentiation was complete in 7 days. Before use in lipolysis studies, cells were washed twice with medium consisting of (mM) NaCl (137.0), KCl (5.4), Na_2HPO_4 (0.34), KH_2PO_4 (0.44), NaHCO_3 (4.2), HEPES (10.0), CaCl_2 (1.26), MgSO_4 (0.81), glucose (11.0), and ascorbate (0.57), pH 7.4, and incubated for 30 min in this medium. In standard protocols, cells were incubated for 4 h at 37 C in the above medium containing 2% fatty acid free BSA, gassed with 5% CO_2 , in the absence or presence of the ligands described in the text. The medium was removed and frozen at -20 C. Fully differentiated cells (>85% adipose cells) from passages 3-9 were used in all experiments.

GIP receptor: RT-PCR analysis

Total RNA was isolated from 3T3-L1 cells (passage 7) or rat epididymal fat using a modification of the method of Chomczynski and Sacchi (22) (Trizol; Gibco BRL, Life Technologies, Burlington, Ontario, Canada). Total RNA (2 μ g) was reverse transcribed in a 20 μ l reaction mixture containing 0.5 mM dNTPs, 30 pmol 3' gene specific primer, 200 U Superscript II RNase H⁻ Reverse Transcriptase (Gibco BRL), 10 U RNAase inhibitor (RNA Guard; Pharmacia Biotech, Québec, Canada), 1 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl_2 . Following RT, 2 μ l of the RT mix was amplified in a 50 μ l PCR reaction containing 67 mM Tris-HCl, 3.0 mM MgSO_4 , 166 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM β -mercaptoethanol, pH 8.3, with 200 μ M dNTPs, 10 pmol of each primer (FCL5': 5'-ACCTGTACGAGAACACGCGAGTGC-3' and FCL3': 5'-GTCTGGAGTAGAGGTCCGTGTA-3'), and 1 U of *Taq* DNA Polymerase. The PCR reaction profile included a 5-min initial denaturation step at 94 C followed by 40 cycles of 94 C (45 sec), 59 C (1 min), 72 C (1 min), and a final extension step for 5 min at 72 C.

GIP receptor: ribonuclease protection assay

All reagents used in the ribonuclease protection assay were from Ambion, Inc. (Austin, TX). An antisense probe of approximately 300 nucleotides, complementary to the C-terminal tail of the GIP receptor DNA, was transcribed using the Megascript SP6 kit. A sense control probe consisting of a 200 nucleotide fragment of the antisense probe was transcribed in the same manner. A probe complementary to 80 nucleotides of 18S ribosomal RNA was also transcribed using pTRI RNA 18S antisense Control Template. Probes were labeled with biotin using the Brightstar Psoralen-Biotin Nonisotopic Labeling Kit. Total RNA (5 μ g) from either 3T3-L1 cells or rat adipose tissue was coprecipitated with 190 ng of antisense probe, 50 pg of sense probe and 420 ng of 18S ribosomal probe, resuspended in hybridization buffer, and hybridized at 44 C for 18 h. Single-stranded RNA was digested with RNase A and RNase T1 at 37 C for 30 min using RPA II kit reagents. Double-stranded RNA was precipitated, subjected to electrophoresis on a 5% acrylamide/8M urea gel, and transferred to a positively charged nylon membrane. Biotinylated RNA was detected using a streptavidin-alkaline phosphatase conjugate that binds CDP-Star to give a chemiluminescent signal. The membrane was exposed to film for 2.5 min.

Iodination of GIP and binding analysis

Synthetic porcine GIP (5 μ g) was iodinated by the chloramine-T method, and the ^{125}I -GIP further purified by reverse phase HPLC to a specific activity of 250-350 $\mu\text{Ci}/\mu\text{g}$ (17). Aliquots of tracer were lyophilized and stored at -20 C until use. Binding analysis was performed as described previously (17) with minor modifications. Briefly, 3T3-L1 cells (1.9×10^5 /well) were washed twice in binding buffer (DMEM/F12, 20 mM HEPES and 0.1% BSA, pH 7.4) at 4 C, and incubated for 4 h at 4 C

with ^{125}I -GIP (50,000 cpm) + 100 KIU aprotinin (Trasylol), in the presence or absence of unlabeled synthetic human GIP (final volume 200 μ l). Binding to CHO-K1 cells (2×10^5 /well), transfected with the wild-type GIP receptor (wtGIP-R1) and grown as described previously (17), was analyzed in parallel. Following incubation, cells were washed twice in ice cold buffer, solubilized with 0.1M NaOH (1.0 ml), and transferred to test tubes for counting of cell associated radioactivity. Nonspecific binding was defined as that measured in the presence of an excess of shGIP (1 μ M) and specific binding was expressed as a percentage of binding in the absence of competitor (%B/Bo).

Measurement of glycerol release

Following incubation of 3T3-L1 cells, for the time periods indicated in figure legends, proteins in the incubation medium were precipitated with 1/10 vol. of 7% (wt/vol) ZnSO_4 , incubated on ice for 10 min, and 1/5 vol. 0.1 M NaOH added at room temp. The mixture was centrifuged and the supernatant assayed for glycerol with an enzymatic assay based on the method of Wieland (23) using reagents from Boehringer Mannheim (Mannheim, Germany). Data are expressed as means \pm SEM of glycerol released per well (nmol/well), or nmol change from basal when preincubation of cells with a specific agent resulted in an altered basal secretion. The number of experiments is presented in brackets.

Measurement of cAMP production

In selected experiments, indicated in the *Results* section, glycerol release, and cAMP production were assayed in parallel. Following incubation, medium was removed for glycerol assay, and cells were extracted with 70% ethanol. The extract was dried by vacuum centrifugation, and cAMP levels measured by RIA (Biomedical Technologies, Stoughton, MA) as previously described (17). Data are expressed as means \pm SEM of cAMP production in pmol/well. Number of experiments is presented in brackets.

Data analysis

Binding, glycerol release and cAMP data were analyzed using the nonlinear regression analysis program Prism (GraphPad, San Diego, CA). Statistical analysis was performed using Student's *t* test. Maximal cAMP and mean EC_{50} values were compared using one-way ANOVA, followed by Dunnett's multiple comparison test. $P < 0.05$ was considered significant.

Materials and Methods

Porcine GIP₁₋₄₂ was from Bachem California, Inc. (Torrance, CA) and Human GIP₁₋₄₂ was synthesized by Hukabel, Montréal, Canada. MDL 12330A, IBMX and wortmannin were obtained from Research Biochemicals International. Tissue culture reagents were from Gibco BRL, Life Technologies. All other chemicals, of reagent or molecular biology grade were from Fisher Scientific International, Inc. (Vancouver, Canada).

Results

The 3T3-L1 cell GIP receptor: expression and binding

RT-PCR was performed on RNA extracted from differentiated 3T3-L1 cells and rat epididymal fat. Primers were designed to allow amplification of the carboxy terminal region of the GIP receptor. A PCR product was detected after amplification of complementary DNA (cDNA) from both extracts with the predicted size (526 bp) (Fig. 1A). With the RNase protection assay a band was detected for extracts from both 3T3-L1 cells and adipose tissue corresponding to a transcript of the appropriate size for the C-terminal region of the GIP receptor (Fig. 1B)

Binding of ^{125}I -GIP to 3T3-L1 cells was significantly less than to wtGIP-R1 transfected CHO-K1 cells, or to β cell ($\beta\text{TC-3}$) tumor cells (unpublished observations). The IC_{50} value of 26.7 ± 0.7 nM ($n = 5$) was approximately 10.5-fold

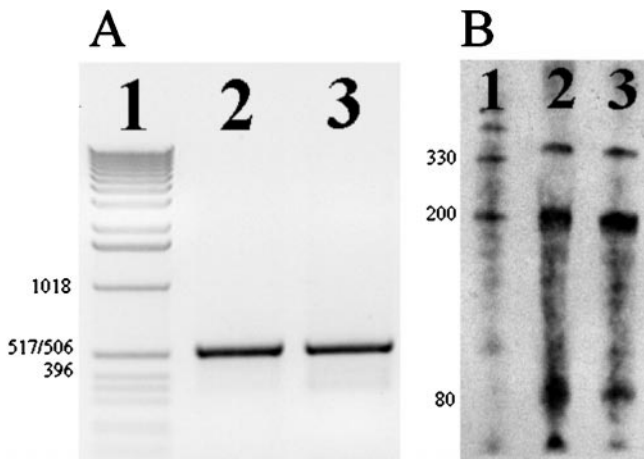


FIG. 1. Demonstration of the presence of GIP receptor messenger RNA in extracts of 3T3-L1 cells using RT-PCR (A) and ribonuclease protection assay (B). RT-PCR and the RNAase assay were performed as described under *Materials and Methods*. RT-PCR (A): 1 = Molecular weight standards (1 kb ladder); 2 = Amplification of 3T3-L1 cell cDNA; 3 = Amplification of adipocyte cDNA. RNAase Assay: 1 = Molecular weight standards; 2 = 3T3-L1 cell RNA; 3 = adipocyte RNA. *, GIP receptor. Bands at 200 and 80 nucleotides = Sense and RNA 18S RNA.

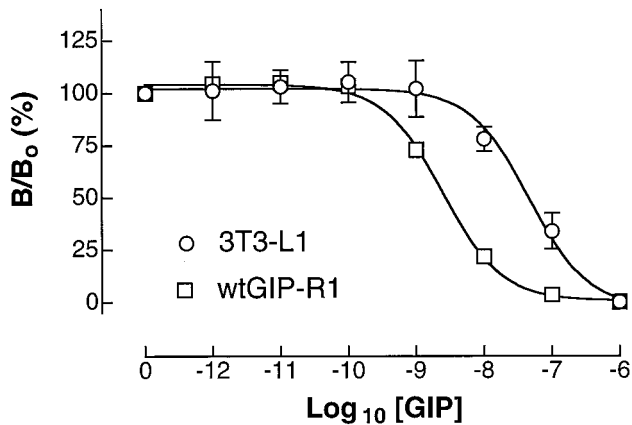


FIG. 2. Displacement of ^{125}I -GIP binding by GIP in 3T3-L1 cells and CHO-K1 cells expressing GIP receptors. Competitive binding studies were performed as described in *Materials and Methods*, and binding expressed as B/B_0 , where B and B_0 = binding in the presence and absence of unlabeled ligand, respectively (wtGIP-R1 cells $n = 4$, 3T3-L1 cells $n = 5$).

greater than that for wtGIP-R1 (2.55 ± 0.44 nM; $n = 4$) (Fig. 2).

Effect of GIP on lipolysis and cAMP production

Under the standard incubation conditions used (4 h, 37°C), GIP-stimulated glycerol release from 3T3-L1 adipocytes in a concentration-dependent manner over the range 10^{-10} – 10^{-6} M (Fig. 3A). Maximal stimulation was approximately 70% of that obtained with isoproterenol (Fig. 3B). Over the same concentration range neither glucagon nor GLP-1 (7–36)amide, structurally related insulinotropic peptides, had any significant effect on glycerol release.

The EC_{50} value for GIP stimulation, averaged over several cell passages, was $3.28 \pm 0.63 \times 10^{-9}$ M ($n = 11$),

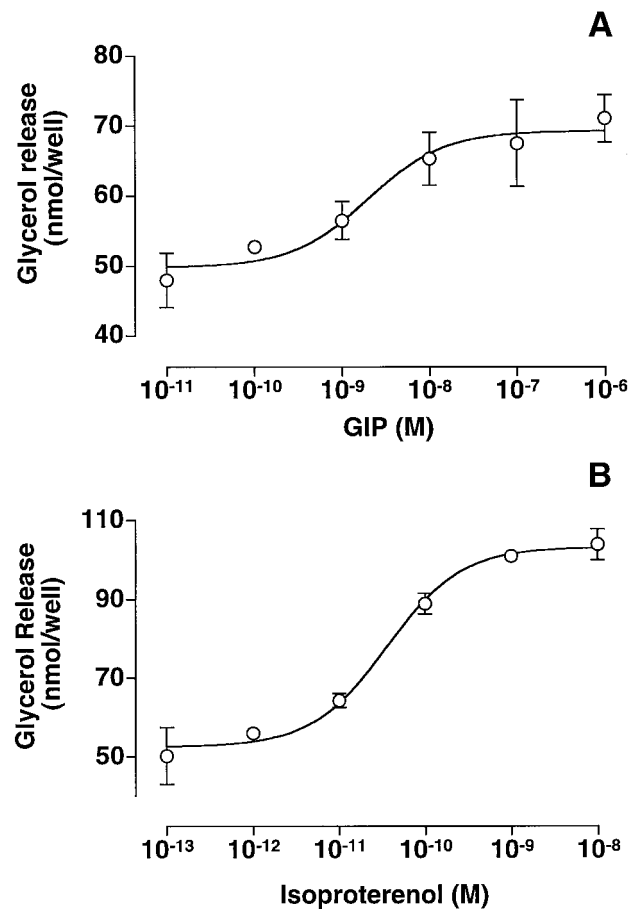


FIG. 3. Glycerol release from 3T3-L1 adipocytes in response to GIP (A) and isoproterenol (B). Cells were incubated under standard conditions for 4 h, as described in *Materials and Methods*, the medium removed and glycerol content measured. $n = 11$ for GIP and 6 for isoproterenol.

whereas with isoproterenol it was two orders of magnitude lower: $4.68 \pm 0.94 \times 10^{-11}$ M ($n = 6$). Addition of low concentrations of IBMX (10^{-6} M) to the medium potentiated glycerol release ($\sim 38\%$) under basal conditions, and in response to 10 nM GIP ($\sim 24\%$), suggesting that the effect of GIP on lipolysis was mediated via stimulation of adenylyl cyclase. However, when cells were incubated for time periods in excess of 30 min with concentrations of IBMX sufficient to maximally block phosphodiesterase activity (1–2 mM), subsequent responses to GIP were reduced or abolished. Preincubation with forskolin (50 μM) resulted in similar reductions in responsiveness, indicating that it was probably due to cAMP-mediated desensitization. Therefore, when examining the effect of IBMX on cAMP responses only 30 min exposures were used. Under these conditions, basal cAMP levels, in the absence and presence of IBMX, were 2.75 ± 0.06 and 25.18 ± 0.86 pmol/well respectively. GIP resulted in concentration-dependent increases in cAMP ($n = 6$): at concentrations of 10^{-9} , 10^{-8} , and 10^{-7} M GIP cAMP levels were 3.51 ± 0.18 , 7.63 ± 0.27 and 18.51 ± 2.04 pmol/well in the absence of IBMX, and 44.94 ± 2.19 , 179.03 ± 11 and 376 ± 35.2 pmol/well in its presence.

Effect of inhibition of adenylyl cyclase on GIP-stimulated glycerol release

To establish that GIP-stimulated release of glycerol was associated with increased cAMP production, a study was made on the effect of the adenylyl cyclase inhibitor MDL 12330A on both parameters. A strong link between GIP-induced cAMP production and glycerol release was obtained in a study in which both parameters were measured over time in the presence and absence of 10^{-4} M MDL 12330A (Fig. 4). Glycerol secretion continued throughout the 120 min. experimental period (Fig. 4A). cAMP accumulation reached a peak within 15 min (Fig. 4B). In the presence of 10^{-9} M GIP cAMP levels returned to basal by 60 min whereas, with GIP at higher concentrations, cAMP remained elevated, although at levels less than those at 15 min. MDL 12330A almost completely abolished cAMP responses (Fig. 4D), apart from those in the presence of 10^{-7} M GIP. Glycerol production was significantly reduced by MDL 12330A at all time periods (Fig. 4C; $P < 0.05$).

Effect of insulin on GIP-stimulated glycerol release

Preincubation of cells for 30 min with insulin reduced basal glycerol release, and inhibited responses to both GIP (10^{-8} M) and isoproterenol (10^{-10} M) in a concentration-dependent manner (Fig. 5A). Significant inhibition ($P < 0.05$) was obtained at an insulin concentration of 10^{-11} M ($n = 6-7$; $P < 0.05$), and 50% inhibition of responses occurred at in-

ulin concentrations of 1.07×10^{-10} M with GIP and at 0.75×10^{-10} M with isoproterenol. Maximum inhibition, obtained with an insulin concentration of 10^{-8} M, was $71.6 \pm 6.1\%$ with GIP and $85.2 \pm 5.5\%$ with isoproterenol. When cells were preincubated with a low concentration of insulin (10^{-10} M) and GIP responses subsequently measured in its presence, responses were reduced throughout the GIP concentration range (Fig. 5B).

Effect of wortmannin on insulin-induced inhibition of responses to GIP

To determine whether the inhibitory effect of insulin on GIP-induced glycerol release was associated with the stimulation of phosphatidylinositol (PI) 3'-kinase, the effect of the selective inhibitor wortmannin on responses to GIP was tested. Incubation of 3T3-L1 adipocytes with wortmannin at concentrations of 10^{-9} - 10^{-7} M had no effect on basal glycerol release: rates were 47.8 ± 0.8 nmol per well in the absence and 48.8 ± 2.3 nmol per well ($n = 6$) in the presence of 10^{-7} M wortmannin. GIP (10^{-6} M)-stimulated glycerol release was likewise unaffected: 64 ± 3.6 nmol per well in the absence and 66.5 ± 3.5 nmol per well in the presence of wortmannin. In agreement with the previous experiments (Fig. 5), insulin (10^{-8} M) completely inhibited glycerol release in response to 10^{-9} M GIP, and inhibited by $>60\%$ responses to 10^{-8} and 10^{-7} M GIP (Fig. 6). When cells were preincubated with wortmannin (10^{-7} M) for 30 min and subsequently incubated in the presence of insulin plus GIP, re-

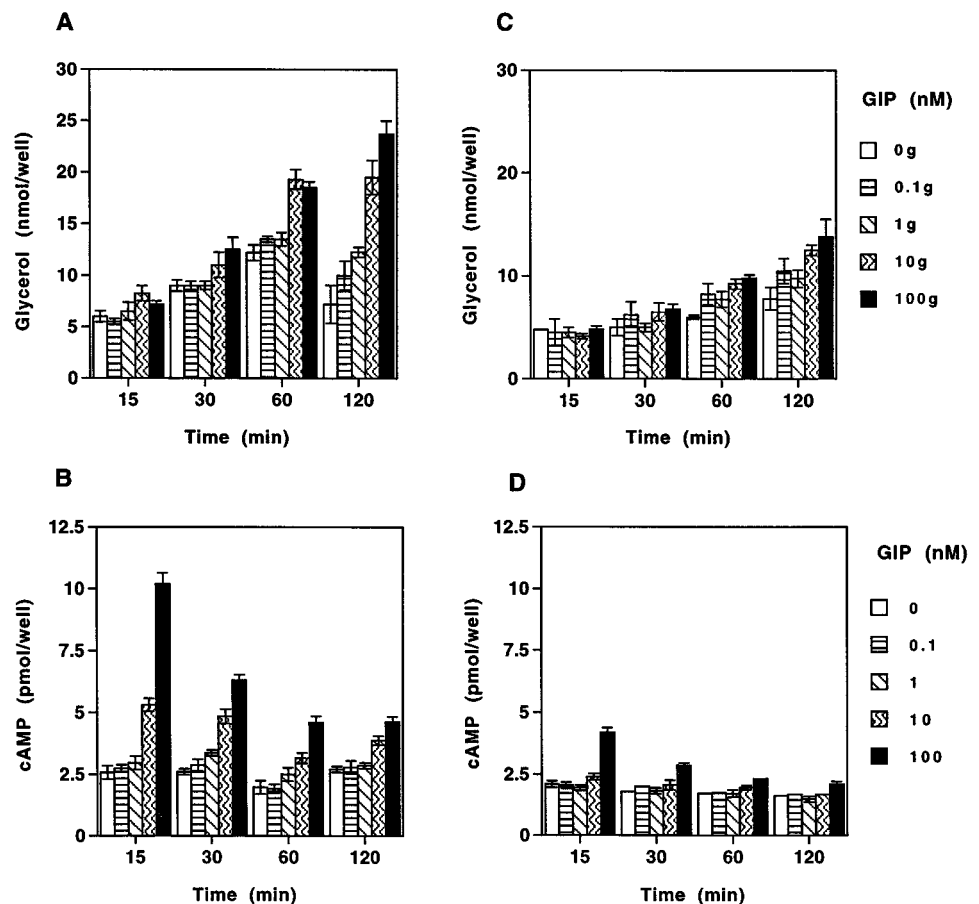


FIG. 4. Time-responses of glycerol and cAMP to GIP in 3T3-L1 adipocytes in the absence and presence of MDL 12330A. Cells were preincubated in the absence (A and B) or presence (C and D) of MDL 12330A (10^{-4} M) for 30 min, followed by incubation for the indicated time periods with the addition of GIP (10^{-10} - 10^{-6} M) ($n = 6$). At the indicated times medium was removed for glycerol measurement (A and C), and the cells extracted for cAMP assay (B and D). Both glycerol and cAMP responses in the presence of MDL 12330A were significantly reduced at all concentrations of GIP over the complete time period ($P < 0.05$).

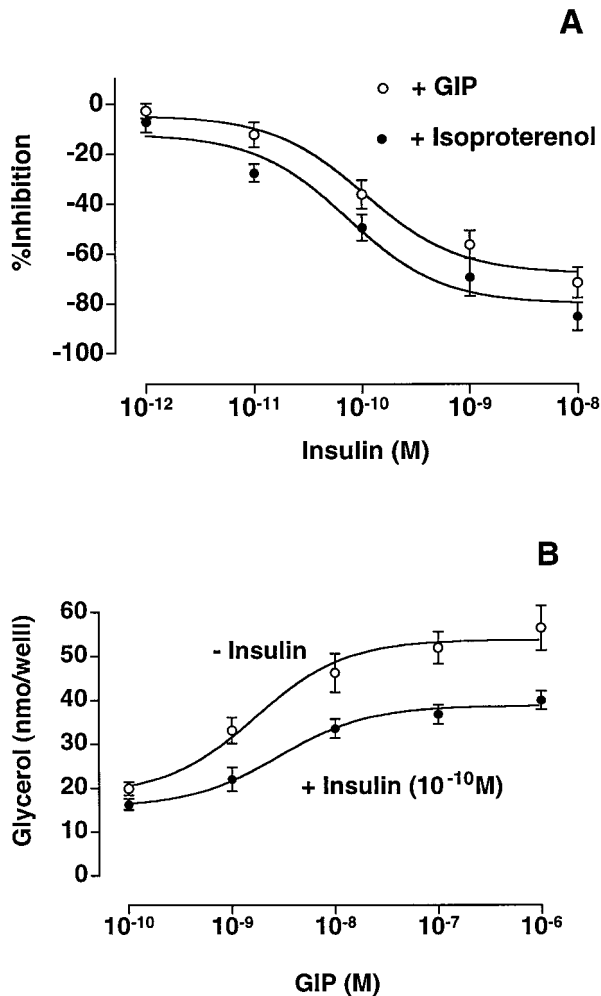


FIG. 5. Inhibition of glycerol responses to GIP and isoproterenol in 3T3-L1 adipocytes by varying concentrations of insulin (A) and concentration-response to GIP in the presence and absence of insulin (B). For (A) glycerol was measured in the medium from cells preincubated with insulin for 30 min, followed by a 4 h incubation in the presence of GIP (10^{-8} M) ($n = 6$). For (B) cells were preincubated with insulin (10^{-10} M), followed by the addition of GIP (10^{-10} – 10^{-6} M) ($n = 6$ – 8). Data are expressed as % inhibition of responses in the absence of insulin. In (A) responses were significantly inhibited at concentrations $>10^{-11}$ M insulin, and in (B) GIP responses were inhibited throughout the concentration range ($P < 0.05$).

sponses to 10^{-8} and 10^{-7} M GIP were not significantly different from those to GIP alone (Fig. 6), indicating almost complete abolition of insulin's inhibitory action.

Discussion

There is convincing evidence in the literature that GIP is involved in the clearance of postprandial circulating triglycerides (11–12), and such a function is consistent with fat being the most powerful stimulus for GIP secretion (1, 2). In contrast, the literature regarding direct effects of GIP on fat cell triglyceride metabolism is less definitive. Dupre and co-workers (16) reported that GIP was nonlipolytic on adipocytes from rat epididymal fat pad, but that it inhibited both glucagon-stimulated lipolysis and cAMP production. In contrast, Ebert and Creutzfeldt (15), using a similar adipocyte

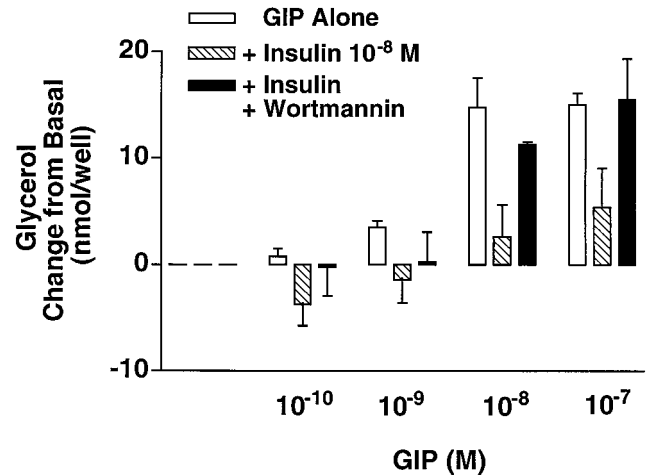


FIG. 6. Effect of wortmannin on insulin inhibition of GIP-stimulated glycerol release. Cells were preincubated with insulin or insulin + wortmannin for 30 min, followed by a 4 h incubation with the addition of GIP (10^{-8} M) ($n = 6$). Insulin resulted in a reduction in basal glycerol release. Therefore, to allow comparison, data were calculated as the change from basal levels in the absence or presence of wortmannin. Following wortmannin treatment responses to GIP were not significantly different from responses to GIP in the absence of insulin.

preparation, found that GIP stimulated cAMP production and induced small increases in glycerol release. Both groups reported that GIP displaced 125 I-glucagon binding from adipocytes, implying that GIP interacted with the glucagon receptor (15, 16). An important implication from these studies was that the predominant effect of GIP on lipolysis was mediated via the glucagon receptor. It had been observed by us earlier (reviewed in Refs. 1 and 24) that pancreatic islets isolated by collagenase digestion were only weakly responsive to GIP, presumably due to receptor damage by the enzymatic digestion. Because rat adipocytes are isolated using a similar procedure, reduced receptor content may explain the previous inability to observe specific GIP effects on lipolysis. An additional factor may have been the use of high concentrations of phosphodiesterase inhibitors because, as described in this study, such treatment results in greatly reduced responsiveness, possibly due to receptor desensitization.

The 3T3-L1 cell line has been used extensively for studying the mode of action of lipolytic and lipogenic hormones. Differentiation of 3T3-L1 preadipocytes is accompanied by increases in proteins characteristic of mature adipocytes including catecholamine-stimulated adenylyl cyclase (25), hormone-sensitive lipase (26), and cAMP phosphodiesterase (27). The differentiated cells are also responsive to many lipolytic hormones (20), and these cells were therefore considered appropriate for examining a possible lipolytic action for GIP.

The 3T3-L1 adipocytes express a GIP receptor with similar characteristics to that from the rat pancreatic islet (17, 18) although it appears that, under the conditions of culture used, the receptor population is mainly in a lower affinity state. It is clear that GIP is a fairly potent lipolytic hormone in 3T3-L1 adipocytes, and that it acts primarily via stimulation of cAMP production. The 3T3-L1 adipocytes used in the current study were found to be unresponsive to glucagon, in

agreement with earlier studies (20, 28), and GIP effects could not, therefore, have been mediated via the glucagon receptor. Recently, GLP-1 (7–36) was described as having insulin-like effects in 3T3-L1 cells, reducing cAMP production (29), and increasing glucose uptake and expression of the glucose transporters GLUT1 and GLUT4 (30), whereas GIP was without effect. In contrast, we did not observe any effect of GLP-1(7–36) on either cAMP or glycerol production (data not shown). Interestingly, Montroze-Rafizadeh *et al.* (29) were unable to demonstrate the presence in 3T3-L1 cells of the G protein-coupled GLP-1 receptor identified in pancreatic islets, and proposed the existence of a second receptor type. The differences in peptide-induced cAMP responses between the two cultured cell preparations could be due to the conditions of culture and differentiation, since in our studies the degree of responsiveness to GIP was found to be passage-dependent.

A strong link between GIP stimulation of adenylyl cyclase and lipolysis was demonstrated by the use of MDL 12330A (alternatively termed RMI 12330A), a compound that has been shown to inhibit adenylyl cyclase in a number of cell types in parallel with the inhibition of cAMP-mediated responses. This includes myocardial function (31), glucose-induced insulin biosynthesis (32), and induction of retinal *N*-acetyltransferase activity (33). In the time studies MDL 12330A, at a concentration of 10^{-4} M, reduced cAMP responses considerably, and this was paralleled by a reduction in GIP-stimulated glycerol release. The concentration required to inhibit adenylyl cyclase is similar to that previously shown to be necessary in heart cells (31). These results, in combination with those demonstrating potentiation by inhibition of phosphodiesterase, provide strong evidence in support of a causal link between cAMP and GIP-induced glycerol production.

Beck and Max (reviewed in Ref. 10) reported that GIP stimulated FA and glucose uptake into adipocytes from epididymal fat pads, and that the former involved an insulin-dependent, but cAMP-independent, pathway. Because the experiments on 3T3-L1 cells were performed in the absence of insulin, it was considered possible that the observed catabolic effects of GIP were a consequence of this absence. However, studies on the effect of insulin on GIP-stimulated glycerol release revealed that addition of insulin resulted in a concentration-dependent inhibition of both GIP- and isoproterenol-stimulated lipolysis. When cells were incubated in the presence of 10^{-10} M insulin, a concentration equivalent to that found circulating early postprandially (34), responses were significantly reduced over the complete GIP concentration range. Therefore, GIP is probably only capable of stimulating lipolysis during fasting, and in the period immediately following glucose absorption.

Insulin's suppressive effect on catecholamine-induced lipolysis has been shown to involve activation of cAMP phosphodiesterase (27, 35), resulting in reduced cAMP levels, and dephosphorylation of hormone-sensitive lipase via activation of a protein phosphatase (36). Additionally, insulin-receptor interaction has been shown to result in phosphorylation of tyrosine residues in G protein-coupled receptors, including the β_2 -adrenergic receptor, an effect that is believed to be directly mediated by the intrinsic tyrosine kinase

activity of the insulin receptor (37, 38). Although the mouse GIP receptor has not been completely sequenced as yet, the rat receptor contains a tyrosine residue in the third intracellular loop, and a second in the C-terminal tail, which are potential phosphorylation sites (Tyr₃₃₂ and Tyr₄₅₄). It is difficult to predict, without evidence of definitive tyrosine kinase consensus sequences (39), but it is possible that tyrosine phosphorylation of the GIP receptor is involved in insulin's antagonistic action.

It has been demonstrated that wortmannin, a selective inhibitor of phosphatidylinositol (PI) 3'-kinase, blocks insulin-induced inhibition of isoproterenol-stimulated lipolysis, suggesting that this enzyme is involved in insulin's inhibitory effect on hormone-stimulated lipase (40). Wortmannin has also been reported recently to cause partial inhibition of GIP-stimulated insulin secretion from HIT cells (41) and GIP-activated MAP kinase in GIP receptor-transfected CHO cells (42). In contrast, in the present study, wortmannin was not found to have any effect on GIP-induced lipolysis, suggesting that the signal-transduction pathways activated by GIP are cell specific. However, preincubation of 3T3-L1 adipocytes with wortmannin blocked insulin-mediated inhibition of GIP-induced lipolysis, implicating PI-3-kinase. The question of specificity is a concern with wortmannin, because it has been reported to exert various nonspecific effects. However, PI-3-kinase involvement in the antilipolytic action of insulin is well established (43, 44), and it seems likely that this enzyme is involved in insulin's effect on GIP-stimulated lipolysis. The pathway involved, however, is unclear. It is currently accepted that following insulin binding, insulin receptor substrates (IRSs) are tyrosine phosphorylated, and interact with the p85 subunit of PI 3'-kinase via src-homology 2 (SH2) domains (45). Subsequent events are not, however, clearly delineated. The phosphatidylinositol (PI) products, PI-3-P, PI-3,4-P₂ and PI-3,4,5-P₃ are clearly the most important second messengers. PI-3,4,5-P₃ has been shown to activate isoforms of protein kinase C (PKC) (46), and the rat GIP receptor contains the PKC consensus sequence Val-Val-Val-Arg-Arg-Ser-Glu-Lys-Gly in the second intracellular loop (243–251), suggesting one possible pathway. Alternatively, because at least one of the multiple forms of PI-3-kinase (46) acts as a dual specificity kinase, possessing both lipid and serine kinase activities (46), phosphorylation of serines in the intracellular loops or C-terminal tail could be involved in the PI 3'-kinase-mediated effect.

In summary, the current study provides strong evidence for a lipolytic effect of GIP in 3T3-L1 adipocytes, and a wortmannin-sensitive inhibition by insulin. Although the current results need to be replicated with primary adipocytes *in vitro*, and *in vivo* lipolytic responses to GIP must be established, a possible physiological for GIP may be associated with the recent demonstration that circulating fatty acids are essential for optimal glucose stimulation of insulin secretion following fasting (47). GIP could be capable of stimulating lipolysis under conditions in which insulin levels are of insufficient magnitude to inhibit its action, and this may ensure that levels of circulating FFAs are optimal for glucose- and GIP-stimulated insulin secretion. Such a proposal is open to experimental verification.

References

- Brown JC, Buchan AMJ, McIntosh CHS, Pederson RA 1989 Gastric inhibitory polypeptide. In: Schultz SG, Makhlouf GM, Rauner BB (eds) Handbook of Physiology, Section 6 (The Gastrointestinal System). American Physiology Society, Bethesda, pp 403–430
- Pederson RA 1993 Gastric inhibitory polypeptide. In: Walsh J, Dockray G (eds) Gut Peptides. Raven Press, New York, pp 217–259
- Habener JF 1993 The incretin notion and its relevance to diabetes. *Endocrinol Metab Clin North Am* 22:775–794
- Gutniak MK, Holst JJ, Linde B, Efendic S 1994 Subcutaneous injection of the incretin hormone glucagon-like peptide 1 abolishes postprandial glycemia in NIDDM. *Diabetes Care* 17:1039–1044
- McGarry JD 1998 Glucose-fatty acid interactions in health and disease. *Am J Clin Nutr* [67:500S–504S
- Ross SA, Shaffer EA 1981 The importance of triglyceride hydrolysis for the release of gastric inhibitory polypeptide. *Gastroenterology* 80:108–111
- Yoshidome K, Miyata M, Izukura M, Mizutani S, Sakamoto T, Tominaga H, Matsuda H 1995 Secretion of gastric inhibitory polypeptide in patients with bile duct obstruction. *Scand J Gastroenterol* 30:586–589
- Ebert R, Creutzfeldt W 1980 Reversal of impaired GIP and insulin secretion in patients with pancreatogenic steatorrhea following enzyme substitution. *Diabetologia* 19:198–204
- O'Dorisio TM, Cataland S, Stevenson M, Mazzaferri EL 1976 Gastric inhibitory polypeptide (GIP). Intestinal distribution and stimulation by amino acids and medium-chain triglycerides. *Am J Dig Dis* 21:761–765
- Beck B 1989 Gastric inhibitory polypeptide: a gut hormone with anabolic functions. *J Mol Endocrinol* 2:169–174
- Wasada T, McCorkle K, Harris V, Kawai K, Howard B, Unger RH 1981 Effect of gastric inhibitory polypeptide on plasma levels of chylomicron triglycerides in dogs. *J Clin Invest* 68:1106–1107
- Ebert R, Nauck M, Creutzfeldt W 1991 Effect of exogenous or endogenous gastric inhibitory polypeptide (GIP) on plasma triglyceride responses in rats. *Horm Metab Res* 23:517–521
- Eckel RH, Fujimoto WY, Brunzell JD 1979 Gastric inhibitory polypeptide enhanced lipoprotein lipase activity in cultured preadipocytes. *Diabetes* 28:1141–1142
- Hauer H, Glatting G, Kaminska D, Pfeiffer EF 1988 Effects of gastric inhibitory polypeptide on glucose and lipid metabolism of isolated rat adipocytes. *Ann Nutr Metab* 32:282–288
- Ebert R, Creutzfeldt W 1987 Metabolic effects of gastric inhibitory polypeptide. *Front Horm Res* 16:175–185
- Dupre J, Greenidge N, McDonald TJ, Ross SA, Rubinstein D 1976 Inhibition of actions of glucagon in adipocytes by gastric inhibitory polypeptide. *Metabolism* 25:1197–1199
- Wheeler MB, Gelling RW, McIntosh CHS, Georgiou J, Brown JC, Pederson RA 1995 Functional expression of the rat pancreatic islet glucose-dependent insulinotropic polypeptide (GIP) receptor: ligand binding and intracellular signaling properties. *Endocrinology* 136:4629–4639
- Gelling RW, Wheeler MB, Xue J, Gyomai S, Nian C, Pederson RA, McIntosh CHS 1997 Localization of the domains involved in ligand binding and activation of the glucose-dependent insulinotropic polypeptide receptor. *Endocrinology* 138:2640–2642
- Yeaman SJ 1990 Hormone-sensitive lipase—a multipurpose enzyme in lipid metabolism. *Biochim Biophys Acta* 1052:128–132
- Rosen OM, Smith CJ, Hirsch A, Lai E, Rubin CS 1979 Recent studies of the 3T3-L1 adipocyte-like cell line. *Recent Prog Horm Res* 35:477–499
- Rentsch J, Chiesi M 1996 Regulation of *ob* gene mRNA levels in cultured adipocytes. *FEBS Lett* 379:55–59
- Chomczynski P, Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
- Wieland O 1974 Glycerol U.V. method. In: Burgmeyer HU (ed) Methods of Enzymatic Analysis. Academic Press, New York, vol 4:1404–1409
- McIntosh CHS, Wheeler MB, Gelling RW, Brown JC, Pederson RA 1996 GIP receptors and signal-transduction mechanisms. *Acta Physiol Scand* 157:361–365
- Lai E, Rosen OM, Rubin CS 1981 Differentiation-dependent expression of catecholamine-stimulated adenylyl cyclase. Roles of the β -receptor and G/F protein in differentiating 3T3-L1 adipocytes. *J Biol Chem* 256:12866–12874
- Kawamura M, Jensen DF, Wancewicz EV, Joy LL, Khoo JC, Steinberg D 1981 Hormone-sensitive lipase in differentiated 3T3-L1 cells and its activation by cyclic AMP-dependent protein kinase. *Proc Natl Acad Sci USA* 78:732–736
- Elks ML, Manganiello VC, Vaughan MJ 1983 Hormone-sensitive particulate cAMP phosphodiesterase activity in 3T3-L1 adipocytes. Regulation of responsiveness by dexamethasone. *J Biol Chem* 258:8582–8587
- Hirsch AH, Rosen OM 1984 Lipolytic stimulation modulates the subcellular distribution of hormone-sensitive lipase in 3T3-L1 cells. *J Lipid Res* 25:665–677
- Montrose-Rafizadeh C, Yang H, Wang Y, Roth J, Montrose MH, Adams LG 1997 Novel signal transduction and peptide specificity of glucagon-like peptide receptor in 3T3-L1 adipocytes. *J Cell Physiol* 172:275–283
- Wang Y, Kole HK, Montrose-Rafizadeh C, Perfetti R, Bernier M, Egan JM 1997 Regulation of glucose transporters and hexose uptake in 3T3-L1 adipocytes: glucagon-like peptide-1 and insulin interactions. *J Mol Endocrinol* 19:241–248
- Grupp G, Grupp IL, Johnson CL, Matlib MA, Rouslin W, Schwartz A, Wallick ET, Wang T, Wisler P 1980 Effects of RMI 12330A, a new inhibitor of adenylyl cyclase on myocardial function and subcellular activity. *Br J Pharmacol* 70:429–442
- Simpson AM, Tuch BE 1995 Control of insulin biosynthesis in the human fetal beta cell. *Pancreas* 11:48–54
- Gan J, Alonso-Gomez AL, Avendano G, Johnson B, Iuvone PM 1995 Melatonin biosynthesis in photoreceptor-enriched chick retinal cell cultures: role of cyclic AMP in the K^+ -evoked, Ca^{2+} -dependent induction of serotonin N-acetyltransferase activity. *Neurochem Int* 27:147–155
- Flakoll P, Carlson MG, Cherrington A 1996 Physiologic action of insulin. In: LeRoith D, Taylor SI, Olefsky JM (eds) Diabetes Mellitus. Lippincott-Raven, Philadelphia, pp 121–132
- Degerman E, Beltrag P, Manganiello VC 1997 Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem* 272:6823–6826
- Strålfors P, Honnor RC 1989 Insulin-induced dephosphorylation of hormone-sensitive lipase. Correlation with lipolysis and cAMP-dependent protein kinase activity. *Eur J Biochem* 182:379–385
- Karoor V, Baltensperger K, Paul H, Czech MP, Malbon CC 1995 Phosphorylation of tyrosyl residues 350/354 of the β -adrenergic receptor is obligatory for counterregulatory effects of insulin. *J Biol Chem* 270:25305–25308
- Baltensperger K, Karoor V, Paul H, Ruoho A, Czech MP, Malbon CC 1996 The β -adrenergic receptor is a substrate for the insulin receptor tyrosine kinase. *J Biol Chem* 271:1061–1064
- Geahlen R, Harrison ML 1990 Protein-tyrosine kinases. In: Kemp BE (ed) Peptides and Protein Phosphorylation. CRC Press, Boca Baton, FL, pp 239–253
- Lam K, Carpenter CL, Ruderman NB, Friel JC, Kelly KL 1994 The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1. Stimulation by insulin and inhibition by wortmannin. *J Biol Chem* 269:20648–20652
- Straub SG, Sharp GWG 1996 Glucose-dependent insulinotropic polypeptide stimulates insulin secretion via increased cyclic AMP and $[Ca^{2+}]_i$ and a wortmannin-sensitive signalling pathway. *Biochem Biophys Res Commun* 224:369–374
- Kubota A, Yamada Y, Yasuda K, Someya Y, Ihara Y, Kagimoto S, Watanabe R, Kuroe A, Ishida H, Seino Y 1997 Gastric inhibitory polypeptide activates MAP kinase through the wortmannin-sensitive and -insensitive pathways. *Biochem Biophys Res Commun* 236:171–175
- Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M 1994 Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with the selective inhibitor wortmannin. *J Biol Chem* 269:3568–3573
- Evans JL, Honer CM, Womelsdorf BE, Kaplan EL, Bell PA 1995 The effects of wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase, on insulin-stimulated glucose transport, GLUT4 translocation, antilipolysis, and DNA synthesis. *Cell Signal* 7:365–376
- White MF 1997 The insulin signalling system and the IRS proteins. *Diabetologia* 40:S2–S17
- Toker A, Cantley LC 1997 Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* 387:673–676
- Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S, McGarry JD 1996 Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J Clin Invest* 97:2728–2735