

Role of glucose in chronic desensitization of isolated rat islets and mouse insulinoma (β TC-3) cells to glucose-dependent insulinotropic polypeptide

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Abstract

It is well documented that the release of insulin from isolated perfused islets attenuates over time, despite a continued glucose stimulation. In the current study we have shown that potentiation of insulin release by the intestinal hormone glucose-dependent insulinotropic polypeptide (GIP) is also attenuated after its continuous application. In less than 20 h of maintained stimulus with either hyperglycaemia (11.0 mM glucose) or GIP (10 nM) under hyperglycaemic conditions, insulin release returned to basal values. This was not due to loss of islet viability or reduction in the releasable pool of insulin granules, as 1 mM isobutylmethylxanthine was able to stimulate equivalent insulin release under both conditions. Further examination of chronic GIP desensitization was examined in cultured mouse insulinoma (β TC-3) cells. GIP-stimulated cAMP production was not greatly affected by the prevailing glucose conditions, suggesting that the

glucose dependence of GIP-stimulated insulin release occurs distally to the increase in intracellular cAMP in β TC-3 cells. The GIP-stimulated cAMP response curve after desensitization was of similar magnitude at all glucose concentrations, but GIP pretreatment did not affect forskolin-stimulated cAMP production. Desensitization of the cAMP response in β TC-3 cells was shown not to involve induction of dipeptidyl peptidase IV or pertussis toxin-sensitive G-proteins, activation of protein kinase C or protein kinase A, or modulation of phosphodiesterase activity. Homologous desensitization of the insulin-potentiating activity of GIP was found to affect both GIP-stimulated and forskolin-stimulated insulin release, indicating desensitization of distal steps in the stimulus-exocytosis cascade.

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Introduction

The primary hormones of the enteroinsular axis (incretins) are glucose-dependent insulinotropic polypeptide (gastric inhibitory polypeptide, GIP) and glucagon-like peptide-1 (GLP-1) (D'Alessio 1997). GIP is released from K cells of the duodenum and proximal jejunum in response to luminal nutrients whereupon it acts on the endocrine pancreas as a humoral signal (Pederson 1994). The incretins have an additive effect in augmenting glucose-induced insulin release from β -cells (Nauck *et al.* 1993a).

In type II diabetes mellitus (non-insulin dependent diabetes mellitus, NIDDM), GIP responsiveness is reduced, whereas responsiveness to GLP-1 is maintained (Nauck *et al.* 1993b, Elahi *et al.* 1994). This may be in part due to chronically high circulating GIP concentrations in some populations of these patients (Brown *et al.* 1975, Ross *et al.* 1977, Elahi *et al.* 1984, Jones *et al.* 1989).

However, GIP desensitization mechanisms have been only poorly characterized (Fehmann & Habener 1991, Tseng *et al.* 1996, Tseng & Zhang 1998a,b). In contrast, desensitization to GLP-1 has been extensively studied in insulin-secreting cell lines (Fehmann & Habener 1991, Gromada *et al.* 1996, Thorens & Widmann 1996) and at the molecular level. Thorens' group have demonstrated the control of GLP-1 receptor responsiveness through phosphorylation of distinct serine doublets in the carboxy (C)-terminal tail of the GLP-1 receptor, resulting in homologous and heterologous desensitization (Widmann *et al.* 1995, 1997).

The GIP receptor is a member of the class II superfamily of serpentine G-protein coupled receptors, to which homologous receptors for glucagon and GLP-1 also belong (Usdin *et al.* 1993). Activation of the GIP receptor results in stimulation of adenylyl cyclase and an increase in intracellular calcium ($[Ca^{2+}]_i$) (Lu *et al.* 1993, Wheeler

et al. 1995, Moens *et al.* 1996). Furthermore, GIP-stimulated exocytosis from islet cells has been determined to be dependent upon protein kinase A (PKA) action at a level of the secretory pathway distal to the increase in $[Ca^{2+}]_i$ (Ding & Gromada 1997). Desensitization of the rat GIP receptor has been localized to the C-terminal tail of the receptor (Tseng & Zhang 1998a, Wheeler *et al.* 1999). However, homologous desensitization of GIP-stimulated cAMP in β -cell models or isolated islets has not been demonstrated.

The present work was aimed at further characterizing β -cell desensitization, in response to both continuing stimuli (glucose and/or GIP) and repetitive stimulation by GIP, using perfused rat islets and mouse insulinoma cells. Studies examining desensitization in the murine β TTC-3 cell line were limited to homologous desensitization, as mouse β -cells reportedly do not desensitize to continuing stimulation with glucose (Zawalich *et al.* 1998). Hence, desensitization of the potentiating effect of GIP was examined by measuring both cAMP production in and insulin release from β TTC-3 cells.

Materials and Methods

Islet preparation and perfusion system

Islets were isolated from male Wistar rats (275–325 g) by collagenase digestion using a modification of the technique described by Van der Vliet *et al.* (1988). Islets were loaded into chambers (70–80 per chamber) and perfused with Hana HB104 perfusion medium (Bolaffi *et al.* 1988) in an Endotronics Acusyst-s culture/perfusion apparatus (Endotronics Inc., Coon Rapids, MN, USA) set to deliver medium to the perfusion chambers at 6.0 ml/h per chamber. This medium consisted of RPMI-1640 (Gibco, Burlington, Ont., Canada) supplemented with 0.07% human serum albumin (HSA; Sigma, Oakville, Ont., Canada), 0.0025% human transferrin (Sigma), 25 nM NaSelenite (Sigma), 20 μ M ethanolamine HCl (Sigma), 1% PBS (Mg^{2+} - and Ca^{2+} -free; Sigma) and penicillin (50 U/ml; Gibco). Glucose concentration was controlled by adding appropriate amounts of D-glucose (BDH Chemicals, Toronto, Ont., Canada) to give concentrations of 4.4 mM and 11.0 mM glucose in the media. Media were temperature controlled (37 °C) and pH was maintained at 7.4 by gassing media with 5% CO_2 /95% air. Polysulphone microchambers (200 μ l) and tubing were autoclaved before use. HPLC-purified monocomponent natural porcine GIP_{1–42} (CHS McIntosh, Physiology Department, UBC, Vancouver, BC, Canada) and 3-isobutyl-1-methylxanthine (IBMX; Research Biochemicals Intl., Natick, MA, USA) were added to the perfusion system through a side arm infusion pump (Harvard Apparatus Co., South Natick, MA, USA). Perfusate was collected distal to the islet-containing chambers at 10–60-min intervals and stored at –20 °C.

All fractions were assayed for immunoreactive insulin using a radioimmunoassay described previously (Pederson *et al.* 1982).

Perfusion procedures

Islets were sandwiched between layers of 150 μ l and 50 μ l Cytodex-3 beads (Amersham-Pharmacia, Baie d'Urfé, Que., Canada) in order to provide a support bed for islets and to prevent turbulent media flow. Four perfusion procedures were carried out as indicated in Fig. 1. Procedures were designed to examine the effect of GIP on the third phase of insulin release and desensitization of the GIP response in subthreshold glucose conditions. In all procedures, 1.0 mM IBMX was administered for 1 h, 5 h before completion of the experiment. Insulin secretion profiles were presented graphically as immunoreactive insulin release, normalized to the average insulin released during the control (4.4 mM glucose) condition during the first 4 h of perfusion.

Cell culture

β TTC-3 cells were obtained from Dr S Efrat (Efrat *et al.* 1988). Cells were cultured in low glucose (5.5 mM) DMEM (Gibco), 12.5% horse serum (Cansera, Rexdale, Ont., Canada), 2.5% fetal calf serum (Cansera), and antibiotics (Gibco). Cells were harvested with trypsin/EDTA (Gibco) and seeded into 24-well plates (Falcon, Becton Dickinson, Mississauga, Ont., Canada) at a density of 5×10^5 cells per well. Cells were used in cAMP stimulation and insulin release experiments 48 h later. Before all experiments, β TTC-3 cells were cultured for 6 h in 1.0 mM glucose DMEM with serum and antibiotics. β TTC-3 cells used in these experiments were of passages 20–25.

cAMP desensitization studies

In cAMP desensitization studies carried out on β TTC-3 cells, cells were washed twice with 37 °C cAMP buffer (DMEM (Gibco), 25 mM Hepes (Sigma), 0.1% BSA (Sigma) pH 7.4, and glucose: 0, 5.5 or 11.0 mM). Cells were then incubated for 1 h in cAMP buffer of varying glucose concentrations, supplemented with 1% Trasylol (aprotinin; Bayer, Etobicoke, Ont., Canada), with or without 100 nM GIP (synthetic human GIP_{1–42}; Bachem, Torrance, CA, USA). Treated cells were rinsed twice with appropriate glucose-containing cAMP buffer over a 10-min washout period. Cells were then stimulated for 30 min with a range of GIP concentrations (320 pM to 1 μ M) in triplicate, as indicated in the figures, in cAMP buffer supplemented with 0.5 mM IBMX. Cells stimulated under these conditions give steady-state cAMP concentrations (Gespach *et al.* 1984, and our unpublished observations). Forskolin (10 μ M; Sigma) was used as a

positive control. Cells were lysed in ice-cold 70% ethanol, dried on a Speed Vac (Savant, Holbrook, NY, USA), and then assayed for intracellular cAMP content by radioimmunoassay (Biomedical Technologies Inc., Stoughton, MA, USA). Control experiments were performed in Krebs–Ringer bicarbonate (KRB) buffer. Cells were similarly incubated for 1 h in 0 or 5.5 mM glucose KRB (gassed, pH 7.4), before a 30-min test period measuring basal, 100 nM GIP-stimulated or 10 μ M forskolin-stimulated cAMP production. The effect of glucose on cAMP production and desensitization were examined in parallel, such that one independent experiment consisted of six experimental conditions on cells all prepared at the same time. cAMP data are expressed as pmol per well or normalized to production of cAMP stimulated by 10 μ M forskolin.

As apparent desensitization could be explained by degradation of extracellular GIP by induction of the enzyme dipeptidyl peptidase IV (DPIV) or activation of pertussis toxin-sensitive G-proteins, their possible involvement was examined. Cells were prepared as above, pretreated with 100 nM GIP in HEPES-buffered 5.5 mM glucose DMEM containing 0.1% BSA, and subsequently stimulated with 10 nM GIP in the presence or absence of the DPIV inhibitor isoleucine-thiazolidide (50 μ M; Ile-Thia, K_i = 130 nM; Probiobdrug, Halle, Germany) or pertussis toxin (100 ng/ml during washout and 500 ng/ml during stimulation; Sigma) in the same buffer supplemented with 0.5 mM IBMX. The effect of IBMX on GIP desensitization was similarly tested. Cells were pretreated in the presence or absence of 100 nM GIP, followed by a 10-min washout period; during the subsequent stimulation period with 10 nM GIP, the concentration of IBMX was varied between 0 and 4 mM.

Negative feedback of signal transduction kinases (PKC and PKA) has also been suggested as a possible mechanism of receptor desensitization (Böhm *et al.* 1997). The broad specificity kinase inhibitors staurosporine (PKC K_i = 0.7 nM; Calbiochem, La Jolla, CA, USA) and H89 (PKA K_i = 48 nM; Calbiochem) were used at 100 nM and 5 μ M (respectively) – concentrations giving relatively specific and complete inhibition of PKC and PKA. In these experiments, inhibitor was included 15 min before addition of the 100 nM GIP pretreatment, and throughout the desensitization period, but not during the stimulation period. Intracellular cAMP was measured as above.

Insulin release experiments

Insulin release experiments were performed in KRB buffer as described for other clonal β -cell lines (Lu *et al.* 1993). Cells were washed twice with the appropriate glucose containing KRB and preincubated at 37 °C for 1 h in the presence or absence of 100 nM GIP in KRB supplemented with 1% Trasylol and in 5% CO₂. As with the cAMP studies, cells were washed over a 10-min period

and then either stimulated a further 30 min in KRB with glucose (0 mM, 5.5 mM or 11 mM) with or without 10 nM GIP or 10 μ M forskolin. Media were removed for insulin radioimmunoassay.

Data analysis

Graphs show the mean \pm S.E.M. with the number of experiments indicated in the figure legends. Statistical analyses performed were analysis of variance (ANOVA) and the Neuman–Keuls test where indicated. Significance was set at the 5% level. Data were analysed using the graphing and statistics program, Prism (GraphPad, San Diego, CA, USA).

Results

The control insulin release profile from isolated rat islets perfused under high glucose conditions (11.0 mM glucose); Fig. 1A depicts the onset of the second phase of insulin secretion during the first 5 h of increased glucose, followed by a return to baseline insulin values over the next 20 h, despite continued glucose stimulation. This latter phase has been termed the third phase of insulin release (Grotsky 1989) – a phase of secretagogue desensitization. Administration of 10 nM GIP at the peak of phase II under the same conditions potentiated the insulin release, as expected, however, the administration of GIP failed to prevent the onset of desensitization. In both cases, stimulation of islets with IBMX resulted in similar insulin responses after the desensitization period, demonstrating islet viability and discounting insulin depletion as the cause of the decrease in insulin release (Fig. 1A). During perfusion of isolated islets with 4.4 mM glucose for 20 h before an 11.0 mM glucose challenge, hourly baseline insulin secretion rates were approximately 1.7–3.6% of total cell content (Fig. 1B). Figure 1B shows the insulinotropic effects of GIP under hyperglycaemic conditions, compared with the effects of glucose alone. After GIP was administered for 20 h at subthreshold glucose concentrations, increasing the glucose concentration stimulated insulin release, but this was less than the insulin release stimulated by glucose alone, and 1 mM IBMX failed to elicit the same magnitude of insulin release achieved with perfusion procedures in which GIP and/or glucose was administered for only 25 h. It appears that chronic GIP administration at subthreshold glucose concentrations was able to desensitize rat islets to glucose and further GIP stimulation, and to IBMX.

Possible mechanisms of GIP desensitization were studied using β TC-3 cells as a β -cell model. GIP receptors have been demonstrated to be expressed in this cell line, and stimulation of insulin release by GIP is glucose-dependent (Kieffer *et al.* 1993). cAMP production in

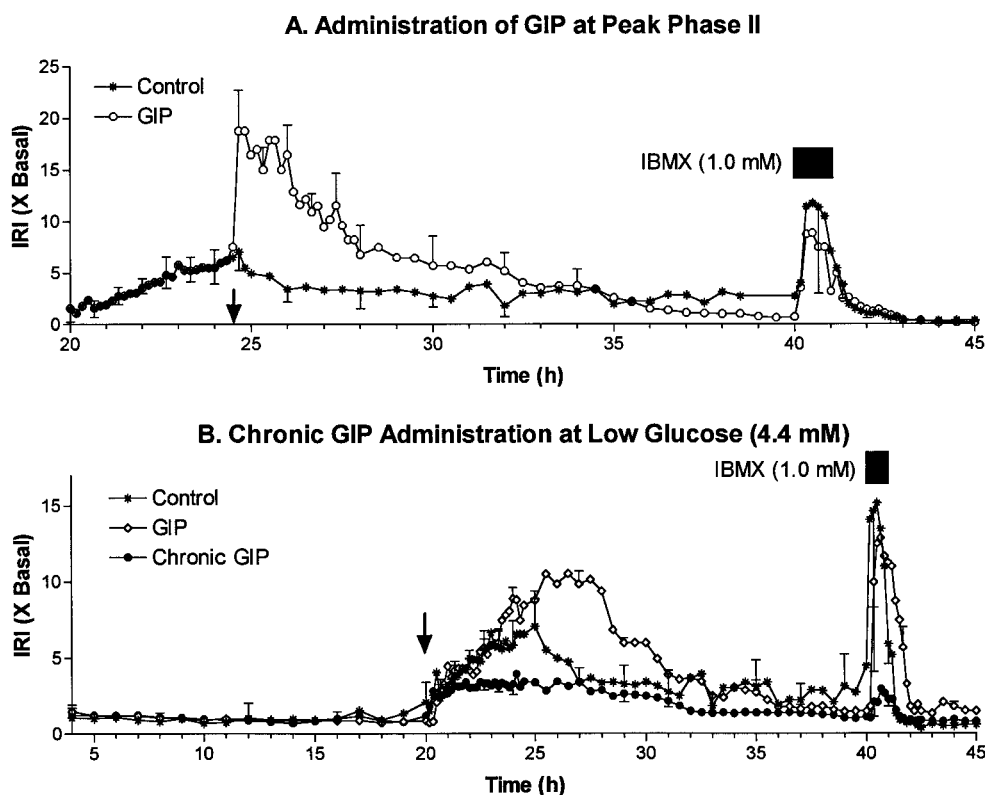


Figure 1 Insulin release from perifused isolated rat islets in response to glucose and GIP. (A) Islets were perfused with 4.4 mM glucose medium for 20 h before glucose concentrations were increased to 11 mM. The arrow indicates when 10 nM GIP was added to the perfusion medium (○), rather than glucose alone (*). (B) Islets were perfused with 4.4 mM glucose medium without (* and ◇) or with (●) 10 nM GIP. After 20 h (arrow) the glucose concentration was increased to 11 mM (control: *), and 10 nM GIP was begun (◇) or continued (●). Data represent the immunoreactive insulin (IRI) released per hour normalized to basal insulin release during the first 4 h of low glucose perfusion (mean ± S.E.M.; $n=3-4$); S.E.M. are shown only every 5 data points, for clarity. Refer to text for specific methods.

response to GIP was found to increase in a concentration-dependent manner (Fig. 2A–D), and was only slightly affected by glycaemic conditions. The maximal cAMP produced in response to GIP was moderately blunted with increasing ambient glucose conditions (Table 1), but sensitivity to GIP was not affected (EC_{50} values: 0 mM glucose: 12.5 ± 4.8 nM; 5.5 mM glucose: 9.5 ± 2.0 nM; 11 mM glucose: 10.9 ± 4.4 nM). At each glucose concentration, however, the desensitized cAMP response was similar after pretreatment of cells with 100 nM GIP for 1 h (Table 1). cAMP production stimulated by 10 μ M forskolin was altered neither by glycaemic condition nor by pretreatment with 100 nM GIP for 1 h (Table 1). A timecourse for desensitization of GIP-stimulated cAMP indicated that a mild but significant reduction in cAMP production could be observed with 10 min of 100 nM GIP pretreatment, and the apparent desensitization was more pronounced with increasing duration of pretreatment (Fig. 3A).

Previous studies have reported failure of GLP-1 or GIP to stimulate cAMP production in the absence of glucose in rodent insulinoma cell lines when performed in Krebs' Ringer solution (Fehmman & Habener 1991, Lu *et al.* 1993). When it was found that GIP was able to produce a concentration-dependent production of cAMP in β TC-3 cells in 0 mM glucose DMEM (Fig. 2A,B), experiments were repeated in KRB to rule out the involvement of more complex constituents. GIP (100 nM) and forskolin (10 μ M) yielded similar responses in the presence of either 0 mM or 5.5 mM glucose, respectively ($P>0.05$) that were similar in magnitude to those obtained in experiments performed in DMEM (data not shown).

Degradation of extracellular ligand is a well accepted mechanism for attenuation of hormone stimulation and could contribute to reduced responses to GIP. Under perfusion conditions, ligand is continually replenished and thus this mechanism is unlikely to play a significant role. However, induction of DPIV, the primary enzyme

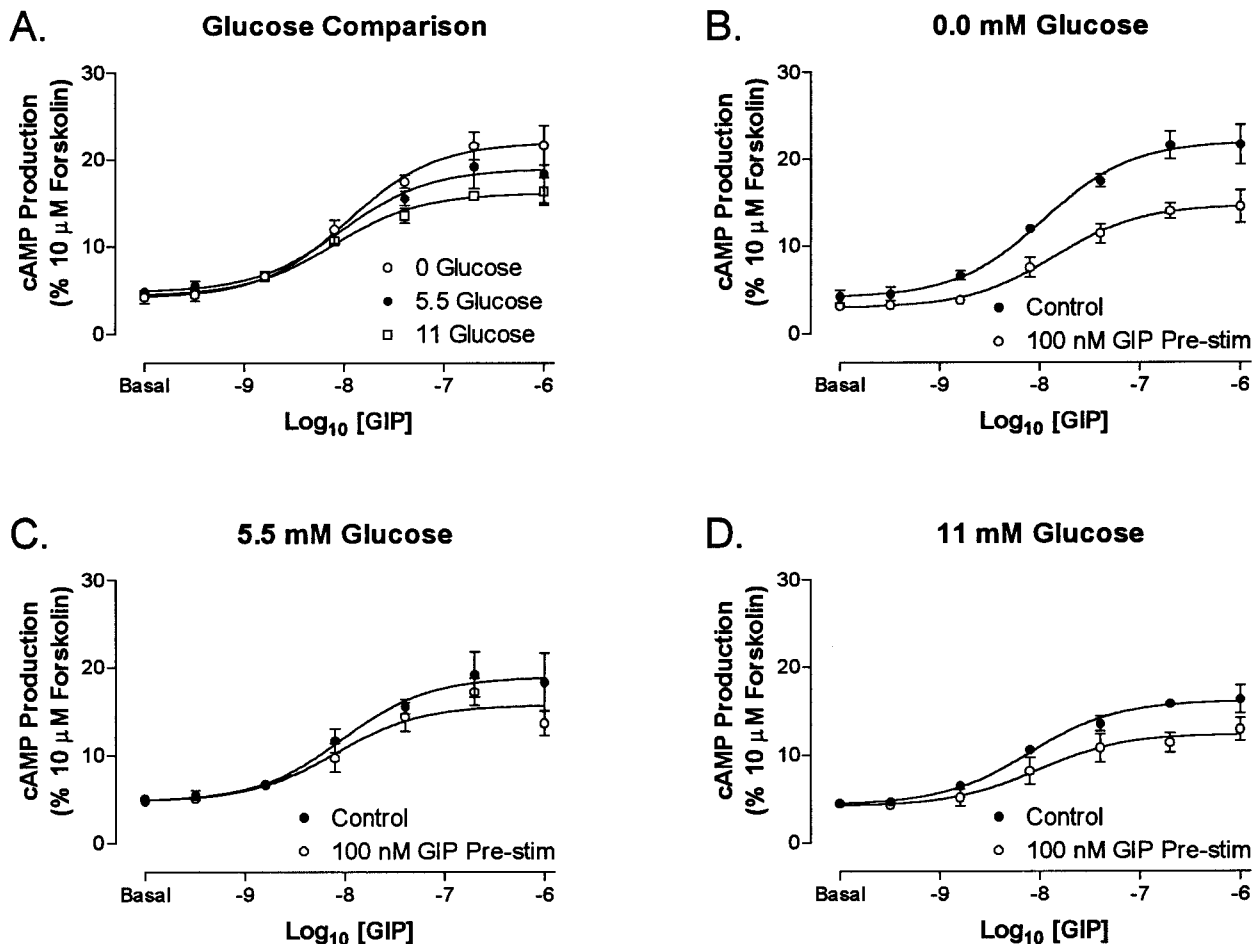


Figure 2 Effect of glycaemic conditions on GIP-stimulated cAMP production in β TC-3 cells. (A) Comparison of GIP-stimulated cAMP production under various glucose conditions. (B–D) Effect of pretreating cells with 100 nM GIP before obtaining concentration–response curves. Glucose had no effect on forskolin-stimulated cAMP production. Data represent the mean \pm S.E.M. of three independent experiments. Refer to text for specific methods.

responsible for incretin inactivation (Pauly *et al.* 1996), could contribute to the desensitized state observed in β TC-3 cells with static incubations. Therefore, the potent

reversible non-hydrolysable transition state inhibitor Ile-Thia was used to examine this possibility (Pederson *et al.* 1998, Pauly *et al.* 1999). Ile-Thia did not affect basal,

Table 1 Summary of cAMP production in β TC-3 cells in response to GIP and forskolin. Cells were incubated with GIP (320 pM–1 μ M) or 10 μ M forskolin for 30 min in the presence of 0.5 mM IBMX; cAMP was measured by RIA. Data represent mean \pm S.E.M. of three independent experiments. (See Fig. 3 for complete concentration–response curves)

	Glucose (mM)	Control (pmol cAMP/well)	100 nM GIP-pretreated* (pmol cAMP/well)
Maximal response to GIP	0.0	70.5 \pm 7.9	52.4 \pm 10.3
	5.5	66.3 \pm 8.1	55.7 \pm 9.1
	11.0	55.2 \pm 7.8	44.4 \pm 7.6
Response to 10 μ M forskolin	0.0	331.0 \pm 40.8	347.1 \pm 29.1
	5.5	346.3 \pm 16.8	328.3 \pm 25.8
	11.0	337.3 \pm 30.0	342.6 \pm 36.6

*Cells were prestimulated with 100 mM GIP for 1 h followed by a 10-min washout period.

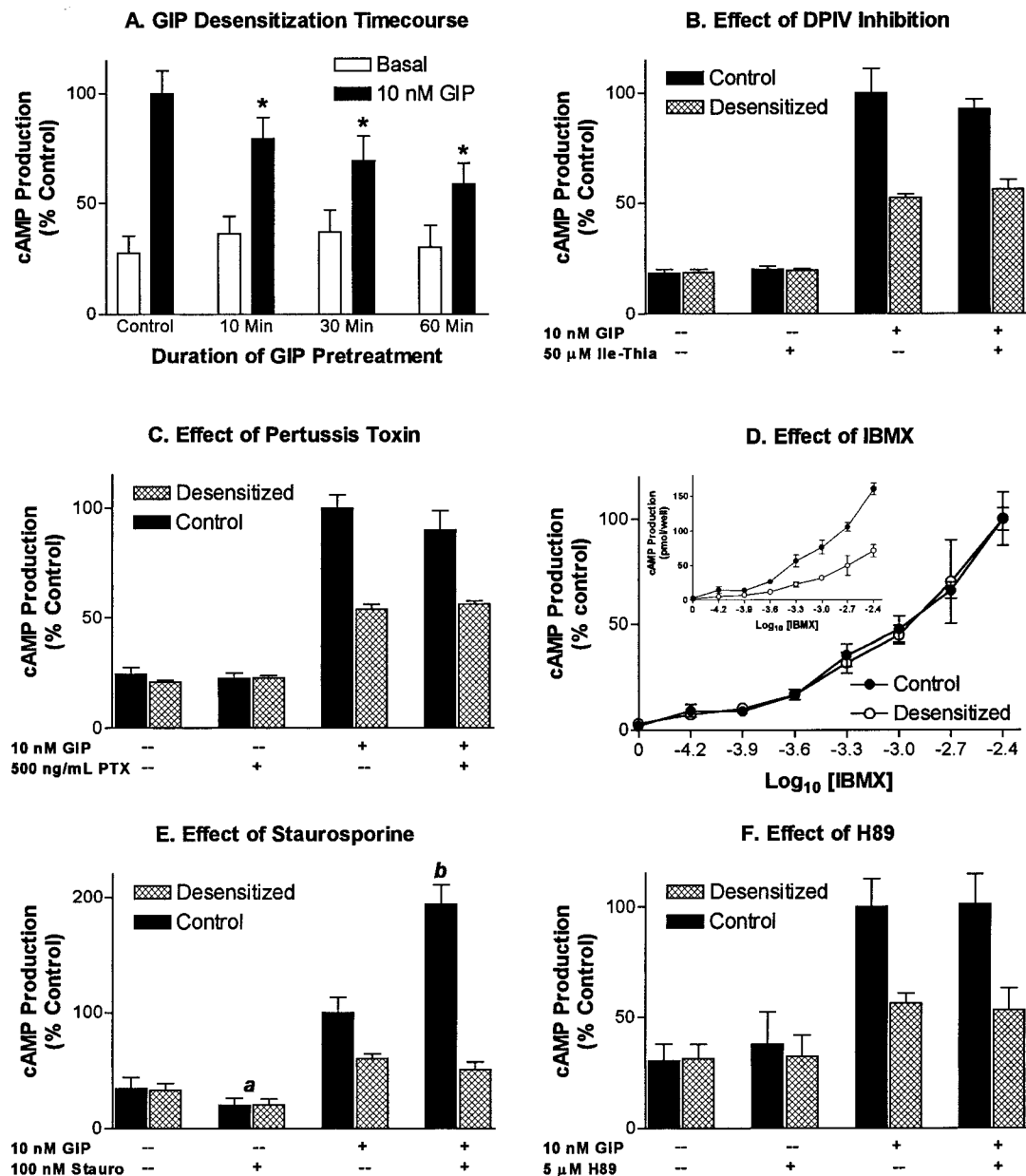


Figure 3 Timecourse of homologous desensitization of GIP-stimulated cAMP production and effect of various inhibitors on desensitization in β TC-3 cells. (A) Cells were pretreated with or without 100 nM GIP for indicated times, followed by a 10-min washout period and subsequent stimulation with 10 nM GIP. * $P < 0.05$ compared with control. Subsequent figures show GIP-stimulated cAMP production with or without pretreatment with 100 nM GIP for 60 min, followed by a 10-min washout period and subsequent stimulation with 10 nM GIP. Except where indicated, 0.5 mM IBMX was used during the stimulation period. (B) Effect of Ile-Thia, a potent DPIV inhibitor, administered during the stimulation period. (C) Effect of pertussis toxin (100 ng/ml during washout period and 500 ng/ml during stimulation) on GIP-stimulated cAMP production. (D) Effect of IBMX on apparent desensitization of GIP-stimulated cAMP production. Data were normalized to the response observed in the control or desensitized state at 4 mM IBMX. The inset shows the same data presented as pmol cAMP/well. (NB: The IBMX concentration is plotted using a logarithmic scale.) (E and F) Effects of 100 nM staurosporine or 5 μ M H89 respectively on homologous desensitization of GIP-stimulated cAMP formation, administered 15 min before and during the GIP pretreatment. ^a $P < 0.05$ compared with non-stimulated, non-staurosporine, ^b $P < 0.01$ compared with stimulated, non-staurosporine. Data represent the mean \pm S.E.M. of four independent experiments. Refer to text for specific methods.

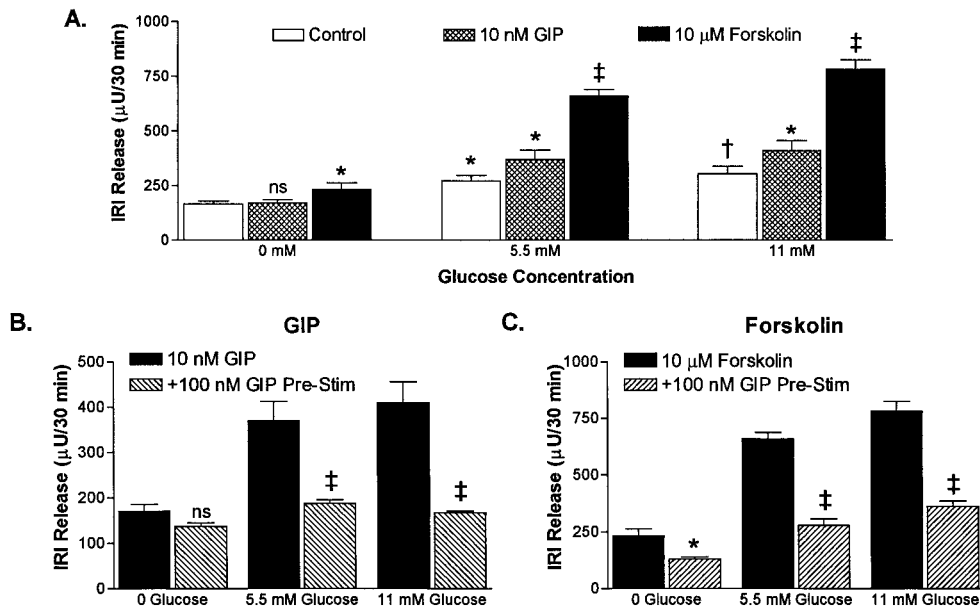


Figure 4 Release of immunoreactive insulin (IRI) from β TC-3 cells. (A) Comparison of glucose, GIP and forskolin-stimulated insulin release. (B) Effect of pretreating cells with 100 nM GIP before 10 nM GIP stimulation. (C) Effect of pretreating cells with 100 nM GIP before 10 μ M forskolin stimulation. Data represent mean \pm S.E.M. of at least four independent experiments. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ for comparisons of GIP and forskolin-stimulated insulin release with euglycaemic controls, and for comparisons of glucose-stimulated insulin release with basal release in the absence of glucose. Refer to text for specific methods.

stimulated or desensitized GIP cAMP responses (Fig. 3B). Recently, Kesper *et al.* (1999) found that chronic exposure of INS-1 cells to GIP resulted in induction of G_{α_i} G-proteins. An increase in inhibitory G-protein complement could also diminish the cAMP response to GIP. However, pertussis toxin had no effect on basal, stimulated or desensitized GIP cAMP responses in β TC-3 cells (Fig. 3C).

Prior work examining desensitization of the glucagon receptor has suggested that alterations in phosphodiesterase (PDE) activity could also lead to an apparent reduction in cAMP production by hormones (Attramadal *et al.* 1988). It was hypothesized that, if PDE were modulated by pretreatment with GIP, then a change in sensitivity to IBMX would be observed relative to control cells. To test the possibility of modulation of PDE activity in the desensitized state, the effect of IBMX concentration was tested under both control and GIP-pretreated conditions (Fig. 3D). Intracellular cAMP concentrations increased with increasing concentration of IBMX over the range tested; however, maximal inhibition of PDE was not achieved. When cAMP was normalized to the maximal value observed in either the control or the desensitized state, no alteration in sensitivity to IBMX was observed. Higher concentrations of IBMX would probably result in non-specific effects.

Signal transduction kinases PKC and PKA have been shown to contribute to receptor desensitization in other hormone models (Böhm *et al.* 1997). These kinases can form a negative feedback loop whereby surface receptors are phosphorylated, thus blunting their response to further stimulation. Broad specificity inhibitors of PKC (staurosporine) and PKA (H89) were used during the GIP pretreatment period to block this feedback, if present (Fig. 3E and F). Staurosporine acted to reduce significantly the basal cAMP concentrations in non-stimulated cells ($P < 0.05$) and to enhance GIP-stimulated cAMP production (1.9-fold; $P < 0.01$), but did not inhibit attenuation of the cAMP response by pretreatment with GIP. In contrast, H89 had no effect on basal or stimulated cAMP values, or desensitization of the cAMP response to GIP.

The degree of glucose-stimulated insulin release from β TC-3 cells in KRB was similar to that previously observed (Kieffer *et al.* 1993), with significant insulin released by 5.5 and 11 mM glucose ($163 \pm 16\%$ and $182 \pm 21\%$ basal release in 0 mM glucose respectively; $P < 0.05$ and $P < 0.01$ compared with basal) (Fig. 4A). In the absence of glucose, GIP failed to stimulate insulin release ($102 \pm 10\%$ basal), but forskolin stimulated a small but significant release ($140 \pm 18\%$ basal; $P < 0.05$). In the presence of increased glucose, 10 nM GIP potentiated insulin release (5.5 mM glucose: $223 \pm 26\%$ basal; 11 mM

glucose: $247 \pm 28\%$ basal; $P < 0.05$), as did $10 \mu\text{M}$ forskolin (5.5 mM glucose: $397 \pm 17\%$ basal; 11 mM glucose: $472 \pm 26\%$ basal; $P < 0.001$) (Fig. 4A). In contrast to the moderate effect of GIP pretreatment on cAMP production, pretreatment of cells with 100 nM GIP completely abolished the potentiating effect of GIP on insulin secretion under conditions of increased glucose (5.5 mM glucose: $113 \pm 4\%$ basal; 11 mM glucose: $101 \pm 3\%$ basal; $P < 0.001$ compared with euglycaemic controls) (Fig. 4B). Furthermore, whereas GIP pretreatment yielded no effect on forskolin-stimulated cAMP production, the same treatment significantly reduced forskolin-stimulated insulin release to values roughly half the magnitude of that in euglycaemic controls (5.5 mM glucose: $168 \pm 17\%$ basal; 11 mM glucose: $218 \pm 14\%$ basal; $P < 0.001$ compared with euglycaemic controls).

Discussion

In confirmation of previous work (Kieffer *et al.* 1993, Jia *et al.* 1995), GIP was shown in the current study to exert its insulin-potentiating effects only in the presence of increased glucose, with both perfused islets and $\beta\text{TC-3}$ cells (Figs 1 and 4). Chronic exposure of islets to 11 mM glucose resulted in an insulin secretory profile characterized by desensitization of the response over time (Fig. 1A) (phase III of insulin release; Grodsky 1989). Retention of the insulin response to IBMX demonstrated both viability of the islets and that attenuation of the insulin release was not due to depletion of intracellular insulin stores. When GIP was included in the perfusion medium, it potentiated the insulin response to glucose, but did not prevent the onset of phase III. Rather, islets exhibited desensitization to both GIP and glucose. Under conditions of low glucose, GIP did not stimulate insulin secretion (Fig. 1B). When the glucose concentration of the perfusate was increased after prolonged GIP application, GIP failed to potentiate insulin secretion (Fig. 1B). These results indicate that, unlike the insulinotropic action of GIP, desensitization of islets to the incretin are independent of the ambient glucose concentration.

In order to understand the basis of desensitization more clearly at the level of intracellular signalling cascades, the $\beta\text{TC-3}$ insulinoma cell line was used. The effect of glucose on homologous desensitization of β cells to GIP was studied with respect to cAMP production and the subsequent effect – insulin release. In contrast to the findings of previous previous work on HIT-T15 cells (Lu *et al.* 1993), we demonstrated that $\beta\text{TC-3}$ cells exhibited GIP-concentration-dependent effects on cAMP in the absence of glucose, and with physiological levels of glycaemia (Fig. 2). Glucose alone appeared to have very mild effects on cAMP production, slightly reducing the cellular responsiveness to GIP with increasing glucose. Prior work on the glucose dependence of incretin-stimulated cAMP

in HIT-T15 cells found that neither GLP-1 nor GIP could increase intracellular cAMP in the absence of glucose, although forskolin-stimulated cAMP was unaffected (Fehmann & Habener 1991, Lu *et al.* 1993). The apparent conflict between the glucose dependence of the cAMP data from HIT and $\beta\text{TC-3}$ cell models may be species dependent and will probably be clarified by similar experiments on alternative insulinoma cells (Poitout *et al.* 1996) or purified β -cells (Moens *et al.* 1996). Regardless of these factors, the necessity for the presence of glucose for GIP to potentiate insulin release was observed in $\beta\text{TC-3}$ cells (Fig. 4), indicating that the glucose dependence of GIP was intact.

Homologous desensitization of GIP-mediated effects was first described by Fehmann & Habener (1991), who examined only the effects of GIP pretreatment on insulin release from HIT cells, not second messenger cascades. Recently, researchers have shown homologous desensitization of GIP-stimulated cAMP from non-glucose-responsive heterologous expression models (Tseng & Zhang 1998b); the current study examined desensitization of both cAMP and insulin release in a single glucose-responsive cell line. Desensitization of GIP-stimulated cAMP in $\beta\text{TC-3}$ cells resulting from GIP pretreatment was at best moderate (Fig. 2). The magnitude of the desensitized response was similar under all glycaemic conditions, although, as a result of the slight inhibitory effect of glucose on GIP-stimulated cAMP formation, the desensitization appeared to be greater in the absence of glucose. Using an identical procedure, we found that the degree of desensitization observed in the absence of glucose corresponds well with that observed in non-glucose-sensitive CHO-K1 cells transfected with the rat pancreatic islet GIP receptor (unpublished observations).

In order to elucidate the point in the signal transduction cascade at which desensitization of the cAMP response to GIP was occurring, we used various inhibitors (Fig. 3). The apparent reduction in cAMP produced after GIP pretreatment could be explained by degradation of extracellular ligand, activation of pertussis-sensitive G-proteins, modulation of adenylyl cyclase or PDE activity, or negative feedback by signal transduction kinases (PKC and PKA). Inhibition of DP-IV, the primary enzyme responsible for degradation of GIP *in vivo* (Kieffer *et al.* 1995), had no effect on GIP-stimulated cAMP or desensitization in $\beta\text{TC-3}$ cells; neither did pertussis toxin. GIP pretreatment reduced only GIP-stimulated cAMP production, not forskolin-stimulated cAMP production, suggesting that adenylyl cyclase activity was unaffected by GIP pretreatment. Furthermore, PDE sensitivity to IBMX was unchanged in the desensitized state, ruling out an increase in PDE activity resulting in a reduction of intracellular cAMP. Staurosporine did affect basal and GIP-stimulated cAMP in control cells, indicating that PKC probably has a role in regulating normal β -cell responsiveness to GIP, but it did not affect homologous desensitization to GIP. PKA

inhibition did not affect responsiveness of β TC-3 cells to GIP. These results, taken together, suggest that homologous desensitization of GIP-stimulated cAMP occurs at the receptor level, and may involve regulators of G-protein signalling (Tseng & Zhang 1998b), receptor sequestration (Wheeler *et al.* 1999) or unidentified processes.

Both glucose and GIP produced changes in insulin release from β TC-3 cells similar to those previously published (Kieffer *et al.* 1993). Parallel experiments on desensitization of insulin release show that the same procedure used for the cAMP studies yields a significant reduction in insulin release. Pretreatment of cells with GIP dramatically returned 10 nM GIP-stimulated insulin to basal values in either 5.5 or 11 mM glucose conditions. It would be difficult to attribute the dramatic alteration in insulin release to the moderate attenuation of GIP-stimulated cAMP by GIP pretreatment, therefore implicating desensitization of distal steps in the stimulus-exocytosis coupling cascade. This hypothesis is supported by similar experiments utilizing forskolin. Forskolin potentiates glucose-induced insulin release via direct activation of adenylyl cyclase and cAMP production. In contrast to GIP-stimulated cAMP production, pretreatment with GIP had no effect on the forskolin-stimulated accumulation of cAMP (Table 1). Nevertheless, GIP pretreatment significantly blunted forskolin-mediated insulin release to near basal values (Fig. 4). This is consistent with the finding that chronic administration of GIP under subthreshold glycaemic conditions blunted both glucose- and IBMX-induced insulin release (Fig. 1B). Further support of this hypothesis is provided by the recent finding that chronic pretreatment of INS-1 cells with GIP also blunted glucose induced insulin release (Kesper *et al.* 1999).

GIP has been found to augment depolarization-induced exocytosis from individual mouse β -cells via a PKA-dependent mechanism (Ding & Gromada 1997). Consistent with results using cAMP analogues and forskolin, GIP exerts its action on exocytosis at a level distal to the increase in intracellular calcium via PKA (Åmmälä *et al.* 1993, Ding & Gromada 1997). Evidence also exists that cAMP interacts directly with the secretory machinery, sensitizing it to $[Ca^{2+}]_i$ (Åmmälä *et al.* 1993, Renström *et al.* 1997). GIP also reportedly acts through phosphatidylinositol 3-kinase (PI3-kinase), as indicated by wortmannin-sensitive activation of mitogen-activated protein (MAP) kinase and stimulation of insulin release (Straub & Sharp 1996, Kubota *et al.* 1997). Thus modulation of these enzymes or any element of the stimulus-secretion machinery by PKA, PI3-kinase, MAP kinase or other uncharacterized mediators of GIP effects could account for the profound effect of GIP prestimulation on glucose, GIP, IBMX and forskolin-stimulated insulin responses. Indeed, the related incretin hormone, GLP-1, has been implicated in the tyrosine phosphorylation of several proteins, one of which was identified as the

synaptic-associated protein of 25 kDa (SNAP-25) (Zhou & Egan 1997), supporting the notion of direct modulation of the exocytotic machinery.

In conclusion, desensitization of islets to glucose and GIP was initially examined. GIP augmented glucose-stimulated insulin release, but did not prevent the onset of phase III. Prolonged exposure to GIP resulted in a loss of GIP-stimulated insulin secretion, regardless of the perfusate glucose concentration. Examination of signal transduction cascades in a β -cell model (β TC-3) showed that, in these cells, stimulation of cAMP by GIP was independent of glucose concentration, whereas insulin release was dependent on prevailing glycaemic conditions. Desensitization of the GIP receptor was mild, but the same procedure produced profound effects on insulin secretion in response to either GIP or forskolin. Collectively, the results implicate desensitization of distal elements in the secretory pathway as being rate limiting in desensitization to GIP.

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