Actions of glucagon-like peptide-1 on K_{ATP} channel-dependent and -independent effects of glucose, sulphonylureas and nateglinide

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Abstract

This study examined the effects of glucagon-like peptide-1 (GLP-1) on insulin secretion alone and in combination with sulphonylureas or nateglinide, with particular attention to K_{ATP} channel-independent insulin secretion. In depolarised cells, GLP-1 significantly augmented glucose-induced K_{ATP} channel-independent insulin secretion in a glucose concentration-dependent manner. GLP-1 similarly augmented the K_{ATP} channel-independent insulin-releasing effects of tolbutamide, glibenclamide or nateglinide. Downregulation of protein kinase A (PKA)- or protein kinase C (PKC)-signalling pathways in culture revealed that the K_{ATP} channel-independent effects of sulphonylureas or nateglinide were critically dependent upon intact PKA and PKC signalling. In contrast, GLP-1 exhibited a reduced but still significant insulin-releasing effect following

PKA and PKC downregulation, indicating that GLP-1 can modulate K_{ATP} channel-independent insulin secretion by protein kinase-dependent and -independent mechanisms. The synergistic insulin-releasing effects of combinatorial GLP-1 and sulphonylurea/nateglinide were lost following PKA- or PKC-desensitisation, despite GLP-1 retaining an insulin-releasing effect, demonstrating that GLP-1 can induce insulin release under conditions where sulphonylureas and nateglinide are no longer effective. Our results provide new insights into the mechanisms of action of GLP-1, and further highlight the promise of GLP-1 or similarly acting analogues alone or in combination with sulphonylureas or meglitinide drugs in type 2 diabetes therapy.

Journal of Endocrinology (2006) **190**, 889–896

Introduction

The starting point for type 2 diabetes therapy is a change in lifestyle, especially diet. Unfortunately, dietary and lifestyle measures alone achieve adequate glycaemic control in only a minority of patients. Thus, oral hypoglycaemic drugs are routinely prescribed for type 2 diabetic patients. Some of these drugs, such as metformin and thiazolidenediones, primarily exert their effects on extra-pancreatic insulin-target tissues (Krentz & Bailey 2005), while others directly target the pancreatic β -cell and induce insulin secretion.

Historically, the most prevalent insulin-releasing drugs have been the sulphonylureas, which potently stimulate insulin secretion by a direct action on the pancreatic β -cell. *In vitro* studies have demonstrated that the insulin-releasing effects of sulphonylureas are primarily mediated via pancreatic β -cell K_{ATP} channels (Ashcroft & Gribble 1999, Bryan *et al.* 2005). The binding of sulphonylureas to the sulphonylurea receptor (SUR)1 subunit of these channels results in closure of the Kir6.2 pore, which in turn evokes membrane depolarisation, influx of extracellular calcium and, ultimately, exocytosis of insulin (Ashcroft & Gribble 1999, Bryan *et al.* 2005). In addition to this mechanism, several studies have reported direct intracellular effects of sulphonylureas upon exocytosis independent of

K_{ATP} -channel function (Flatt *et al.* 1994, Eliasson *et al.* 1996, Tian *et al.* 1998, Ball *et al.* 2000a, Kamp *et al.* 2003).

Despite their potent insulin-releasing effects, clinical studies have shown that over time sulphonylurea therapy has a tendency to fail (Matthews *et al.* 1998). *In vitro* data confirm that over time, prolonged exposure of insulin-secreting cells to sulphonylureas dramatically decreases their ability to stimulate insulin secretion (Rabuzzo *et al.* 1992, Ball *et al.* 2000a,b, 2004a). These findings highlight the need for a new generation of insulin-releasing drugs.

One such novel drug is nateglinide, a D-phenylalanine derivative, considered to act as a prandial insulin releaser and which has recently been approved for clinical use. Nateglinide elicits insulin-releasing effects in a similar manner to sulphonylureas, in that it blocks K_{ATP} -channel activity (Hu 2002), and has also been shown to exert a K_{ATP} channel-independent effect on insulin secretion (Ball *et al.* 2004b). Recent data have suggested that a similar β -cell desensitisation compared with sulphonylureas may occur following prolonged exposure to nateglinide (Ball *et al.* 2004b).

In vivo, insulin secretion can be stimulated by glucagon-like peptide-1 (GLP-1), a hormone of the enteroinsular axis, released into the circulation from the gut after meal ingestion. This insulin-releasing action has led to GLP-1 and more stable

GLP-1 analogues being considered as potential therapeutic agents for type 2 diabetes (Holst 2002, Drucker 2003, Green *et al.* 2004). Furthermore, GLP-1 has been shown to enhance the growth, differentiation, proliferation and survival of pancreatic β -cells (Egan *et al.* 2003), qualities that make GLP-1 an attractive candidate for diabetes therapy. The effects of GLP-1 on insulin release are mediated by binding to a specific G-protein-coupled receptor on the β -cell plasma membrane, resulting in the activation of adenylate cyclase. This causes an increase in β -cell cAMP production, which in turn leads to activation of protein kinase A (PKA) and insulin secretion (MacDonald *et al.* 2002, Gromada *et al.* 2004). GLP-1 also enhances insulin secretion by interacting with multiple cellular processes, including regulation of ion-channel activity, intracellular calcium handling and insulin exocytosis (MacDonald *et al.* 2002, Gromada *et al.* 2004).

This study was designed to provide a detailed analysis of the effects of GLP-1 on insulin secretion alone and in combination with sulphonylureas or nateglinide. Particular attention was paid to its effects on insulin-secreting cells exposed to a depolarising concentration of KCl, in order to examine its effects in conditions where K_{ATP} -channel closure was unable to modulate insulin release. Additionally, we determined the effects of GLP-1 in combination with sulphonylureas and nateglinide in depolarised and non-depolarised cells. Finally, we examined how the insulin-releasing effects of GLP-1, sulphonylureas and nateglinide were affected under conditions designed to prohibit signalling via PKA- and protein kinase C (PKC)-dependent pathways. Our results provide new insights into the mechanism(s) of action of GLP-1 and have implications for the ongoing quest to develop improved type 2 diabetes therapies.

Materials and Methods

Chemicals

Reagents of analytical grade and deionised water (Purite Ltd, Thame, Oxon, UK) were used. RPMI-1640 tissue culture medium, foetal bovine serum and antibiotics were obtained from GibcoBRL (Paisley, Strathclyde, UK), rat insulin standard was from Novo-Nordisk (Bagsvaerd, Denmark) and [^{125} I]bovine insulin was from Lifescan (Watford, UK). GLP-1 was obtained from American Peptide Company (Sunnyvale, CA, USA). Nateglinide was a gift from Novartis Pharmaceuticals Corporation (Summit, NJ, USA). All other chemicals were from Sigma and BDH Chemicals Ltd (Poole, Dorset, UK).

Cell culture and measurement of insulin release

Clonal pancreatic BRIN-BD11 cells (passage numbers 20–30) were used for this study. The origin and characteristics of this glucose-responsive insulin-secreting cell line, including metabolic, membrane potential and calcium ion-channel

properties, are described in detail elsewhere (McClenaghan *et al.* 1996, Chapman *et al.* 1999, Salgado *et al.* 2000, Brennan *et al.* 2002, Van Eylen *et al.* 2002). BRIN-BD11 cells were grown in RPMI-1640 tissue culture medium containing 11.1 mmol/l glucose and 0.3 g/l L-glutamine and supplemented with 10% (v/v) foetal calf serum, 100 IU/ml penicillin and 0.1 g/l streptomycin at 37 °C with 5% CO₂ and 95% air. The tissue culture media were removed and replaced with fresh media every 24 h. Cells were washed with Hanks' balanced saline solution prior to detachment from tissue culture flasks with the aid of 0.025% (w/v) trypsin containing 1 mM EDTA, and seeded at 1.5×10^5 cells/well into 24-multiwell plates. Monolayers of cells were then cultured for 18 h at 37 °C. Culture medium was then replaced with 1 ml Krebs Ringer bicarbonate (KRB) buffer, consisting of (in mM) 115 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.28 CaCl₂, 1.2 KH₂PO₄, 25 Hepes and 8.4% (w/v) NaHCO₃ (pH 7.4) supplemented with 0.1% (w/v) BSA and 1.1 mmol/l glucose. After 40-min preincubation at 37 °C, the buffer was replaced with 1 ml KRB test buffer containing glucose and test agents as detailed in the figures. Previous studies with BRIN-BD11 cells have demonstrated concentration-dependent effects of the drugs tested on insulin secretion (Ball *et al.* 2002a,b, 2004b, Green *et al.* 2004). Optimal working concentrations for the present study were chosen as 200 μ M for tolbutamide, glibenclamide and nateglinide and 10 μ M for GLP-1. After 20-min incubation at 37 °C, aliquots of test buffer were removed and stored at –20 °C for insulin RIA.

Statistical analysis

Results are presented as means $\pm$ S.E.M. for a given number of observations (*n*). Groups of data were compared using Bonferroni's-modified *t*-test. Differences were considered significant if $P < 0.05$.

Results

Unlike sulphonylureas and nateglinide, GLP-1 enhances K_{ATP} channel-dependent and -independent insulin secretion in a glucose concentration-dependent manner

In the presence of 1.1 mM glucose and a physiologic concentration (4.7 mM) of KCl, tolbutamide (1.9-fold, $P < 0.001$), glibenclamide (2.4-fold, $P < 0.001$) and nateglinide (1.8-fold, $P < 0.001$) each significantly increased insulin secretion (Fig. 1A). At this glucose concentration, GLP-1 evoked a smaller increase (1.5-fold, $P < 0.01$) in insulin secretion than these three drugs (Fig. 1A). At 16.7 mM glucose, tolbutamide, glibenclamide and nateglinide retained similar insulin-releasing effects at 1.1 mM glucose, eliciting 2.0-, 2.2- and 1.7-fold increases in insulin secretion respectively (Fig. 1A). In contrast, GLP-1 was more potent at this elevated glucose concentration, evoking a 2.2-fold compared with 1.5-fold increase ($P < 0.001$) in insulin release (Fig. 1A).

Figure 1 Unlike sulphonylureas and nateglinide, glucagon-like peptide-1 (GLP-1) enhances K_{ATP} channel-dependent and -independent insulin secretion in a glucose concentration-dependent manner. Following 40-min preincubation with a buffer containing 1.1 mM glucose, effects of tolbutamide (200 μ M), glibenclamide (200 μ M), nateglinide (200 μ M) and GLP-1 (10 nM) were tested during a 20-min incubation period in the presence of 1.1 or 16.7 mM glucose (A) or 1.1 or 16.7 mM glucose plus 30 mM KCl (B). Values are means $\pm$ S.E.M. ($n=6$). * $P<0.05$, † $P<0.01$, ‡ $P<0.001$ compared with respective effect in the presence of 1.1 mM glucose; § $P<0.01$, || $P<0.001$ versus effect in the absence of tolbutamide, glibenclamide, nateglinide or GLP-1.

Figure 1B shows the effects of sulphonylureas, nateglinide and GLP-1 on insulin secretion in the presence of 30 mM KCl to completely depolarise the BRIN-BD11 cell plasma membrane and stimulate maximum Ca^{2+} influx. Under these conditions, insulin release at a basal glucose concentration (1.1 mM) was increased by tolbutamide (1.6-fold, $P<0.001$), glibenclamide (1.5-fold, $P<0.001$) and nateglinide (1.6-fold, $P<0.001$; Fig. 1B). Raising the glucose concentration to 16.7 mM resulted in slight (1.1-fold) increase in insulin output in the presence of each of these three agents (Fig. 1B). At 1.1 mM glucose and 30 mM KCl, GLP-1 was less potent than tolbutamide, glibenclamide or nateglinide, evoking a 1.2-fold ($P<0.01$) increase in insulin secretion. Equimolar GLP-1 evoked a 1.5-fold response over that of 16.7 mM glucose and 30 mM KCl alone ($P<0.001$) and under these conditions, the insulin-releasing effect of GLP-1 was significantly higher (1.4-fold, $P<0.001$) compared with that observed with 1.1 mM glucose and 30 mM KCl (Fig. 1B).

GLP-1 augments K_{ATP} channel-dependent and -independent insulin-releasing effects of sulphonylureas and nateglinide

In the presence of 16.7 mM glucose and a non-depolarising concentration of KCl, insulin release was significantly ($P<0.001$) stimulated by 200 μ M tolbutamide (2.0-fold), glibenclamide (2.2-fold) and nateglinide (1.7-fold; Fig. 2A). Addition of 10 nM GLP-1 led to significantly increased insulin release in the presence of tolbutamide (1.5-fold, $P<0.001$), glibenclamide (1.6-fold, $P<0.001$) and nateglinide (2.0-fold, $P<0.001$; Fig. 2A).

Under depolarising conditions (16.7 mM glucose and 30 mM KCl), tolbutamide (1.3-fold increase), glibenclamide (1.3-fold) and nateglinide (1.4-fold) each exerted insulin-releasing effects (Fig. 2B). Addition of GLP-1 augmented the insulin-releasing effects of tolbutamide leading to a 1.8-fold ($P<0.001$) increase compared with tolbutamide alone. Insulin release in the presence of glibenclamide (1.9-fold, $P<0.001$) and nateglinide (1.2-fold, $P<0.05$) were also significantly enhanced following addition of GLP-1 (Fig. 2B).

Figure 2 GLP-1 augments K_{ATP} channel-dependent and -independent insulin-releasing effects of sulphonylureas and nateglinide. Following 40-min preincubation with a buffer containing 1.1 mM glucose, effects of GLP-1 (10 nM) on insulin secretion induced by tolbutamide (200 μ M), glibenclamide (200 μ M) or nateglinide (200 μ M) and GLP-1 (10 nM) were tested during a 20-min incubation period in the presence of either 16.7 mM glucose (A) or 16.7 mM glucose plus 30 mM KCl (B). Values are means $\pm$ S.E.M. ($n=6$). * $P<0.001$ compared with respective effect in the absence of GLP-1; † $P<0.05$, ‡ $P<0.001$ versus effect on the absence of tolbutamide, glibenclamide or nateglinide.

Acute and long-term effects of forskolin and PMA on K_{ATP} channel-dependent and -independent insulin secretion

Forskolin and phorbol 12-myristate 13-acetate (PMA) are potent insulin secretagogues, acting primarily via PKA/cAMP and PKC pathways respectively. Forskolin (25 μ M) increased insulin secretion at 16.7 mM glucose (4.4-fold, $P < 0.001$; Fig. 3A). Likewise, PMA (10 nM) exerted marked insulin-releasing effects at stimulatory glucose concentrations (4.9-fold, $P < 0.001$; Fig. 3A). Under depolarising conditions (16.7 mM glucose and 30 mM KCl), a significant K_{ATP} channel-independent increase in insulin secretion was evoked both by forskolin (1.6-fold, $P < 0.001$) and by PMA (2.0-fold, $P < 0.001$; Fig. 3B).

The insulin-releasing effects of GLP-1 are largely attributed to PKA-signalling pathways. To determine if the PKA/cAMP and/or PKC-signalling pathways also mediate the K_{ATP} channel-independent effects of GLP-1, and to examine their involvement in the K_{ATP} channel-independent effects of sulphonylureas and nateglinide, experiments were performed following downregulation of these pathways by culture with either 25 μ M forskolin or 10 nM PMA. As shown in Fig. 4, overnight culture with forskolin abolished the acute effects of forskolin, whereas responsiveness to PMA was retained with a 2.9-fold ($P < 0.001$) increase in insulin secretion under depolarising conditions. In cells exposed to PMA overnight, subsequent acute responsiveness to PMA was abolished, whilst the insulin-releasing effects of forskolin were retained with a 1.3-fold ($P < 0.001$) increase in secretion under

Figure 3 Like GLP-1, forskolin and PMA potentiate K_{ATP} channel-dependent and -independent insulin secretion. Following 40-min preincubation with a buffer containing 1.1 mM glucose, effects of GLP-1 (10 nM), forskolin (Forsk; 25 μ M) or PMA (10 nM) were tested during a 20-min incubation period in the presence of (A) 16.7 mM glucose or (B) 16.7 mM glucose (G) plus 30 mM KCl. Values are means $\pm$ S.E.M. ($n = 6$). * $P < 0.001$ compared with effect in the absence of GLP-1, forskolin or PMA; † $P < 0.001$ versus effect in the presence of 16.7 mM glucose.

Figure 4 Culture with forskolin or PMA abolishes subsequent acute secretory responsiveness to the same drug. Cells were cultured for 18 h in the absence or presence of either 25 μ M forskolin or 10 nM PMA. Following 40-min preincubation with a buffer containing 1.1 mM glucose, effects of 25 μ M forskolin or 10 nM PMA were tested during a 20-min incubation period in the presence of 16.7 mM glucose (G) plus 30 mM KCl. * $P < 0.001$, † $P < 0.001$, ‡ $P < 0.01$, § $P < 0.001$ compared with effect in the absence of forskolin or PMA during acute incubation; || $P < 0.001$ versus effect following 18-h culture under standard culture conditions. The inset shows the same data with insulin release expressed as percentage of cellular insulin (Ins) content.

depolarising conditions (Fig. 4). These long-term effects of forskolin and PMA were not a result of depletion of cellular insulin content, as when the secretory data were expressed as a percentage of cellular insulin content, the pattern of secretory responsiveness was unchanged (Fig. 4, inset).

GLP-1, but not sulphonylureas or nateglinide, retains insulin-releasing activity in depolarised cells following prolonged exposure to forskolin or PMA

In depolarised cells at stimulatory glucose, GLP-1 evoked a 1.6-fold increase ($P < 0.001$) of insulin release (Fig. 5). Following 18-h culture with 25 μ M forskolin, secretory responsiveness to GLP-1 was significantly reduced (84% reduction, $P < 0.005$; Fig. 5). However, insulin-releasing effects of GLP-1 were not completely abolished, with the peptide inducing a 1.6-fold ($P < 0.001$) increase in insulin secretion compared with no peptide (Fig. 5). Similar effects were observed following culture with 10 nM PMA. Despite the secretory responsiveness being reduced by 78% ($P < 0.001$, Fig. 5), GLP-1 induced a 1.4-fold increase in insulin release ($P < 0.001$) compared with control (Fig. 5).

Tolbutamide (1.3-fold, $P < 0.01$), glibenclamide (1.3-fold, $P < 0.01$) and nateglinide (1.5-fold, $P < 0.001$) elicit significant K_{ATP} channel-independent increases in insulin secretion in depolarised cells (Fig. 6A–C). Unlike GLP-1, the insulin-releasing actions of tolbutamide, glibenclamide and nateglinide were completely abolished in cells exposed to 25 μ M forskolin or 10 nM PMA (Fig. 6A–C).

Figure 5 GLP-1 retains insulin-releasing activity in depolarised BRIN-BD11 cells following prolonged exposure to forskolin or PMA. Cells were cultured for 18 h in the absence or presence of either 25 μ M forskolin or 10 nM PMA. Following 40-min pre-incubation with a buffer containing 1.1 mM glucose, effects of 10 nM GLP-1 were tested during a 20-min incubation period in the presence of 16.7 mM glucose (G) plus 30 mM KCl. * $P < 0.01$, [†] $P < 0.001$ compared with effect in the absence of GLP-1; [‡] $P < 0.001$ versus effect following 18-h culture under standard culture conditions.

Augmentation of GLP-1-induced insulin release by sulphonylureas/nateglinide in depolarised cells is abolished following prolonged exposure to forskolin or PMA

Figure 7 shows the effects of prolonged exposure to forskolin or PMA on the combined secretory effects of GLP-1 and sulphonylureas/nateglinide in 30 mM KCl depolarised cells at 16.7 mM glucose. Under standard culture conditions, the insulin-releasing effects of GLP-1 were significantly augmented by tolbutamide (1.4-fold, $P < 0.001$), glibenclamide (1.5-fold, $P < 0.001$) and nateglinide (1.2-fold, $P < 0.05$; Fig. 7A–C). In depolarised cells previously exposed to 25 μ M forskolin, insulin release in response to GLP-1 plus tolbutamide was significantly enhanced (1.5-fold, $P < 0.001$) compared with the absence of both agents, but was not higher than the response to GLP-1 alone (Fig. 7A). Likewise, insulin release in response to GLP-1 plus glibenclamide or nateglinide exceeded that observed in the absence of these agents in depolarised forskolin-cultured cells (1.5- and 1.7-fold respectively, $P < 0.001$), but was not greater than the response to GLP-1 alone (Fig. 7B and C).

The responses of depolarised cells cultured previously for 18 h in the presence of 10 nM PMA were very similar to those observed with forskolin-pretreated cells. Combinations of GLP-1 with tolbutamide (1.3-fold increase, $P < 0.01$), glibenclamide (1.4-fold, $P < 0.001$) or nateglinide (1.6-fold,

Figure 6 K_{ATP} channel-independent insulin-releasing effects of sulphonylureas and nateglinide are abolished by prolonged exposure to forskolin or PMA. Cells were cultured for 18 h in the absence or presence of either 25 μ M forskolin or 10 nM PMA. Following 40-min preincubation with a buffer containing 1.1 mM glucose, effects of tolbutamide (200 μ M; A), glibenclamide (200 μ M; B) and nateglinide (200 μ M; C) were tested during a 20-min incubation period in the presence of 16.7 mM glucose (G) plus 30 mM KCl. * $P < 0.01$, [†] $P < 0.001$ compared with effect in the absence of tolbutamide, glibenclamide or nateglinide; [‡] $P < 0.001$ versus effect following 18-h culture under standard culture conditions.

$P < 0.01$) evoked greater insulin-secretory responses than observed with 16.7 mM glucose and 30 mM KCl alone (Fig. 7A–C). However, in PMA-pretreated cells, addition of tolbutamide, glibenclamide or nateglinide in the presence of GLP-1 did not stimulate insulin release beyond that observed with GLP-1 alone (Fig. 7A–C).

Discussion

Stable analogues of GLP-1 and related GLP-1 mimetics have many functional characteristics, which promise to provide a new generation of improved anti-diabetic drugs (Holst 2002,

Figure 7 Augmentation of GLP-1-induced insulin release by sulphonylureas/nateglinide in depolarised cells is abolished following prolonged exposure to forskolin or PMA. Cells were cultured for 18 h in the absence or presence of either 25 μ M forskolin or 10 nM PMA. Following 40-min preincubation with a buffer containing 1.1 mM glucose, effects of 10 nM GLP-1 plus either tolbutamide (200 μ M; A), glibenclamide (200 μ M; B) or nateglinide (200 μ M; C) were tested during a 20-min incubation period in the presence of 16.7 mM glucose (G) plus 30 mM KCl. * $P < 0.01$, † $P < 0.001$ compared with effect in the presence of 16.7 mM glucose and 30 mM KCl alone; ‡ $P < 0.001$ versus effect following 18-h culture under standard culture conditions; § $P < 0.05$, ¶ $P < 0.001$ versus effect in the absence of tolbutamide, glibenclamide or nateglinide.

Drucker 2003, Green *et al.* 2004). Prominent amongst the desirable effects of GLP-1 are its potent insulin-releasing actions, which have been extensively studied with regard to K_{ATP} channel-dependent insulin secretion. This study primarily aimed to examine and provide a mechanistic understanding of the effects of GLP-1 on K_{ATP} channel-independent insulin secretory pathways and interactions with sulphonylureas and meglitinide drugs.

Initial experiments confirmed that in BRIN-BD11 cells, as in pancreatic islets, GLP-1 evokes insulin release in a glucose concentration-dependent manner. Glucose is known to retain

an insulin-releasing action in depolarised pancreatic β -cells (Henquin 2000). This K_{ATP} channel-independent effect of glucose, referred to by some authors as the amplifying pathway, is increasingly thought to play an important role in the regulation of phase 2 insulin secretion (Henquin 2000). Our observation that GLP-1 exhibits glucose-dependent insulin-releasing effects in depolarised cells is novel, and extends our understanding of the β -cell actions of GLP-1. Our data support the observations of Gromada *et al.* (1998), who reported that GLP-1 potentiated exocytosis at a site distal to a rise in the cytoplasmic Ca^{2+} concentration. K_{ATP} channel-independent stimulation of insulin secretion by drugs has previously been reported for sulphonylureas and nateglinide (Ball *et al.* 2000a, 2004b), and attributed to novel actions at intracellular sites in the β -cell (Renstrom *et al.* 2002, Ball *et al.* 2004b). However, it is notable that the glucose dependency of GLP-1 clearly distinguishes its actions from sulphonylureas and nateglinide, which stimulate K_{ATP} channel-independent insulin release by a glucose insensitive mechanism. Such observations accord with the reduced likelihood of hypoglycaemic episodes with GLP-1 versus sulphonylureas or nateglinide treatment.

When sulphonylurea or nateglinide was tested in combination with GLP-1, insulin release was markedly increased, to levels greater than those seen in response to any of these agents alone. This is consistent with clinical data indicating that GLP-1 can increase the insulin-releasing effect of glibenclamide in type 2 diabetic patients (Gutniak *et al.* 1996). Our data predict a similar clinical effect in patients treated with GLP-1 and nateglinide. Strikingly, we observed synergism between GLP-1 and sulphonylureas/nateglinide in both non-depolarised and depolarised cells, indicating that such effects are mediated at both early and late stages of insulin stimulus-secretion coupling. From a mechanistic viewpoint, the observation that GLP-1 dramatically increased K_{ATP} channel-independent insulin release in the presence of sulphonylureas/nateglinide suggests that a single shared pathway does not account for all distal effects on insulin release. We assume that these drug effects are mediated through plasma membrane SUR1 but cannot rule out other actions, especially in the case of glibenclamide, which penetrates the β -cell (Flatt *et al.* 1994).

We hypothesised that protein kinase signalling may also be involved in modulating K_{ATP} channel-independent insulin secretion in response to GLP-1 and nateglinide, since PKC has been shown to play a crucial role in mediating such effects of sulphonylureas (Eliasson *et al.* 1996, Tian *et al.* 1998). To determine if protein kinases were underlying these effects, we first assessed the effects of PKA and PKC activators. Under depolarising conditions, acute exposure to forskolin (activator of adenylate cyclase and thus PKA) or PMA (activator of PKC) augmented the K_{ATP} channel-independent effects of glucose, by an order of magnitude similar to that induced by GLP-1. This confirmed that distal steps in the insulin-secretory pathway mediated by PKA and PKC remained intact in depolarised cells. Using an approach employed

successfully in our laboratory to probe drug actions (Ball *et al.* 2000a,b, 2004a,b), we observed that the acute insulin-releasing effects mediated by forskolin or PMA were largely downregulated by 18 h prior exposure to forskolin or PMA respectively. Such desensitisation provided a method, independent of changes in cellular insulin content, to examine the role of PKA and PKC signalling in the modulation of K_{ATP} channel-independent insulin secretion induced by GLP-1, sulphonylureas and nateglinide.

In depolarised cells cultured overnight with forskolin or PMA to desensitise to PKA or PKC signalling, we observed that neither sulphonylurea nor nateglinide was able to stimulate insulin release. Our data in PMA-treated cells agree with those of Smith *et al.* (1999), who showed that the K_{ATP} channel-independent effects of tolbutamide were blocked by downregulation of PKC. Our results extend these observations and suggest a central role for PKC in nateglinide-mediated exocytosis. However, the present data obtained from forskolin-treated cells indicate that adenylate cyclase and cAMP may also play a role in regulating the K_{ATP} channel-independent effects of both sulphonylureas and nateglinide.

Unlike sulphonylureas and nateglinide, GLP-1 retained a significant insulin-releasing effect following culture of cells for 18 h with either forskolin or PMA. However, the GLP-1 effects were less pronounced than those observed under normal culture conditions. This reduction in GLP-1 potency following forskolin or PMA exposure indicates that both PKA- and PKC-mediated signalling play a significant role in mediating the effects of GLP-1 on K_{ATP} channel-independent insulin release. The effect of pretreatment with PMA may reflect its ability to desensitise GLP-1 receptors (Gromada *et al.* 1996). However, since GLP-1 still retains a significant insulin-releasing effect after 18-h culture with PMA, the effects on K_{ATP} channel-independent insulin secretion appear to involve PKC-independent components. Signalling via cAMP, which is elevated by GLP-1, is thought to have PKA-dependent and -independent components (MacDonald *et al.* 2002, Gromada *et al.* 2004). It also seems that the SUR1 is involved in mediating the PKA-independent effects of GLP-1 upon K_{ATP} channel-independent insulin secretion, since islets isolated from SUR1^{-/-} mice lack the PKA-independent component of exocytosis (Nakazaki *et al.* 2002, Eliasson *et al.* 2003). The significantly reduced potency of GLP-1 in forskolin-treated cells may reflect the observation that the PKA-independent effects of cAMP are only exerted on the readily releasable pool of insulin granules (Renstrom *et al.* 1997).

As anticipated from the earlier results, when GLP-1 was tested in combination with sulphonylureas or nateglinide the previously described synergistic effects on insulin release were abolished, with sulphonylureas or nateglinide no longer able to evoke a greater secretory response than GLP-1 alone. The similarities between the data obtained with nateglinide and sulphonylureas in this study provide further support for the idea that nateglinide shares intracellular K_{ATP} channel-independent signalling pathways with sulphonylureas (Ball *et al.* 2004b, 2005).

This is consistent with the observation that the binding site for nateglinide resides on the intracellular side of SUR1 and shares at least one point of interaction with the binding site for the sulphonylurea tolbutamide (Hansen *et al.* 2002). Our data suggest that GLP-1 may continue to be an effective insulin-releasing agent in patients experiencing secondary failure of sulphonylureas or nateglinide.

Acknowledgements

These studies were supported in part by the Research and Development Office of the Northern Ireland Department of Personal Health and Social Services. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 9 May 2006

Received in final form 15 June 2006

Accepted 21 June 2006

Made available online as an Accepted Preprint

30 June 2006