EXPERIMENTAL STUDY

Nateglinide, but not repaglinide, stimulates growth hormone release in rat pituitary cells by inhibition of K\(^+\) channels and stimulation of cyclic AMP-dependent exocytosis

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Abstract

Objective: GH causes insulin resistance, impairs glycemic control and increases the risk of vascular diabetic complications. Sulphonylureas stimulate GH secretion and this study was undertaken to investigate the possible stimulatory effect of repaglinide and nateglinide, two novel oral glucose regulators, on critical steps of the stimulus–secretion coupling in single rat somatotrophs.

Methods: Patch-clamp techniques were used to record whole-cell ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) and delayed outward K\(^+\) currents, membrane potential and Ca\(^{2+}\)-dependent exocytosis. GH release was measured from perfused rat somatotrophs.

Results: Both nateglinide and repaglinide dose-dependently suppressed K\(_{\text{ATP}}\) channel activity with half-maximal inhibition being observed at 413 nM and 13 nM respectively. Both compounds induced action potential firing in the somatotrophs irrespective of whether GH-releasing hormone was present or not. The stimulation of electrical activity by nateglinide, but not repaglinide, was associated with an increased mean duration of the action potentials. The latter effect correlated with a reduction of the delayed outward K\(^+\) current, which accounts for action potential repolarization. The latter effect had a K\(_d\) of 19 \(\mu\)M but was limited to 38% inhibition. When applied at concentrations similar to those required to block K\(_{\text{ATP}}\) channels, nateglinide in addition potentiated Ca\(^{2+}\)-evoked exocytosis 3.3-fold (K\(_d\) = 3 \(\mu\)M) and stimulated GH release 4.5-fold. The latter effect was not shared by repaglinide. The stimulation of exocytosis by nateglinide was mimicked by cAMP and antagonized by the protein kinase A inhibitor Rp-cAMPS.

Conclusion: Nateglinide stimulates GH release by inhibition of plasma membrane K\(^+\) channels, elevation of cytoplasmic cAMP levels and stimulation of Ca\(^{2+}\)-dependent exocytosis. By contrast, the effect of repaglinide was confined to inhibition of the K\(_{\text{ATP}}\) channels.

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Introduction

Type 2 diabetes involves both β-cell dysfunction and peripheral insulin resistance but overt type 2 diabetes only occurs when the pancreatic β-cells secrete amounts of insulin that are unable to maintain euglycemia (1). Insulin secretagogues such as the sulphonylureas remain the best-documented and most commonly used class of oral anti-diabetics (2). However, some problems have been associated with the use of these drugs, such as hypoglycemic episodes due to chronic β-cell stimulation. It is unclear whether this is also the cause of the secondary failure, i.e. the patients cease to respond to sulphonylurea treatment, frequently occurring after long-term therapy (3). It also remains unestablished as to what extent secondary failure reflects death of the insulin-secreting β-cells or their failure to secrete insulin in response to sulphonylurea stimulation but the UK Prospective Diabetes Study revealed that the decline of β-cell function in patients receiving traditional sulphonylurea treatment was not accelerated compared with those treated with the insulin sensitizer metformin (4). Thus, secondary failure may be ascribed to a variety of factors (3), one of which may relate to the persistent, excessive hepatic glucose production induced by, among other factors, the counter-regulatory hyperglycemic hormones growth hormone (GH) and glucagon, which oppose...
the hypoglycemic action of insulin (5). Additionally, these counter-regulatory hormones have the ability to impede insulin-stimulated glucose metabolism (6, 7). It is of interest that chronic exposure of β-cells to the long-acting sulphonylurea glibenclamide eventually reduces the insulinotropic action of the compound by reducing the number of functional ATP-sensitive K⁺ (K<sub>ATP</sub>) channels in the plasma membrane (8).

Insulin secretagogues should ideally stimulate insulin secretion only at elevated blood glucose concentrations and without concomitant acceleration of the release of other hormones, including GH. However, studies on dispersed somatotrophs have revealed that they contain both sulphonylurea-sensitive K<sub>ATP</sub> channels and that GH release from these cells was stimulated by glibenclamide (9). Stimulation of GH release was likewise observed in humans after administration of glipizide in vivo (10).

Because of the problems associated with sulphonylurea therapy, several new anti-diabetic drugs chemically unrelated to the sulphonylureas have been developed during recent years. These include repaglinide, which is a carbamoyl-ethoxy benzoic acid derivative like meglitinide, and nateglinide, a D-form derivative of phenylalanine. We have previously demonstrated that repaglinide differs from the sulphonylureas in stimulating insulin secretion solely by closure of the K<sub>ATP</sub> channels (11, 12), whereas the latter group of compounds act by both closure of the K<sub>ATP</sub> channels and stimulation of Ca²⁺-dependent exocytosis (11, 13, 14). We now report that nateglinide, unlike repaglinide, is also a potent stimulator of GH release from rat pituitary cells and we have therefore compared the actions of repaglinide and nateglinide on a number of functional parameters. We demonstrate that the strong stimulatory effect of nateglinide on GH release can be attributed to inhibition of K<sub>ATP</sub> channels, delayed rectifying K⁺ channels and potentiation of Ca²⁺-evoked exocytosis by a cAMP-dependent mechanism.

Materials and methods

**Cell preparation**

Pituitary somatotrophs from Sprague–Dawley male albino rats (Møllegaard, Lille Skensved, Denmark) were used for the electrophysiological studies. The rats were decapitated and the pituitary glands removed. The anterior lobe was isolated and placed in ice-cold Gey’s medium (Gibco, Paisley, Strathclyde, UK) supplemented with 0.25% D-glucose, 2% non-essential amino acids and 1% bovine serum albumin (BSA). The cells were prepared as described previously (15). Based on the hormonal contents, we estimate that our preparations contain ≈70% somatotrophs. This percentage agrees favorably with the fraction of endocrine cells (16 out of 22 cells) responding to extracellular application of the GH-releasing hexapeptide (GHRP-6), which is specific for somatotrophs, with an increased exocytosis in response to 500 ms voltage pulses from −70 mV to 0 mV using the perforated patch configuration. The amplitude of the capacitance increase rose from a basal value of 27±8 pF to 101±19 pF (P < 0.01; n = 16). Cells responding to GHRP-6 had a capacitance (C<sub>m</sub>) of 3.9±0.3 pF. In the six cells that did not respond, C<sub>m</sub> was 5.2±0.4 pF. The remaining cells constitute mainly lactotrophs (10%), fibroblasts and endothelial cells (20%). After isolation, the cells were plated onto plastic Petri dishes and maintained for up to 2 days in Dulbecco’s modified Eagle’s medium supplemented with 5 mM HEPES, 4 mM glutamine, 0.075% Na₂CO₃, 0.1% non-essential amino acids, 2.5% fetal calf serum, 3% horse serum, 10% fresh rat serum, 1 mM triiodothyronine and 40 μg/l dexamethasone (pH 7.30 at 37°C and 8% CO₂). All tissue culture media were obtained from Life Technologies (Paisley, Strathclyde, UK).

**Electrophysiology**

Patch pipettes were pulled from borosilicate glass capillaries, coated with Sylgard (Dow Corning, Wiesbaden, Germany) at their tips and fire-polished before use. The pipette resistance (when filled with the pipette-filling solutions) was 2–4 MΩ. Membrane potential and currents were measured using the perforated patch whole-cell configuration, Axopatch 200B (Axon Instruments, Foster City, CA, USA) or EPC9 (Heka Electronics, Lambrecht/Pfalz, Germany) patch-clamp amplifiers and the software pClamp (version 6.0; Axon Instruments) or Pulse (Heka Electronics). The voltage and current signals were filtered at 1 kHz, sampled at a rate of 2–5 kHz using a Digidata AD-converter and the software pClamp (Axon Instruments) and stored in a computer pending analysis. The whole-cell K<sub>ATP</sub> conductance was estimated by applying 10 mV hyper- and depolarizing voltage pulses (duration: 200 ms; pulse interval: 2 s) from a holding potential of −70 mV using the standard whole-cell configuration of the patch-clamp technique. The delayed K⁺ current was recorded during depolarization from −70 mV to +20 mV (duration: 200 ms; pulse interval: 5 s). Exocytosis was monitored as increases in cell membrane capacitance using the standard whole-cell configuration of the patch-clamp technique and an EPC9 patch-clamp amplifier and the Pulse software. The interval between two successive points was 0.4 s. The measurements of cell capacitance were initiated <5 s following establishment of the whole-cell configuration. Exocytosis was elicited by infusion of Ca²⁺–EGTA buffers through the recording electrode.

**Solutions**

The extracellular medium consisted of (in mM) 138 NaCl, 5.6 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 5 HEPES (pH
7.4 with NaOH) and 5 d-glucose. The volume of the recording chamber was 0.4 ml and perfused at a rate of 1.5 ml/min. For recordings of whole-cell K_ATP-channel activity (standard whole-cell), the pipette solution consisted of (in mM) 125 KCl, 30 KOH, 10 EGTA, 1 MgCl2, 5 HEPES, 0.3 Mg-ATP and 0.3 K-ADP (pH 7.15 with KOH). The pipette solution for measurements of delayed rectifying K+ currents (standard whole-cell) consisted of (in mM) 125 KCl, 30 KOH, 1.2 MgCl2, 5 HEPES and 3 Mg-ATP (pH 7.15 with KOH). The membrane potential recordings were performed using the perforated patch technique and in these experiments the pipette solution was composed of (in mM) 76 K2SO4, 10 NaCl, 10 KCl, 1 MgCl2, 5 HEPES (pH 7.35 with KOH). Electrical contact was established by adding 0.24 mg/ml amphotericin B to the pipette solution. Perforation required a few minutes and was considered satisfactory when the G_series was stable and >35–40 nS. The electrode solution for measurements of exocytosis (standard whole-cell) consisted of (in mM) 125 K-glutamate, 10 KCl, 10 NaCl, 1 MgCl2, 5 HEPES, 3 Mg-ATP, 10 EGTA and 0 or 5 CaCl2. The free Ca2+ concentrations of the resulting buffers were estimated as 10 nM and 220 nM using the binding constants of Martell & Smith (16). Nateglinide, nateglinide and human GH-releasing hormone 1-29 (GHRH) were synthesized at Novo Nordisk A/S (Bagsvaerd, Denmark). The specific protein kinase A (PKA) inhibitor Rp-cAMPS was obtained from BIOLOG Life Science Institute (Bremen, Germany). All other chemicals were purchased from Sigma (Poole, Dorset, UK). For measurements of membrane potential and exocytosis, the temperature of the extracellular solution in the recording chamber was +33 °C.

**Measurements of cAMP levels**

Cells (50,000 cells per well) were first preincubated in 0.1 ml for 1 h in Hanks’ balanced salt solution with 20 mM glucose and 10 mg/ml BSA and subsequently stimulated with 1 nM GHRH alone or in the combined presence of GHRH and 100 nM repaglinide or 0.1 mM nateglinide for 15 min. The reaction was terminated by the addition of HCl (final concentration 50 mM). The samples were neutralized with NaOH and assayed for cAMP using a cAMP [125I]scintillation proximity assay (Amersham International plc, Amersham, Bucks, UK).

**GH release**

Cultured cells were harvested and re-suspended in 1.0 ml perifusion medium containing (in mM) 115 NaCl, 4.7 KCl, 1.2 K2HPO4, 1.2 MgSO4, 2.56 CaCl2, 20 NaHCO3, 20 HEPES, 1 d-glucose and 10 mg/ml BSA, pH 7.40. From this cell suspension, 0.1 ml was used for protein determination (17, 18) and the remaining 0.9 ml (~1.3 x 10^6 cells) were washed by centrifugation and placed on top of 2 mm pre-swollen Sephadex G10 in the perfusion chamber (a Nuclepore 13 mm Pop-Top holder from Nuclepore Corporation, Pleasanton, CA, USA) and maintained at +37 °C. After a 30-min adjustment period in basal medium, test substances were added. Samples from each fraction of effluent were saved (~20 °C) for GH radioimmunoassay (18). The lag period for a new medium to reach the chamber was 43 s and has been corrected for. Cell viability (Evans Blue exclusion) was >97% after the perifusions. All glass and plastic surfaces in contact with tissue or cells were siliconized with Sigmacote (Sigma).

**Data analysis**

The exocytotic rate is presented as the increase in cell capacitance occurring during the first 60 s following establishment of the whole-cell configuration excluding any rapid changes occurring during the initial ~10 s required for equilibration of the pipette solution with cytosol. The duration of the action potentials was measured as the time spent at and above the half-maximal amplitude of the spike using MATLAB software (Mathworks Inc., Natick, MA, USA). Action potential frequencies were calculated by dividing the number of spikes detected (>200 spikes) with the total time analyzed (>2 min). Stimulation of GH secretion was expressed as per cent of the average secretion rate during the 5 min prior to and during the first 2 min after GHRH application. Results are presented as mean values±S.E.M. for the number of experiments indicated. Statistical significances were evaluated using Student’s t-test for paired (Figs 2 and 7) or unpaired observations (Figs 5 and 6).

**Results**

**Nateglinide and repaglinide stimulate electrical activity in rat somatotrophs**

Figure 1a shows membrane potential recordings from single rat pituitary somatotrophs. In the presence of 5 mM glucose alone, the membrane potential was approximately −60 mV and action potentials were generated at a low frequency (if at all). Addition of repaglinide (0.1 mM) evoked a brief burst of action potentials. In this particular cell, each action potential was associated with an after-hyperpolarization of 10–15 mV, which transiently approached −75 mV (Fig. 1a,i). Similar after-hyperpolarizations were observed in a total of three of six other cells. By contrast, nateglinide (0.1 mM) when applied to the same cell resulted in a depolarization and a sustained stimulation of electrical activity (Fig. 1a,ii). Rather different results were obtained in somatotrophs already exposed to 1 nM GHRH. In all five cells tested, spontaneous electrical activity was seen invariably before the addition of...
repaglinide (Fig. 1b,i) or nateglinide (Fig. 1b,ii). Under these conditions, both compounds failed to affect the frequency of action potentials (Fig. 2b). However, nateglinide produced a $27\pm8\%$ ($P<0.05$; $n=5$) reversible increase in the duration of the action potentials (Fig. 2a) and they often came in groups (bursts; Fig. 1c).

**Repaglinide and nateglinide inhibit K$_{ATP}$ channels**

Previous experiments have demonstrated that K$_{ATP}$ channels sensitive to anti-diabetic sulphonylureas are present in pituitary cells (9). Figure 3a shows the changes in membrane conductance in a single rat pituitary cell monitored using a voltage protocol consisting of $\pm10$ mV voltage excursions from the holding potential ($-70$ mV) as previously described (19) and intracellular dialysis with 0.3 mM ATP and 0.3 mM ADP to activate the K$_{ATP}$ channels. The lighter trace in Fig. 3a (*Control*) indicates the conductance determined shortly ($<15$ s) after establishment of the whole-cell configuration. The current amplitude of 4 pA corresponds to an input conductance of 0.4 nS (or 0.18 nS/pF). In a series of 11 experiments, a mean value for the starting value of $0.14\pm0.05$ nS/pS was observed. Following the wash-out of the cytosol and replacement with the pipette solution, the membrane conductance increased 15-fold and eventually stabilized at $1.2\pm0.2$ nS/pF. Addition of the specific K$_{ATP}$ channel antagonist glibenclamide (100 nM) caused a $>95\%$ inhibition of the whole-cell K$^+$ current (data not shown; $n=5$). Increasing the ATP concentration in

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**Figure 1** Effects of repaglinide and nateglinide on the membrane potential ($V_m$) recorded from individual rat pituitary cells exposed to 5 mM glucose and 1 nM GHRH. The recordings were obtained using the perforated patch whole-cell configuration. (a) Effects of repaglinide (100 nM, i) and nateglinide (100 $\mu$M, ii) on membrane potential and electrical activity in the presence of 5 mM glucose alone. (b) Same as (a) but 1 nM GHRH was present throughout the recordings. Nateglinide and repaglinide were added to the bath solution during the periods indicated by the horizontal lines above the voltage traces. (c) Expanded segments of the trace in (b) taken in the absence (left) and presence (right) of nateglinide. The recordings in (a) and (b) were obtained from different cells and are representative of five cells under each experimental condition.

**Figure 2** Effects of repaglinide (repa) and nateglinide (nateg) on GHRH-stimulated action potential frequency and duration. Histograms summarize (a) the duration ($t$) or (b) frequency ($f$) of the action potentials before and following the application of repaglinide (100 nM; left) and nateglinide (100 $\mu$M; right) in the continuous presence of 1 nM GHRH. The analysis was performed on the experiments presented in Fig. 1 and for the whole length of the drug application. Data are mean values $\pm$ S.E.M. of five different experiments. $^*P<0.05$ compared with GHRH alone.
the pipette-filling solution to 3 mM completely inhibited the wash-out $K^\text{+}$ current (0.12±0.06 nS/pS; $n = 5$; data not shown) as expected for a current flowing through $K_{\text{ATP}}$ channels. Increasing concentrations of repaglinide and nateglinide were then applied to determine the dose-inhibition properties. At a concentration of 0.5 nM, repaglinide inhibited the $K_{\text{ATP}}$ channels by 5%. Higher concentrations produced progressively stronger inhibition, which amounted to 23% and 90% at 5 nM and 50 nM respectively. Similar responses were obtained with nateglinide but 30-fold higher concentrations were required (not shown). The effects of several concentrations of repaglinide and nateglinide on the $K_{\text{ATP}}$-channel activity are summarized by the dose-inhibition curves in Fig. 3b. The data points were approximated to the Hill equation and least squares fits yielded values of $K_d$ and the co-operativity factor ($n$) for repaglinide of 13±3 nM and 1.3±0.3 (five cells). The corresponding values for nateglinide amounted to 413±94 nM and 1.1±0.1 (five cells) respectively.

**Nateglinide inhibits delayed outward $K^+$ current**

We next explored the effects of repaglinide and nateglinide on the delayed outward $K^+$ current (20). Figure 4a shows that nateglinide (0.1 mM) reduced delayed
outward K\(^+\) current activity by 38%. This effect was partially reversible and the current returned to 83% of the control amplitude following removal of the drug from the extracellular medium. By contrast, repaglinide did not affect the delayed outward current even at a concentration as high as 100 \(\mu\)M (Fig. 4b). The inhibitory action of nateglinide on delayed outward K\(^+\) current activity was dose dependent (Fig. 4b). A least-squares fit yielded values of \(K_d\) and \(n\) of 19 \(\mu\)M and 1.7 respectively. Approximately 60% of the delayed outward current was resistant to nateglinide but inhibited by 10 mM tetraethylammonium chloride (91\(\pm\)9%; \(n = 5\)).

**Figure 5** Effects of repaglinide and nateglinide on Ca\(^{2+}\)-evoked exocytosis in rat pituitary cells. (a) Increases in cell capacitance elicited by intracellular infusion with a Ca\(^{2+}\)–EGTA buffer with a free Ca\(^{2+}\) concentration of 0.22 \(\mu\)M in the absence (control) or presence of repaglinide (100 nM) or nateglinide (100 \(\mu\)M) in the pipette solution. Throughout the recording, the cell was clamped at –70 mV in order to avoid activation of the voltage-dependent Ca\(^{2+}\) channels that would otherwise interfere with the measurement. (b) Relationships between the concentration of repaglinide or nateglinide and the average rates of increase in cell capacitance (\(\Delta C_m/\Delta t\)) measured 10–60 s after establishment of the whole-cell configuration. (Inset) Changes in cell capacitance in cells dialyzed with 0.2, 2 and 20 \(\mu\)M nateglinide. For nateglinide, the line represents the best fit to the mean data using the Hill equation. Data are mean values\(\pm\)S.E.M. of five experiments. *\(P < 0.05\), **\(P < 0.005\) compared with control.

**Figure 6** Nateglinide stimulates PKA-dependent exocytosis in rat pituitary cells. (a) Histogram summarizing the average rates of increase in cell capacitance measured 10–60 s after establishment of the standard whole-cell configuration in the absence (control) and presence of 100 \(\mu\)M nateglinide. (b) Average increases in exocytosis under control conditions, in the presence of 100 \(\mu\)M cAMP or 100 nM of the phorbol ester PMA, in the combined presence of 100 \(\mu\)M cAMP and 100 \(\mu\)M nateglinide in the absence and presence of 100 nM PMA. (c) Average rates of exocytosis under control conditions, in the presence of 500 \(\mu\)M of the phosphodiesterase inhibitor IBMX, in the combined presence of 500 \(\mu\)M IBMX and 100 \(\mu\)M nateglinide in the absence and presence of 100 \(\mu\)M cAMP. Cells were pretreated for 20 min with 500 \(\mu\)M IBMX before initiation of the experiments. (d) Increases in exocytosis in cells pretreated with 100 \(\mu\)M Rp-cAMPS for >20 min in the absence and presence of 100 \(\mu\)M nateglinide. (e) Effects of staurosporine (100 nM), calphostin C (1.5 \(\mu\)M) and following down-regulation of PKC activity with PMA on exocytosis in the absence and presence of 100 \(\mu\)M nateglinide. In the experiments involving the use of staurosporine and calphostin C, the cells were pre-incubated with the compounds at the indicated concentrations for >20 min in the absence and presence of 100 \(\mu\)M nateglinide. PKC activity was also down-regulated by exposing cells for >20 h to 100 nM PMA. The experiments were conducted as described in the legend to Fig. 5. Data are mean values\(\pm\)S.E.M. of five to eight experiments. *\(P < 0.05\), **\(P < 0.005\).
Nateglinide stimulates PKA-dependent exocytosis

It has previously been demonstrated that first and second generation sulphonylureas (including tolbutamide, glibenclamide and glipizide) potentiate Ca\(^{2+}\)-induced exocytosis in pancreatic β-cells (13, 14). This effect may contribute to the insulinotropic action of the drugs mediated by closure of the K\(_{ATP}\) channels. Importantly, the effect of sulphonylureas on exocytosis are not shared by repaglinide (11). Figure 5a shows that nateglinide (0.1 mM) stimulated Ca\(^{2+}\)-evoked exocytosis in rat pituitary cells threefold. Secretion was elicited by dialyzing the cells with a Ca\(^{2+}\)-EGTA buffer with a free Ca\(^{2+}\) concentration of 0.22 mM, which in itself produced a low rate of exocytosis (2 fF/s). The stimulation of exocytosis cannot be accounted for by the effects of nateglinide on the plasma membrane K\(_{ATP}\) channels since the pituitary cells were voltage-clamped and the membrane potential held constant at −70 mV irrespective of the K\(_{ATP}\)-channel activity. Nateglinide did not stimulate exocytosis in cells infused with a Ca\(^{2+}\)-free pipette-filling solution (control: 0 ± 0.2 fF/s; n = 5; nateglinide: 0.3 ± 0.2 fF/s; n = 5). Consistent with the earlier results in β-cells (11), the stimulatory action of nateglinide on exocytosis in somatotrophs was not shared by repaglinide even when applied at a maximally effective concentration on closure of K\(_{ATP}\)-channel activity (100 nM; Fig. 5a).

The effect of nateglinide on exocytosis was concentration dependent (Fig. 5b). No stimulation of exocytosis was observed at 0.2 μM. Higher concentrations of nateglinide accelerated the rate of increase in a concentration-dependent manner (Fig. 5b). Approximating the average data points to the Hill equation yielded values of the association constant (K\(_a\)) and n of 2.7 μM and 1.6 respectively.

It has previously been demonstrated that another novel insulin secretagogue (L-686398: 9-[1,S,2R)-2-fluoro-1-methylpropyl]-2-methoxy-6-(1-piperazinyl)purine hydrochloride) is a potent phosphodiesterase inhibitor (21). To investigate whether such a mechanism could underlie the stimulation of exocytosis by nateglinide, the experiments were repeated in the continuous presence of 0.1 mM cAMP in the pipette solution which itself stimulated exocytosis threefold (Fig. 6b). Under these experimental conditions, nateglinide was unable to produce further stimulation. The lack of exocytotic response in the combined presence of cAMP and nateglinide is not a result of exocytosis already operating at its maximum speed as suggested by the ability of phorbol 12-myristate 13-acetate (PMA; 100 nM) to produce an additional 53% stimulation (P < 0.05; n = 5). PMA itself stimulated exocytosis threefold (Fig. 6b). To further investigate whether inhibition of phosphodiesterase activity could underlie the stimulation of exocytosis by nateglinide, experiments were performed in the continuous

Figure 7 Effects of repaglinide and nateglinide on GH release in the absence and presence of GHRH. (a) GH release was measured from perfused rat somatotrophs before, during and after application of either 1 nM (▲) or 10 nM (▼) GHRH. The arrows and the horizontal bars indicate the application and removal of the test compounds. (b–c) Effects of repaglinide (b, 100 nM; ◇) and nateglinide (c, 10 μM; ●) on GH release elicited by GHRH (1 nM). The lag period for a new medium to reach the chamber was 43 s and has been corrected for. Data are given as the mean values±S.E.M. for six (1 nM GHRH), five (10 nM GHRH) or six (repaglinide and nateglinide) experiments.
presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), which itself increased the rate of exocytosis by 210% \( (P < 0.05; n = 5); \) cells were pretreated with 0.5 mM for 20 min and IBMX was also included in the pipette-filling solution. Under these experimental conditions, nateglinide was unable to exert its stimulatory action (Fig. 6c). Again the lack of exocytotic response in the presence of nateglinide was not the result of depletion of the releasable pool of granules or of the exocytotic machinery having reached its maximal capacity since inclusion of cAMP in the pipette-filling solution consistently produced a further stimulation of exocytosis under these experimental conditions (Fig. 6e). These observations suggested that the stimulation observed in Fig. 6a is attributable to elevation of cytoplasmic cAMP. The latter interpretation is consistent with the observation that the PKA inhibitor Rp-cAMPS prevented nateglinide-induced exocytosis (Fig. 6d; cells were pretreated with 100 \( \mu \)M for >20 min). By contrast, inhibition of protein kinase C (PKC) with staurosporine (cells were pre-incubated with 100 nM for >10 min) or calphostin C (cells were pretreated with 1.5 \( \mu \)M for >20 min) did not affect the ability of nateglinide to stimulate exocytosis (Fig. 6e). Further support for the idea that nateglinide exerts its stimulatory action independently of PKC activation comes from the observation that nateglinide remained stimulatory when applied to pituitary cells in which PKC had been down-regulated by exposure to 100 nM PMA for >20 h (Fig. 6e).

The finding that nateglinide failed to stimulate exocytosis in cells infused with a high cAMP concentration (0.1 mM) in the presence of the PKA inhibitor Rp-cAMPS or after inhibition of phosphodiesterase activity by IBMX led us to investigate its effects on the cellular cAMP content. The basal concentration measured in the well was 4.0±0.5 pM/ml (Table 1). This corresponds to 11 \( \mu \)M taking into account that the well had a volume of 0.1 ml and contained 50,000 cells and that the somatotroph had a volume of 0.72 pl as determined from the cell capacitance (3.9±0.1 pF; \( n = 117 \)) and assuming a specific membrane capacitance of 1 \( \mu \)F/cm² and spheric geometry. We acknowledge that the cAMP concentration quoted above is a lower estimate since the cytoplasm only constitutes part of the total cell volume. Exposing the somatotrophs to 1 nM GHRH for 15 min produced a 15-fold increase in cAMP content (=0.17 mM). Interestingly, nateglinide, but not repaglinide, elevated basal cAMP level: nateglinide producing a further 60% elevation of cAMP. These observations argue that nateglinide principally acts by inhibiting the degradation of cAMP.

**Nateglinide stimulates GH release in the presence of GHRH**

In Figure 7 we finally determined the ability of nateglinide and repaglinide to promote GH release in the absence and presence of GHRH. Under basal conditions, GH release was low and stable. Adding 1 nM GHRH to the control medium produced 48±15% \( (P < 0.001; \) \( n = 6 \)) stimulation of GH release whereas a ten times higher concentration of GHRH stimulated GH release 355±49% \( (Fig. 7a; P < 0.005; n = 5) \). Inclusion of repaglinide (100 nM; Fig. 7b) or nateglinide (10 \( \mu \)M; Fig. 7c) in the perfusion medium did not detectably stimulate GH secretion during a 5-min incubation period over that observed under control conditions. Whereas subsequent addition of 1 nM GHRH resulted in a marginal stimulation of GH release (77±18%) when applied in the presence of repaglinide (only marginally bigger than the response in the absence of repaglinide; 48%; Fig. 7a), the stimulatory action of GHRH in the presence of nateglinide was pronounced and amounted to 450±161% \( (P < 0.05; n = 6) \). The latter stimulation is in fact stronger than that elicited by a tenfold higher concentration of GHRH in the absence of nateglinide (cf. Fig. 7a).

**Discussion**

Our data suggest that the novel anti-diabetic compound nateglinide influences several important regulatory steps in the stimulus-secretion coupling in rat somatotrophs. The stimulatory action of nateglinide on GH release results from the capacity of this drug to (a) inhibit \( K_{ATP} \) channel activity, resulting in membrane depolarization and increased electrical activity, (b) reduce the amplitude of the delayed outward \( K^+ \) current that is responsible for the repolarization of the action potential and thus prolong the action potential, which in turn promotes voltage-gated \( Ca^{2+} \) entry and (c) potentiation of \( Ca^{2+} \)-induced exocytosis at a site distal to the elevation of cytoplasmic \( Ca^{2+} \). The latter effect appears to be mediated by activation of PKA and may result from inhibition of phosphodiesterase activity.

**Table 1** Effects of nateglinide (0.1 mM) and repaglinide (100 nM) on basal and GHRH (1 nM)-stimulated cAMP levels. The data are mean valuesSEM for three individual experiments each repeated five times.

<table>
<thead>
<tr>
<th>Condition (pM/ml)</th>
<th>cAMP</th>
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<tbody>
<tr>
<td>Control</td>
<td>4.0±0.5</td>
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<tr>
<td>Repaglinide</td>
<td>4.2±0.4</td>
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<tr>
<td>Nateglinide</td>
<td>5.9±0.3*</td>
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<tr>
<td>GHRH</td>
<td>60.8±1.8**</td>
</tr>
<tr>
<td>GHRH+ repaglinide</td>
<td>53.3±3.4</td>
</tr>
<tr>
<td>GHRH+ nateglinide</td>
<td>89.8±4.2§</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) against control; ** \( P < 0.001 \) against control; § \( P < 0.05 \) against GHRH.
**Decreased K⁺-channel activity underlies nateglinide-induced stimulation of electrical activity**

Different types of K⁺ currents important for hormone secretion have previously been identified in rat somatotrophs. These include a delayed rectifier K⁺ current, a transient outward K⁺ current, an inwardly rectifying K⁺ current and an ATP-sensitive K⁺ current (9, 20, 22). In the β-cell, KATP channels constitute the main resting conductance maintaining a negative membrane potential. Glucose produces a concentration-dependent inhibition of KATP-channel activity, leading to opening of voltage-gated Ca²⁺ channels, Ca²⁺ influx and stimulation of Ca²⁺-dependent exocytosis (23). Similar results have been obtained in the somatotrophs where an increase in the extracellular glucose concentration resulted in stimulation of GH release (24). The activity of the KATP channel in the somatotrophs is also modulated by sulphonylureas and the K⁺-channel opener diazoxide and the release of GH echoes the effects of these compounds on KATP-channel activity (9). The observed potencies of repaglinide and nateglinide in reducing KATP-channel activity (Fig. 3b) are very similar to those seen in rat α- and β-cells (25, 26).

A potentially important finding is that nateglinide inhibits the delayed outward currents that underlie action potential repolarization. We observed that almost 40% of the delayed outward current was inhibited by nateglinide whereas the remainder was unaffected by the compound. This finding is easiest to explain if the whole-cell current flows through at least two types of voltage-gated K⁺ channels, one of which is sensitive to nateglinide (20). By contrast, repaglinide had no effect on delayed outward K⁺ currents.

Because of the effect of nateglinide on the delayed outward K⁺ current, there is a 30% prolongation of the action potentials. This will presumably be associated with a corresponding stimulation of Ca²⁺ influx since the action potentials are, at least in part, Ca²⁺ dependent (27). However, the increase in spike duration can only account for a small fraction of the total increase in GH release actually observed (+30%). We point out that under these experimental conditions (1 nM GHRH) nateglinide had little effect on electrical activity. We conclude therefore that the major part of the total stimulatory action must be explained in terms of enhanced Ca²⁺-dependent exocytosis.

**Nateglinide potentiates Ca²⁺-dependent exocytosis by activation of PKA**

In addition to the actions on the KATP channels and delayed outward K⁺ current, our data indicated that nateglinide also promotes GH release by a more direct effect on the secretory machinery. As already remarked, the latter effect is likely to be the quantitatively more important one. Interestingly, the ability to stimulate exocytosis is not shared with repaglinide (present study) but the sulphonylureas tolbutamide and glibenclamide (28). A similar difference in the responses to nateglinide and repaglinide has previously also been observed in rat α-cells (25) and rat β-cells (26), where the former produced a more than threefold enhancement of Ca²⁺-dependent exocytosis and the latter did not affect the process at all.

Several pieces of evidence indicate that inhibition of cAMP phosphodiesterase activity mediates the effect of nateglinide on exocytosis and that its action is distinct from that of the sulphonylureas. (a) The observation that nateglinide acts via activation of cAMP-mediated potentiation of exocytosis resulting from inhibition of phosphodiesterase activity is contrary to the finding that the effect of the sulphonylureas is resistant to inhibition of PKA by Rp-cAMPS (13). (b) In the presence of cAMP, nateglinide had no additional stimulatory action. (c) Measurements of cAMP contents revealed that nateglinide increased cAMP levels in both basal and GHRH-stimulated cells. (d) Addition of the phosphodiesterase inhibitor IBMX produced the same degree of stimulation as nateglinide. (e) In the presence of the phosphodiesterase inhibitor, nateglinide had no additional stimulatory action. Collectively, these observations make it justifiable to propose that inhibition of cAMP phosphodiesterase by nateglinide with resultant elevation of cytoplasmic cAMP levels and acceleration of exocytosis contributes to the secretory action of the compound. The ability of nateglinide to enhance Ca²⁺-dependent exocytosis is quantitatively significant and in fact accounts for around 70% of the total stimulatory action. This is probably the main reason why repaglinide, which, like nateglinide, closes KATP channels and induces electrical activity, is a much weaker stimulus of GH release in the presence of GHRH (Fig. 7).

**Conclusion**

Our data suggest that the stimulatory action of nateglinide on GH release is the combined result of the inhibition of the delayed outward current and the KATP channels with resultant stimulation of electrical activity together with the PKA-dependent potentiation of exocytosis. The stimulation of GH secretion predicted to result from the combination of the effects on action potential duration (+40%, Fig. 2a) and exocytosis (+330%, Fig. 6a) is a 4.6-fold enhancement of hormone release. This is in excellent agreement with the 4.5-fold enhancement of GH release observed experimentally (Fig. 7). Such an effect would be undesired since stimulated release of GH will aggravate the diabetic state through its effect on glucose and lipid metabolism (29–32). New therapeutic strategies should consequently aim at reducing the production...
or action of GH in diabetes via a mechanism selective on the β-cell K\textsubscript{ATP} channel.

References


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