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Selectivity of prandial glucose regulators: nateglinide, but not repaglinide, accelerates exocytosis in rat pancreatic A-cells

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Abstract

The effects of the two prandial glucose regulators, repaglinide and nateglinide, on ATP-sensitive K^+ (K_{ATP}) channel activity, membrane potential and exocytosis in single rat pancreatic A-cells were investigated using the patch-clamp technique. K_{ATP} channel activity was reversibly blocked by repaglinide ($K_d = 22$ nM) and nateglinide ($K_d = 410$ nM) and this was associated with membrane depolarisation and initiation of electrical activity. The effect of repaglinide and nateglinide on stimulation of glucagon secretion by direct interference with the exocytotic machinery was investigated by the use of capacitance measurements. Nateglinide, but not repaglinide, at concentrations similar to those required to block K_{ATP} channels potentiated Ca^{2+}-evoked exocytosis 3-fold. In αTC1-9 glucagonoma cells addition of nateglinide, but not repaglinide, was associated with stimulation of glucagon secretion. These results indicate that the fast-acting insulin secretagogue nateglinide is glucagonotropic primarily by stimulating Ca^{2+}-dependent exocytosis. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Exocytosis; Glucagon; K_{ATP} channel; Nateglinide; Repaglinide

1. Introduction

Treatment of human type 2 diabetes with currently available hypoglycemic sulphonylureas is associated with a number of problems (Groop, 1992). Furthermore, specific reduction of post-prandial glucose excursions in type 2 diabetes has been considered to be an unmet therapeutic need (Lefèbvre and Scheen, 1998). To meet these challenges several new oral anti-diabetic drugs have been developed during recent years. These include the fast-acting prandial glucose regulators, repaglinide ((*S*)-(+)-2-ethoxy-4[2-[[3-methyl-1-[2-(1-piperidinyl)-phenyl]-butyl]amino]-2-oxo-ethyl]) (a carbamoylmethyl benzoic acid derivative) and nateglinide (*N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine or A-4166) (a D-form derivative of phenylalanine). The structures of these compounds differ from the sulphonylureas and are shown in Fig. 1. Although the mechanisms underlying the cellular actions of insulin secretagogues, including repaglinide and nateglinide, are not completely established, it is now clear that their ability to inhibit ATP-sensitive K^+ (K_{ATP}) channels represents an important mode of their action (Panten et al., 1989; Gromada et al., 1995). This results in membrane depolarisation, initiation of electrical activity, acceleration of Ca^{2+}-influx and stimulation of Ca^{2+}-dependent exocytosis (Ashcroft and Rorsman, 1989).

It has recently been demonstrated that sulphonylureas, but not repaglinide, in addition to stimulation of insulin secretion by closure of the K_{ATP} channels, also interfere with the secretory machinery at a level distal to an elevation of the cytoplasmic Ca^{2+} concentration in pancreatic B-cells (Eliasson et al., 1996; Fuhlendorff et al., 1998). Here we have characterised the effects of repaglinide and nateglinide on K_{ATP} channel activity and the membrane potential in single rat pancreatic A-cells. With this background we proceeded to investigate to what extent the efficacy as K_{ATP} channel inhibitors was correlated with

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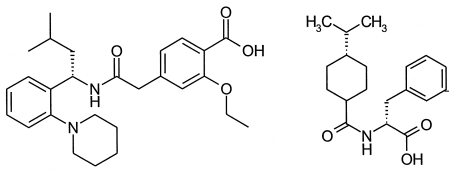

Fig. 1. Structure formulas of repaglinide and nateglinide.

direct stimulation of exocytosis. We demonstrate that it is possible to pharmacologically dissociate the actions on K_{ATP} channels and exocytosis and suggest that compounds with direct effects on exocytosis in A- and B-cells can be used in future studies on granule exocytosis of cells within the endocrine system.

2. Materials and methods

2.1. Preparation of single A-cells

Male Lewis rats (250–300 g; Møllegaard, Lille Skensved, Denmark) were anaesthetised by pentobarbital (100 mg/kg, i.p.), and the pancreas was removed. Islets were isolated by collagenase digestion and dispersed into single cells using dispase. Pancreatic A-cells were separated by fluorescence-activated cell sorting as described elsewhere (Josefsen et al., 1996). Based on the hormone contents and their glucose sensitivity, we estimate that the preparation contains $>80\%$ A-cells and $<3\%$ B-cells (Josefsen et al., 1996; Gromada et al., 1997). The cell suspension was plated on 35-mm-diameter Petri dishes and incubated in a humidified atmosphere for up to 5 days in RPMI 1640 tissue-culture medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin; no changes of the electrophysiological and exocytotic properties were observed during the period of culture.

2.2. Electrophysiology

Patch pipettes were pulled from borosilicate glass capillaries, coated with Sylgard at their tips and fire-polished before use. Whole-cell K_{ATP} currents were recorded with an Axopatch 200B patch clamp amplifier, a Digidata AD-converter (Axon Instruments) and the software pClamp (version 6.0; Axon Instruments, Foster City, CA, USA). The pipette resistance (when filled with the pipette-filling solutions) was 2–4 MΩ. The zero-current potential was adjusted before establishment of the seal with the pipette in the bath. Currents through K_{ATP} channels were estimated by applying 10 mV hyper- and depolarising voltage pulses (duration: 200 ms; pulse interval: 2 s) from a holding potential of −70 mV using the standard whole-cell configuration. Repaglinide and nateglinide were added after the current had reached steady-state and the effects of both drugs are expressed as the fraction of the average currents with control solutions. The membrane potential was measured using the perforated patch whole-cell configuration. Test solutions were applied in random order.

Exocytosis was monitored as increases in cell membrane capacitance using the standard whole-cell configuration of the patch-clamp technique and an EPC-9 patch-clamp amplifier and the Pulse software (HEKA Electronics, Lambrecht/Pfalz, Germany). 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 Ca^{2+}-EGTA buffers through the recording electrode.

2.3. Solutions

The extracellular medium consisted of (in mM) 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4 with NaOH) and 5 D-glucose unless stated otherwise. The volume of the recording chamber was 0.4 ml with a flow rate of 1.5–2 ml/min. The temperature for recordings of the membrane potential and exocytosis was +33°C.

For measurements of whole-cell K^+channel activity, the pipette solution contained (in mM) 125 KCl, 30 KOH, 10 EGTA, 1 $MgCl_2$, 5 HEPES, 0.3 Mg-ATP and 0.3 K-ADP (pH 7.15). For membrane potential measurements the pipette solution was composed of (in mM) 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$ and 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 the voltage-clamp was considered satisfactory when the G_{series} was stable and >35 nS. The electrode solution for measurements of exocytosis consisted of (in mM) 125 potassium glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 Hepes, 3 Mg-ATP, 10 EGTA, 5 $CaCl_2$. The free Ca^{2+} concentration of the resulting buffer was 0.22 μM using the binding constants of Martell and Smith (1971). Repaglinide and nateglinide were synthesised at Novo Nordisk (Bagsvaerd, Denmark). All other chemicals were purchased from Sigma.

2.4. Glucagon release

Glucagon release from αTC1-9 glucagonoma cells (2×10^6 cells) was measured at +37°C in static incubation experiments using an extracellular medium with 1 mM glucose (see above for composition). After 30 min pre-incubation, the cells were exposed to the same medium with test substances added. After 30 min, the incubation medium was aspirated and kept at −20°C until assayed for immunoreactive glucagon using a radioimmunoassay based on antiserum 4304, monoiodinated ^{125}I-labeled porcine glucagon with a specific activity of approximately 70

MBq/nmol (Novo Nordisk) and synthetic human glucagon (Peninsula Europe, Merseyside, St. Helens, UK) as standards. This assay is directed against the 6–15 sequence of the glucagon molecule and therefore measures glucagon as well as its biosynthetic intermediates (Holst, 1980, 1982). The sensitivity and detection limit were approximately 5 pmol/l and the intra-assay coefficient of variation better than 5% at a level of 150 pM. Antiserum and standard or samples were preincubated for 24–48 h before addition of tracer. Free and bound hormone moieties were separated with plasma-coated charcoal.

2.5. Data analysis

Whole-cell K_{ATP} currents were acquired at 2 kHz and filtered at 1 kHz using the internal filters of the amplifiers. Results are presented as mean values ± S.E.M. for indicated number of experiments. Statistical significance was evaluated using Student's *t*-test for unpaired observations.

3. Results

3.1. Effects of repaglinide and nateglinide on A-cell electrical activity

Fig. 2a (top) illustrates spontaneous electrical activity recorded from a single rat A-cell in the absence of glucose, a condition known to be associated with stimulation of glucagon secretion (Gerich, 1983). Addition of 20 mM glucose to the extracellular solution inhibited electrical activity after a delay of ≈ 3 min. This glucose dependence is precisely that expected for glucagon-secreting A-cells and is opposite to that of the insulin secreting B-cell. Application of repaglinide (1 μM; middle) or nateglinide (100 μM; bottom) in the continuous presence of 20 mM glucose was associated with intense electrical activity. The stimulatory effects were reversible within 10 min following removal of the drugs from the perfusion medium. The strong electrical activity in the presence of repaglinide and nateglinide confirm the existence of K_{ATP} channels in rat A-cells (Bokvist et al., 1999; Suzuki et al., 1999).

3.2. Dose–inhibition relationship for K_{ATP} channel blocking action of repaglinide and nateglinide

The concentration dependence of the inhibitory action of repaglinide and nateglinide on K_{ATP} channel activity was evaluated using the voltage protocol previously described by Trube et al. (1986) and intracellular dialysis with 0.3 mM ATP and 0.3 mM ADP to activate the K_{ATP} channels. Fig. 3a (top) shows membrane currents in a single rat A-cell (estimated by 200 ms 10 mV hyper- and depolarising pulses) following complete exchange of the cytoplasm with its high ATP/ADP ratio with the pipette solution and also shows that the current was inhibited by 35% in the presence of 10 nM repaglinide. The dose–inhibition curve for the effects of repaglinide on K_{ATP} channels is shown in Fig. 3a (bottom). The data points were approximated to the Hill equation

$$I/I_{max} = K^n/(K^n + C^n)$$

where I is the membrane current observed at the concentration C, I_{max} the membrane current under control conditions, K the association constant and n the co-operativity factor. A least-squares fit yielded values of K and n of 22 ± 14 nM and 4.8 ± 1.5 ($n = 6$), respectively. Fig. 3b depicts the corresponding currents before and after application of 200 nM nateglinide. Half-maximal inhibition for nateglinide was observed at 410 ± 21 nM and with a co-operativity factor of 4.5 ± 1.9 ($n = 6$).

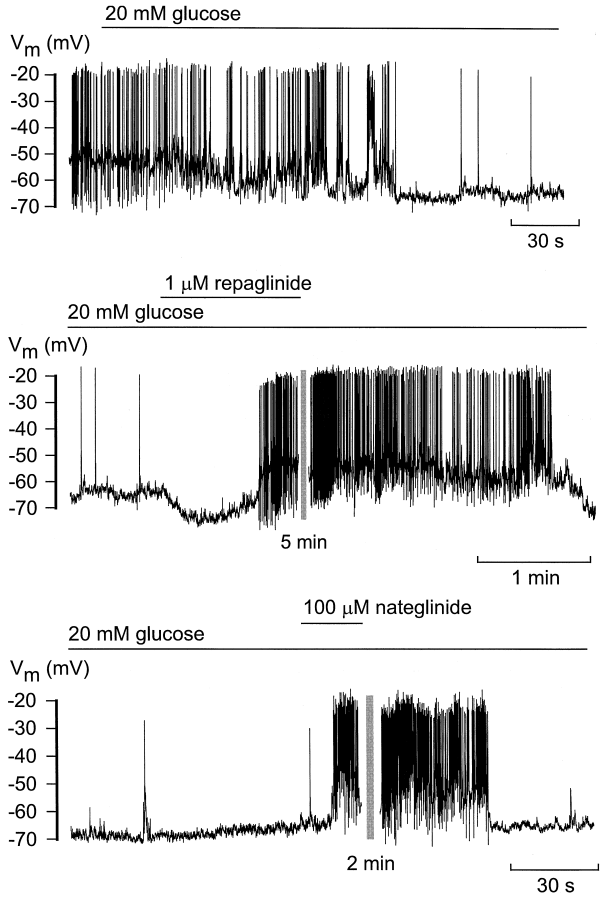

Fig. 2. (a) Effects of repaglinide and nateglinide on membrane potential in isolated rat pancreatic A-cells. Spontaneous electrical activity in an isolated rat pancreatic A-cell and inhibition by 20 mM glucose (top). Repaglinide (1 μM; middle) and nateglinide (100 μM; bottom) were applied in the continuous presence of 20 mM glucose as indicated by the lines above the membrane potential recording. The boxes indicate a 5-min (repaglinide) or 2-min (nateglinide) interval of action potentials which has been left out. The traces are continuous and representative for three different cells.

3.3. Nateglinide, but not repaglinide, accelerates Ca^{2+}-induced exocytosis

Fig. 4a shows that nateglinide (100 μM) stimulates Ca^{2+}-evoked exocytosis in single rat A-cells. Secretion

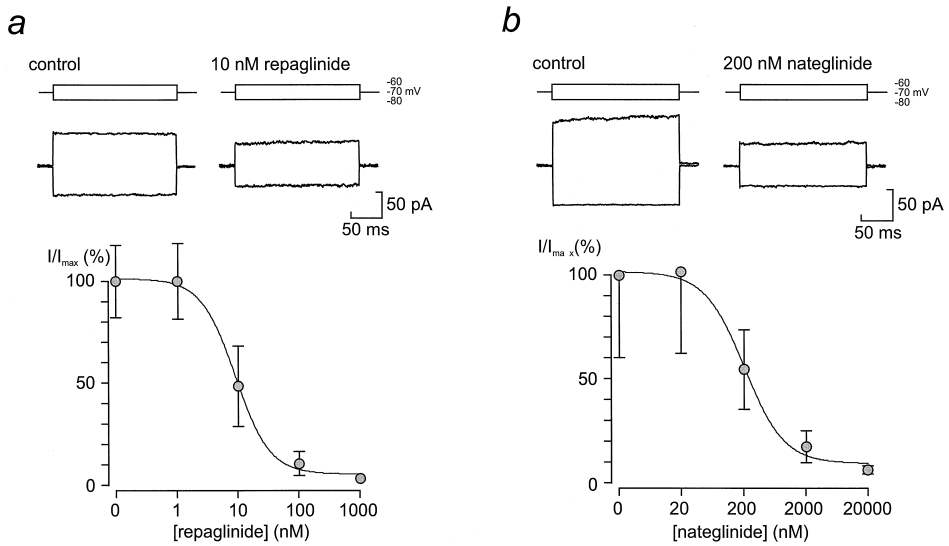

Fig. 3. Concentration dependence of repaglinide and nateglinide on K_{ATP} channel activity in rat A-cells. Changes in K_{ATP} whole-cell current were monitored in the standard whole-cell configuration by the application of 10 mV de- and repolarising voltage pulses (200 ms; 2-s interval) from a holding potential of −70 mV. Once steady-state had been attained (≈ 4 min), the indicated concentrations of repaglinide or nateglinide were applied (top panels). The relationship between repaglinide concentration (a) or nateglinide concentration (b) and relative current amplitude (I/I_{max}) is depicted in the lower panels. The lines represent the best fit to the mean data using the Hill equation. The recordings are representative of four (nateglinide) and six cells (repaglinide).

was elicited by dialysing the cells with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 0.22 μM which in itself produced a rapid but small increase in cell capacitance following establishment of the whole-cell configuration. The stimulation of exocytosis cannot be accounted for by the effects of nateglinide on the K_{ATP} channels since the cells were voltage-clamped and the membrane potential held constant irrespective of the K_{ATP} channel activity. On average (Fig. 4b), nateglinide stimulated the rate of increase in cell capacitance measured over the first 60 s after establishment of the whole-cell configuration by 306% ($P < 0.001$; $n = 3$). Repaglinide failed to stimulate exocytosis when applied at a concentration of 100 nM (Fig. 4a). The same negative results were obtained with a 1000-fold higher concentration (Fig. 4b).

Table 1 shows a comparison of the effects of nateglinide and repaglinide on glucagon release from αTC1-9 glucagonoma cells. All experiments were performed in the

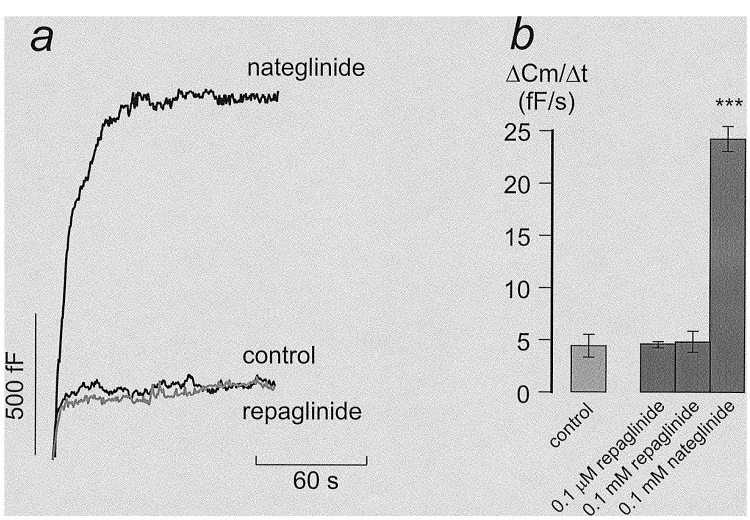

Fig. 4. Differential effects of nateglinide and repaglinide on Ca^{2+}-evoked exocytosis. (a) Increases in cell capacitance in single A-cells elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 0.22 μM in the absence (control) or presence of 0.1 μM repaglinide or 100 μM nateglinide in the recording pipette. Throughout the recordings, the cells were clamped at −70 mV in order to avoid activation of the voltage-dependent Ca^{2+} channels that would otherwise interfere with the measurements. (b) Histogram depicting the average rates of increase in cell capacitance ($\Delta C_m/\Delta t$) measured over the first 60 s after establishment of the whole-cell configuration under control conditions and in the presence of repaglinide (0.1 or 100 μM) or nateglinide (100 μM). Data are mean values ± S.E.M. of 3–5 experiments. $^{***}P < 0.001$.

Table 1
Effects of nateglinide and repaglinide on glucagon release from αTC1-9 glucagonoma cells. All experiments have been obtained in the presence of 1 mM glucose
Data are mean ± S.E.M. of 12 experiments.

Condition	Glucagon release (pM)
Control	586 ± 34
Arginine (15 mM)	1139 ± 113*
Repaglinide (100 nM)	687 ± 68
Nateglinide (0.1 mM)	832 ± 57*

* $P < 0.005$.

presence of 1 mM glucose. Addition of 15 mM arginine was associated with a doubling of the glucagon release. Interestingly, nateglinide but not repaglinide enhanced the release of glucagon, suggesting that the direct stimulatory effect of nateglinide on Ca^{2+}-dependent exocytosis is of physiological relevance. Indeed, it was possible to calculate that 59% of the total stimulation of glucagon release is independent of K_{ATP} channel closure.

4. Discussion

4.1. Cytotropic action of repaglinide and nateglinide involves closure of K_{ATP} channels

Here, we demonstrate that repaglinide and nateglinide both inhibit the A-cell K_{ATP} channels which entrain membrane depolarisation and electrical activity. This finding reinforces previous observations that clonal glucagon-secreting αTC cells express K_{ATP} channels (Rajan et al., 1993; Ronner et al., 1993) and that rat A-cells contain K_{ATP} channels with nucleotide and sulphonylurea sensitivities similar to those of their counterparts in the B-cell (Bokvist et al., 1999). Surprisingly, guinea pig A-cells appears not to express functional K_{ATP} channels (Rorsman and Hellman, 1988). The concentrations of repaglinide required to close the K_{ATP} channels was 10-fold lower than those of nateglinide, reflecting the higher potency of the former compound. Repaglinide also distinguishes itself from nateglinide in its more slow onset and reversibility of its action. These differences are reminiscent to those described for the high- and low-affinity sulphonylureas glibenclamide and tolbutamide in the B-cell (Panten et al., 1989) and we therefore tentatively attribute the differences to nateglinide having a lower affinity than repaglinide for the (respective) receptor(s). Interestingly, the fact that nateglinide (Ikenoue et al., 1997) displaces radioactive glibenclamide suggests that D-phenylalanine derivatives bind to the same receptor as the sulphonylureas, most likely the recently cloned 145 kDa sulphonylurea receptor (Aguilar-Bryan et al., 1995). This does not exclude the presence of additional binding sites for these secretagogues since repaglinide potency correlates better with a high affinity binding site than with the classical glibenclamide binding site (Fuhlendorff et al., 1998).

It is well established that sulphonylureas promote insulin secretion through direct effects on the pancreatic B-cells. By contrast, the results with respect to undesired effect of sulphonylureas on glucagon release vary in the literature, and sulphonylureas have been reported both to stimulate and inhibit A-cell function (Samols et al., 1969; Grodsky et al., 1977; Efendic et al., 1979). In the present study it is demonstrated that nateglinide stimulates glucagon secretion by suppressing K_{ATP} channel activity and by promoting Ca^{2+}-dependent exocytosis. This is consistent with a recent report that nateglinide enhances glucagon release from the perfused rat pancreas (Fujitani et al., 1996).

4.2. Effects of antidiabetic drugs on the exocytotic machinery

In addition to closing K_{ATP} channels, recent evidence suggests that the sulphonylureas, in contrast to repaglinide, also stimulate insulin secretion by a direct interaction with the secretory machinery (Flatt et al., 1994; Eliasson et al., 1996; Fuhlendorff et al., 1998). The latter action is exerted at a level distal to the elevation of cytoplasmic Ca^{2+} and may involve the activity of protein kinase C. Here, we demonstrate that nateglinide shares with the sulphonylureas the capacity to stimulate exocytosis by the direct route in rat A-cells. However, the lack of stimulation by repaglinide of Ca^{2+}-dependent A-cell exocytosis clearly distinguishes the action of repaglinide from that of nateglinide and sulphonylureas. The lack of effect of repaglinide on exocytosis is not the result of limited intracellular uptake of the drug since both compounds were applied directly to the cell interior through the patch pipette. The mechanism by which the sulphonylureas and nateglinide stimulate exocytosis remain unestablished but it is pertinent that 80–90% of the B-cell sulphonylurea binding sites are intracellular and localises to the secretory granules (Carpentier et al., 1986; Ozanne et al., 1995). It has recently been demonstrated that the effects of sulphonylureas on exocytosis in B-cells are secondary to their binding to a 140 kDa high affinity sulphonylurea receptor in the granular membrane which culminates in the activation of a granular Cl^- membrane conductance (Barg et al., 1999). The uptake of ions and water into the granules may provide the energy required for fusion by increasing the hydrostatic pressure within these organelles (Barg et al., 1999). Modulation of ionic fluxes over the granular membrane in rat A-cells may also account for the stimulatory action of nateglinide on exocytosis observed in the present study.

4.3. Clinical implications

The action of nateglinide on exocytosis may be of general clinical significance since nateglinide, but not

repaglinide, also enhances Ca^{2+}-induced secretion in rat pituitary cells and rat B-cells (unpublished observations). This raises the possibility that nateglinide (and sulphonylureas) could influence vesicular trafficking in general. As a consequence, these compounds might have a higher probability of undesired side effects. For example, relative hyperglucagonaemia has been demonstrated to be present in type 2 diabetics (Consoli et al., 1990) and this might impede insulin-stimulated glucose metabolism in the longer term (Rizza et al., 1981). Furthermore, it has been suggested that the failure of some patients to respond to sulphonylureas after long-term therapy may relate, among other things, to the persistent, excessive hepatic glucose production induced by counterregulatory hormones such as glucagon, contributing to fasting hyperglycemia (DeFronzo, 1992). Thus, the finding that repaglinide distinguishes itself by exerting its effects solely via the plasma membrane K_{ATP} channel, as opposed to nateglinide and sulphonylureas that also promote exocytosis by direct interference with the secretory process, obviously opens interesting perspectives for the development of new antidiabetic compounds with a hormone-selection action profile.

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