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Synthesis and characterization of a quinolinonic compound activating ATP-sensitive K^+ channels in endocrine and smooth muscle tissues

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1 Original quinolinone derivatives structurally related to diazoxide were synthesized and their effects on insulin secretion from rat pancreatic islets and the contractile activity of rat aortic rings determined.

2 A concentration-dependent decrease of insulin release was induced by 6-chloro-2-methylquinolin-4(1*H*)-one (HEI 713). The average IC_{50} values were 16.9 ± 0.8 μM for HEI 713 and 18.4 ± 2.2 μM for diazoxide.

3 HEI 713 increased the rate of ^{86}Rb outflow from perifused pancreatic islets. This effect persisted in the absence of external Ca^{2+} but was inhibited by glibenclamide, a K_{ATP} channel blocker. Inside-out patch-clamp experiments revealed that HEI 713 increased K_{ATP} channel openings.

4 HEI 713 decreased ^{45}Ca outflow, insulin output and cytosolic free Ca^{2+} concentration in pancreatic islets and islet cells incubated in the presence of 16.7 or 20 mM glucose and extracellular Ca^{2+}. The drug did not affect the K^+(50 mM)-induced increase in ^{45}Ca outflow.

5 In aortic rings, the vasorelaxant effects of HEI 713, less potent than diazoxide, were sensitive to glibenclamide and to the extracellular K^+ concentration.

6 The drug elicited a glibenclamide-sensitive increase in ^{86}Rb outflow from perifused rat aortic rings.

7 Our data describe an original compound which inhibits insulin release with a similar potency to diazoxide but which has fewer vasorelaxant effects.

8 Our results suggest that, in both aortic rings and islet tissue, the biological effects of HEI 713 mainly result from activation of K_{ATP} channels ultimately leading to a decrease in Ca^{2+} inflow.
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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; FOR, fractional outflow rate; HEI 712, 6-fluoro-2-methylquinolin-4(*1H*)-one; HEI 713, 6-chloro-2-methylquinolin-4(*1H*)-one; K_{ATP}, ATP-sensitive K^+; Kir, inwardly rectifying K^+ channel; SUR, sulphonylurea receptor

Introduction

ATP-sensitive potassium (K_{ATP}) channels were first discovered in cardiac muscle (Noma, 1983) and later found in a wide variety of tissues, including pancreatic β-cells (Ashcroft *et al*., 1984; Cook & Hales, 1984; Rorsman & Trube, 1985), smooth muscle (Standen *et al*., 1989) and brain (Ashford *et al*., 1988). Recent data also revealed the existence of such ionic channels on the inner mitochondrial membrane (Inoue *et al*., 1991). The activity of plasma membrane K_{ATP} channels is controlled by the intracellular ATP/ADP ratio in such a way that an increase of the ratio closes K_{ATP} channels whereas a decrease opens them (see Aguilar-Bryan & Bryan, 1999; Ashcroft & Ashcroft, 1990; Seino, 1999; Miki *et al*., 1999; Nichols & Lederer, 1991). By coupling cell metabolism to membrane potential, K_{ATP} channels play an important role in the control of many physiological processes.

K_{ATP} channels are octameric structures consisting of two types of subunit: an inwardly rectifying K^+ channel subunit (Kir6.x) and a sulphonylurea receptor subunit (SURx) which is a member of the ATP-binding cassette (ABC) transporter protein family (Ashcroft & Gribble, 1998; Aguilar-Bryan & Bryan, 1999; Seino, 1999). The pancreatic β-cell K_{ATP} channel is formed by Kir6.2 and SUR1 subunits, the cardiac and skeletal muscle types by Kir6.2 and SUR2A subunits and the smooth muscle type by Kir6.1/Kir6.2 and SUR2B subunits (Seino, 1999). The Kir6.1 subunit is also present on the inner membrane of mitochondria (Suzuki *et al*., 1997; Szewczyk & Marban, 1999). In each case, SUR and Kir subunits are physically associated in a 4:4 stoichiometry to form functional K_{ATP} channels (Aguilar-Bryan & Bryan, 1999). Different combinations of the SUR

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and Kir subunits constitute K_{ATP} channels with distinct nucleotide sensitivities and pharmacological properties (Seino, 1999).

Diazoxide (7-chloro-3-methyl-*2H*-1,2,4-benzothiadiazine 1,1-dioxide), a compound which activates K_{ATP} channels, is known to possess not only vasodilator properties but also potent hyperglycaemic actions (Quast & Cook, 1989). Therefore, this drug has been used for the treatment of hypertensive emergencies and for the treatment of various forms of hypoglycaemia (Gerber & Nies, 1990; Lebrun *et al*., 1992). Diazoxide exhibits a weak tissue selectivity between vascular smooth muscle and pancreatic β-cells (Antoine *et al*., 1992; de Tullio *et al*., 1996).

Recently, we have developed original quinolinonic compounds (Figure 1) structurally related to diazoxide but chemically different from the pyrido- and benzothiadiazine dioxides that we have previously synthesized (de Tullio *et al*., 1996; Lebrun *et al*., 1996, 2000; Pirotte *et al*., 2000). Our ultimate goal is to generate new chemical entities with a powerful, and hopefully selective, biological activity on insulin secreting cells. Such compounds could be valuable drugs for the treatment of several metabolic disorders (Lebrun *et al*., 2000). This study was made in order to document the activity of these new quinolinonic derivatives on insulin secretion from rat pancreatic islets and on the contractile activity of rat aortic rings. Radioisotopic, fluorimetric and electrophysiological approaches have been used to characterize the mechanism of action of the most potent compound.

Methods

Partition coefficient determinations

The partition coefficients in 1-octanol/phosphate buffer (pH 7.4) were determined by shake-flask technique (Cloux *et al*., 1988), using 5.10^{-5} M stock solutions of drugs in 1-octanol or in phosphate buffer. Drug concentration after partition was determined by u.v. spectrophotometry at the maximum absorbance (HEI 712: buffer, $\lambda = 234$ nm; octanol, $\lambda = 236$ nm; HEI 713: buffer, $\lambda = 238$ nm; octanol, $\lambda = 240$ nm; Diazoxide: buffer, $\lambda = 265$ nm; octanol, $\lambda = 268$ nm) (experiments were performed in triplicate from the 1-octanol stock solution and in triplicate from the buffer stock solution). The results were expressed as the logarithm of the partition coefficient (log P′).

Measurements of insulin release from incubated pancreatic islets

Experiments were performed with pancreatic islets isolated by the collagenase method from fed Wistar rats (Lacy & Kostianowsky, 1967). The laboratory animal care was approved by the ethics committee of the Université Libre de Bruxelles.

Groups of 10 islets, each from the same batch, were preincubated for 30 min at 37°C in 1 ml of a bicarbonate-buffered solution (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, supplemented with 2.8 mM glucose, 0.5% (w v^{-1}) dialysed albumin (fraction V; Sigma Chemical Co, U.S.A.) and equilibrated with a mixture of O_2 (95%) and CO_2 (5%). The islets were then incubated at 37°C for a further 90 min in 1 ml of the same bicarbonate-buffered medium containing 16.7 mM glucose and, in addition, increasing concentrations of diazoxide, HEI712 or HEI713. Experiments were repeated on different islets populations. Insulin release was expressed as percentage of the value recorded in control experiments (100%), i.e. in the absence of drug and presence of 16.7 mM glucose. The release of insulin was measured radioimmunologically, as previous reported (Leclercq-Meyer *et al*., 1985).

Measurements of ^{86}Rb, ^{45}Ca outflow and insulin release from perifused pancreatic islets

The methods used to measure ^{86}Rb (^{42}K substitute) outflow, ^{45}Ca outflow and insulin release from rat perifused pancreatic islets have been described previously (Lebrun *et al*., 1982; 1996). Groups of 100 islets were incubated for 60 min in a bicarbonate buffered medium (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, containing 16.7 mM glucose and either ^{86}Rb ion (0.15–0.25 mM; 50 μCi.ml^{-1}) or ^{45}Ca ion (0.02–0.04 mM; 100 μCi.ml^{-1}). After incubation, the islets were washed four times with a non-radioactive medium and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 ml.min^{-1}). From the 31st to the 90th min of perifusion, the effluent was continuously collected over successive periods of 1 min each. An aliquot of the effluent (0.5 ml) was used for scintillation counting while the remainder was stored at −20°C for insulin radioimmunoassay (Leclercq-Meyer *et al*., 1985). At the end of the perifusion, the radioactive content of the islets was also determined. The outflow of ^{86}Rb or ^{45}Ca ion (c.p.m: min^{-1}) was expressed as a fractional outflow rate (per cent of instantaneous islet content min^{-1}, FOR). The validity of ^{86}Rb

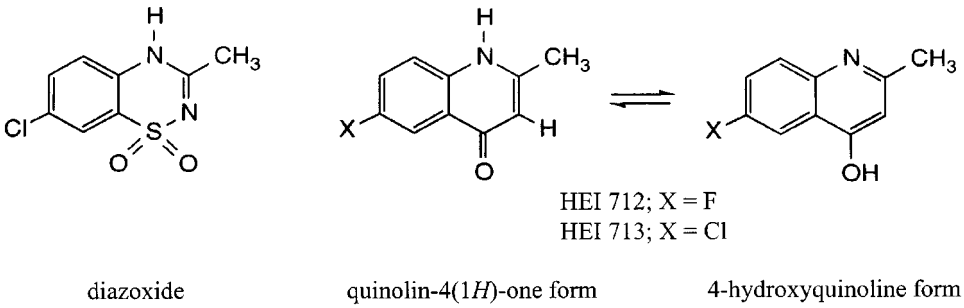


Figure 1 Chemical structure of diazoxide and tautomeric forms of HEI 712 and HEI 713.

as a tracer for the study of K^+ handling in pancreatic islets has been previously assessed (Malaisse *et al*., 1978).

Electrophysiological measurements

Pancreatic islets were isolated from human cadaver donors or, post-operatively, from the non-focal region of a patient with focal hyperinsulinism of infancy (Kane *et al*., 1996; Verkarre *et al*., 1998). The islets were mechanically dispersed and isolated cells maintained under standard tissue culture conditions using RPMI 1640 medium (Sigma, Poole, U.K.) supplemented with 10% (v v^{-1}) foetal calf serum, 100 IU ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin. Experiments were carried out using human β-cells or NES2Y β-cells (MacFarlane *et al*., 2000) transfected with cDNA encoding Kir6.2ΔC26 (Tucker *et al*., 1997). Single channel currents were recorded using the cell-free, inside-out patch-clamp configuration (Hamill *et al*., 1981). Micropipettes (5–10 MΩ) were filled with a Na^+-rich solution of the following ionic composition (in mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.13, $CaCl_2$ 2.5, glucose 2.5, HEPES 10, pH 7.4. The internal face of membrane was bathed with a K^+-rich solution containing (in mM): KCl 140, NaCl 10, $MgCl_2$ 1.13, EGTA 1, 2.5 glucose and HEPES 10, pH 7.2. Ion channel experiments were carried out at room temperature and changes in K_{TAP} channel activity have been expressed as either changes in open-state probability (Pod) or as a function of NO where N is the number of operational channels (Lebrun *et al*., 1996).

Measurements of fura-2 fluorescence from single pancreatic islet cells

Rat pancreatic islets were disrupted in a Ca^{2+}-deprived medium (mM): NaCl 124, KCl 5.4, $MgSO_4$ 0.8, Na_2HPO_4 1, HEPES 10, glucose 2.8, $NaHCO_3$ 14.3 and EGTA 1 (modified from Pipeleers *et al*., 1985) and then centrifuged to remove debris and dead cells. Cells were seeded onto glass coverslips and maintained in tissue culture for 72 h before use. Islet cells were cultured in RPMI 1640 culture medium (Life Technologies, Europe) supplemented with 10% (v v^{-1}) newborn calf serum and containing glutamine (2.3 mM), glucose (16.7 mM), penicillin G (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}). The cells were then incubated with fura-2 AM (2 μM) (Molecular Probes, U.S.A.) for 1 h and, after fura-2 solution elimination, the coverslips with the cells were mounted as the bottom of an open chamber (1 ml) placed on the stage of the microscope. The medium used to perifuse the cells contained (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, glucose 2.8 and was gassed with O_2(95%)/CO_2(5%). Fura-2 fluorescence of single loaded cells was measured by use of dual-excitation microfluorimetry with Spex photometric system (Optilas, Alphen aan den Rijn, The Netherlands). The excitation wavelengths (340 and 380 nm) were alternated at the frequency of 1 Hz. The emission wavelength was set at 510 nm. $[Ca^{2+}]_i$ was calculated as previously described (Lebrun *et al*., 1996). Individual experiments were repeated at least four times, on different cell populations.

Measurements of tension in aorta rings

Adult fed Wistar rats were stunned and exsanguinated. The thoracic aorta was removed, cut into transverse rings (4–5 mm) and adhering fat and connective tissue was removed. After removal of the endothelium, the segments were suspended under 2 g load in an organ bath containing Krebs bicarbonate-buffered solution (20 ml; (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5). The solution maintained at 37°C was continuously oxygenated with a mixture of 95% O_2 and 5% CO_2. After equilibration for 60 min, isometric contractions were measured with a force-displacement transducer. Contractile activity was induced by increasing the extracellular concentration of K^+ (30 or 80 mM KCl). When a plateau of tension was reached, drugs were added to the preparation cumulatively to a maximum concentration of 300 μM. Some experiments were repeated in the continuous presence of 1 or 10 μM glibenclamide.

Measurements of ^{86}Rb outflow from perifused aorta rings

Experiments were performed with thoracic rat aorta rings ($\cong$2 mm length) isolated from fed Wistar rats.

The aorta rings were preincubated for 30 min in a bicarbonate buffered medium (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, containing 5 mM glucose. Following preincubation, the aorta rings were incubated for 60 min in bicarbonate buffered medium containing ^{86}Rb ion (0.15–0.25 mM; 50 μCi.ml^{-1}). Experiments were then performed using the protocol described above for ^{86}Rb outflow from perifused pancreatic islets. The experiments were conducted in the presence of 30 mM extracellular KCl to mimic the experimental conditions used to measure muscle tension.

Although the use of ^{86}Rb as a tracer for the study of K^+ handling in aorta rings underestimates the drugs-induced increases in K^+ permeability the ^{86}Rb method is valid in this tissue at drug concentrations $\geqslant$0.1 mM (Antoine *et al*., 1992; Quast & Baumlin, 1988; Lebrun *et al*., 1990).

Drugs

The quinolinone derivatives were synthesized at the Laboratory of Molecular Engineering, Ecole des Hautes Etudes Industrielles, Lille, France.

Briefly, (Figure 2), 6-Fluoro-2-methylquinolin-4(1*H*)-one (HEI 712) and 6-chloro-2-methylquinolin-4(1*H*)-one (HEI 713) were obtained according to a synthetic process adapted from Mallams & Israelstam (1964). A stirred mixture of ethyl acetoacetate (1.3 g, 0.01 mol) and 4-chloroaniline (1.28 g, 0.01 mol) or 4-fluoroaniline (1.11 g, 0.01 mol) in polyphosphoric acid (5 g) was heated at 130°C for 1 h. After cooling, the solution was neutralized with 2 M NaOH. The solid obtained was collected by filtration and washed with water. The crude product was purified by dissolution in a 5% w v^{-1} aqueous solution of NaOH and the alkaline solution was treated with charcoal, filtered and adjusted to pH 7 with 6 M HCl. The final precipitate was collected by filtration, washed with water and dried.

6-Fluoro-2-methylquinolin-4(1*H*)-one (HEI 712), yields: 1.21 g, 68%; m.p.: 268°C [lit.: 266°C (Franchi & Vigni, 1967)]; IR(KBr) 3280, 1650, 1610, 1560, 1520, 1470 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ ppm 2.78 (s, 3H, CH_3), 7.07 (bs, 1H, 3-H), 7.74 (td, J = 8.4, 2.7 Hz, 8-H), 7.85–8.01 (m, 2H, 5-H + 7-H); anal. ($C_{10}H_8FNO$) calcd.: C: 67.79; H: 4.55; N: 7.91; F: 10.72; found: C: 67.58; H: 4.73; N: 7.73; F: 10.36.

6-Chloro-2-methylquinolin-4(1*H*)-one (HEI 713), yields: 1.23 g, 63%; m.p.: >300°C [lit.: 321°C (Desai & Desai, 1967)]; IR(KBr) 3250, 1640, 1600, 1555, 1510, 1465 cm^{-1}; ^{1}H-NMR ($CDCl_3 + CF_3CO_2H$) δ ppm 2.77 (s, 3H, CH_3), 7.07 (bs, 1H, 3-H), 7.81 (d, J = 9.1 Hz, 1H, 8-H), 7.92 (dd, J = 9.1, 2.3 Hz, 1H, 7-H); 8.31 (d, J = 2.3 Hz, 1H, 5-H); anal. ($C_{10}H_8ClNO$) calcd.: C: 62.03; H: 4.16; N: 7.23; Cl: 18.31; found: C: 62.10; H: 4.22; N: 7.19; Cl: 18.26.

In some experiments, extracellular Ca^{2+} was eliminated by the omission of $CaCl_2$ from the physiological medium and the addition of 0.5 mM ethylene glycol-bis (β-aminoethyl ether) N,N′-tetraacetic acid (EGTA; Sigma Chemical Co). According to the experiments, the media were enriched with glucose (Merck, Darmstadt, Germany), glibenclamide (ICN Biomedicals, Inc. Ohio, U.S.A.), diazoxide (Sigma Chemical Co), HEI 712 or HEI 713.

Glibenclamide, diazoxide, HEI 712 and HEI 713 were dissolved in dimethylsulphoxide which was added to both control and test media. At the final concentrations used (<0.1% for insulin secreting cells and <0.8% for aortic rings), dimethylsulphoxide fails to affect islet function and smooth muscle contractility (Antoine *et al*., 1992; B. Becker *et al*., unpublished observations; Lebrun & Atwater, 1985; Lebrun *et al*., 1996). When high concentrations of extracellular K^+ (>30 mM) were used, the concentration of extracellular NaCl was lowered to keep osmolarity constant.

Calculations

Results are expressed as the mean ± s.e.mean. The magnitude of the increase in ^{86}Rb outflow was estimated in each individual experiment from the integrated outflow of ^{86}Rb observed during stimulation (45th – 68th min) after correction for basal value (40th – 44th min). The inhibitory effect of HEI 713 on ^{45}Ca outflow and insulin release was taken as the difference between the mean value for ^{45}Ca outflow or insulin output recorded in each individual experiment between the 40th – 44th and 60th – 68th min of perifusion. The IC_{50} value for insulin release (concentration giving a 50% reduction of the secretory response to 16.7 mM glucose) and ED_{50} values for rat aortic rings (drug concentrations inducing half-maximum inhibition of the plateau phase induced by KCl) were assessed from concentration-response curves using Datanalyst software (EMKA Technologies, France). The statistical significance of the differences between mean data was assessed by use of Student's *t*-test or by analysis of variance followed by a Scheffe test procedure.

Results

Effects of diazoxide, HEI 712 and HEI 713 on the glucose-induced insulin release from incubated rat pancreatic islets

The addition of increasing concentrations of HEI 713 to pancreatic islets incubated in the presence of 16.7 mM glucose provoked a concentration-dependent decrease in insulin release. After the addition of 1, 10 and 50 μM HEI 713 to the incubation medium, the insulin release was, respectively, 97.52 ± 4.31% ($P>0.5$); 76.20 ± 3.14% ($P<0.05$) and 5.11 ± 0.66% ($P<0.001$) of the control value. The IC_{50} value for HEI 713 averaged 16.9 ± 0.8 μM (Table 1).

The presence of diazoxide in the incubation medium also reduced the glucose-induced insulin release. Thus, in the presence of 1, 10, 50, 100 and 500 μM diazoxide, the release of insulin averaged, respectively, 82.52 ± 3.08% ($P<0.05$), 71.66 ± 2.77% ($P<0.001$), 27.89 ± 1.54% ($P<0.001$), 14.76 ± 0.98% ($P<0.001$) and 8.52 ± 0.70% ($P<0.001$) of that recorded in the presence of glucose (16.7 mM) but absence of drug. The IC_{50} value for diazoxide averaged 18.4 ± 2.2 μM (Table 1).

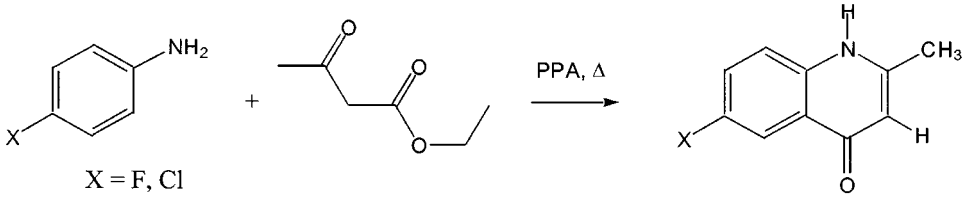
According to the IC_{50} values (Table 1), the inhibitory effect of diazoxide on insulin output was not significantly different ($P>0.5$) from that of HEI 713.

HEI 712 (IC_{50} 75.5 ± 1.7 ($n=24$)), the 6-fluoroquinolinone derivative, was less potent than HEI 713 and diazoxide ($P<0.05$) at inhibiting the secretory response to glucose (Table 1).

Table 1 Effect of HEI 712, HEI 713 and diazoxide on insulin release from rat pancreatic islets, on the contractile activity of rat aortic rings and on the log P′ value

	IC_{50} (μM) pancreatic islets	*ED_{50} (μM) aortic rings*	*log P′*
Diazoxide	18.4 ± 2.2 (38)	23.8 ± 2.4 (10)	1.21
HEI 713	16.9 ± 0.8 (22)	42.5 ± 2.3 (13)	>1.50
HEI 712	75.5 ± 1.7 (24)	223.0 ± 17.6 (6)	1.29

Rat pancreatic islets were incubated in the presence of 16.7 mM glucose and rat aortic rings were depolarized by K^+ (30 mM). IC_{50} and ED_{50} values are expressed as means ± s.e.mean with the number of samples or individual experiments in parentheses. IC_{50} is the drug concentration eliciting 50% reduction in insulin release whilst EC_{50} is the drug concentration (μM) eliciting 50% relaxation of the 30 mM KCl-induced contraction.



PPA = polyphosphoric acid
Δ = 130 °C, 1 h

HEI 712; X = F
HEI 713; X = Cl

Figure 2 Chemical synthesis of HEI 712 and HEI 713.

Effects of HEI 713 on ^{86}Rb, ^{45}Ca outflow and insulin release from perifused rat pancreatic islets

In the presence of 5.6 mM glucose in the basal medium, and whether the experiments were performed in the presence or absence of extracellular Ca^{2+}, the addition of HEI 713 (50 μM) provoked a fast, sustained and rapidly reversible increase in the rate of ^{86}Rb outflow from prelabelled and perifused rat pancreatic islets (Figure 3, upper panel). The increment in ^{86}Rb FOR averaged $2.76\pm0.22\%$ min^{-1} ($n=4$) in the absence and $1.93\pm0.13\%$ min^{-1} ($n=4$) in the presence of external Ca^{2+} ($P<0.05$).

In another series of experiments, we characterized the effect of glibenclamide on the cationic response to HEI 713 in islets exposed throughout to 5.6 mM glucose and extracellular Ca^{2+}. When the perifusate was enriched with the hypoglycaemic sulphonylurea glibenclamide (10 μM), the capacity of HEI 713 (50 μM) to stimulate ^{86}Rb outflow was significantly reduced but not totally abolished (Figure 3, lower panel). Thus, the rise in ^{86}Rb FOR averaged $1.88\pm0.16\%$ min^{-1} ($n=4$) in the absence and $0.20\pm0.01\%$ min^{-1} ($n=5$) in the presence of glibenclamide throughout ($P<0.001$).

In the presence of 16.7 mM glucose and extracellular Ca^{2+} in the perifusing medium, the addition of HEI 713 (50 μM) elicited an immediate and sustained inhibition of both ^{45}Ca outflow and insulin release (Figure 4, upper and lower panel). During exposure to HEI 713 (60th–68th min), the ^{45}Ca outflow and the release of insulin was, respectively, $44.3\pm7.1\%$ ($n=6$, $P<0.001$) and $23.1\pm3.8\%$ ($n=6$, $P<0.001$) of that recorded before the administration of the drug (40th–44th min). The withdrawal of HEI 713 from the perifusate was accompanied by a rapid increase in both ^{45}Ca outflow and insulin release (Figure 4). The latter increases probably reflect relief from inhibitory effects of the drug.

The addition of HEI 713 (50 μM) to islets exposed to 16.7 mM glucose and Ca^{2+} deprived media did not provoke any reduction in ^{45}Ca FOR (data not shown).

To further investigate the effect of HEI 713 on ^{45}Ca outflow, additional experiments were performed at non-insulinotropic glucose concentration. In islets exposed throughout to 2.8 mM glucose, HEI 713 (50 μM) elicited a biphasic and sustained increase in the rate of ^{45}Ca outflow (Figure 5). This stimulatory effect of HEI 713 was reversible and slightly more marked in islets exposed to Ca^{2+} deprived

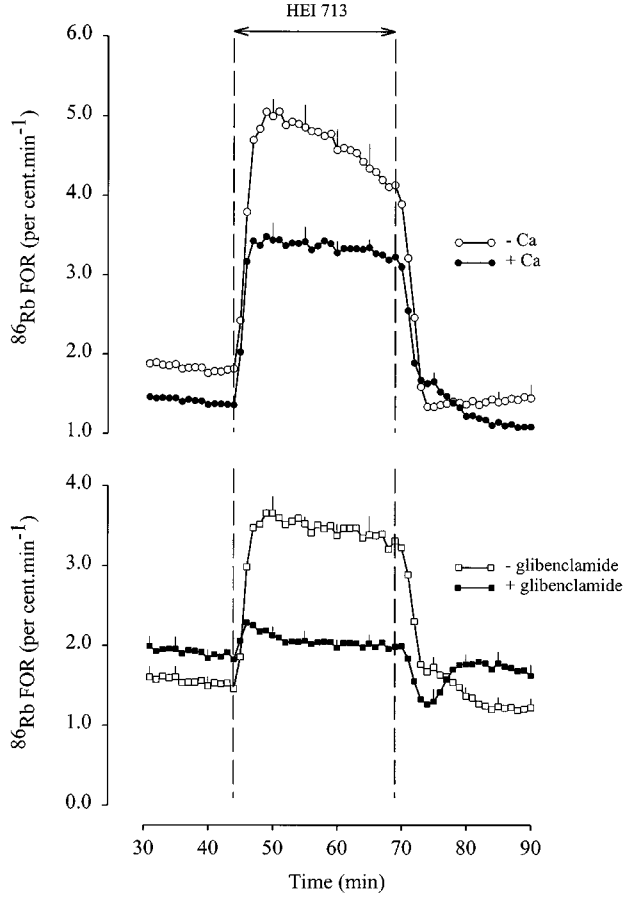


Figure 3 Upper panel: Effect of HEI 713 (50 μM) on ^{86}Rb outflow from pancreatic islets perifused throughout in the presence of 5.6 mM glucose. Basal media contained extracellular Ca^{2+} or were deprived of Ca^{2+} and enriched with EGTA. Lower panel: Effect of HEI 713 (50 μM) on ^{86}Rb outflow from pancreatic islets perifused throughout in the absence or presence of glibenclamide (10 μM). Basal media contained extracellular Ca^{2+} and 5.6 mM glucose. Mean values ($\pm$s.e.mean) refer to 4–5 individual experiments.

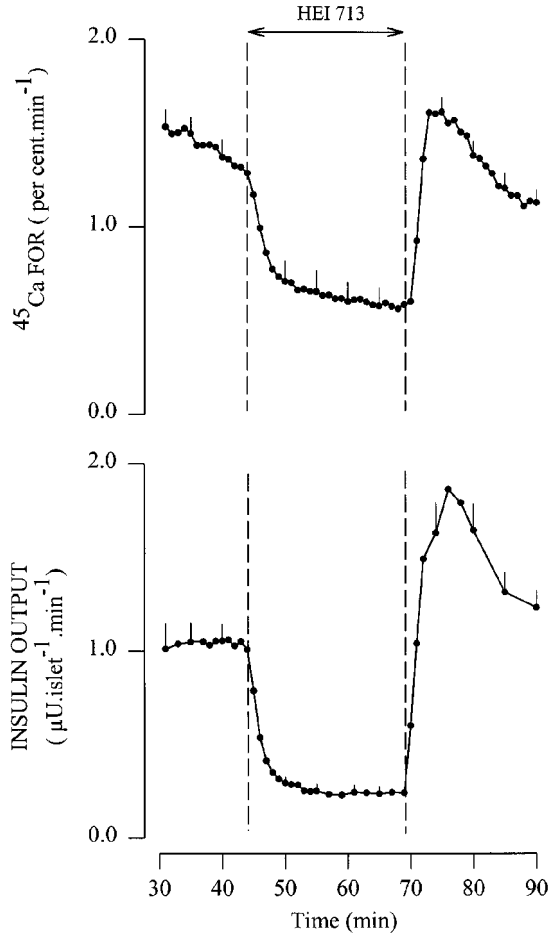


Figure 4 Effect of HEI 713 (50 μM) on ^{45}Ca outflow (upper panel) and insulin release (lower panel) from pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+}. Mean values ($\pm$s.e.mean) refer to six individual experiments.

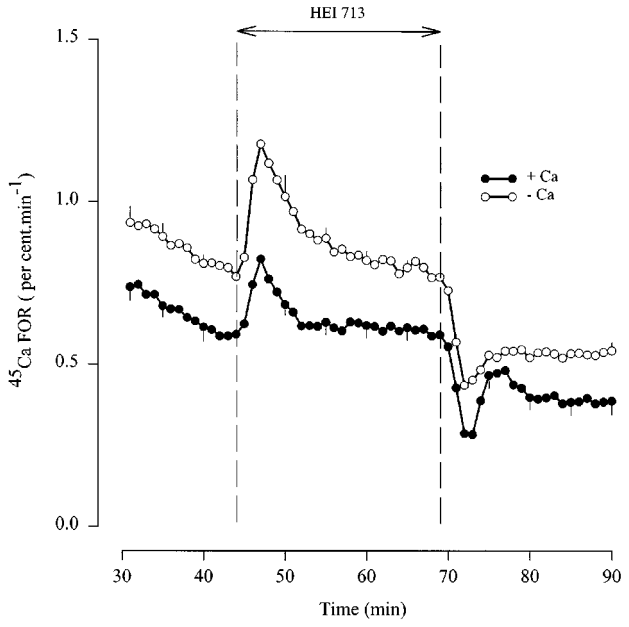


Figure 5 Effect of HEI 713 (50 μM) on ^{45}Ca outflow from pancreatic islets perifused throughout in the presence of 2.8 mM glucose. Basal media contained extracellular Ca^{2+} or were deprived of Ca^{2+} and enriched with EGTA. Mean values ($\pm$s.e.mean) refer to four individual experiments.

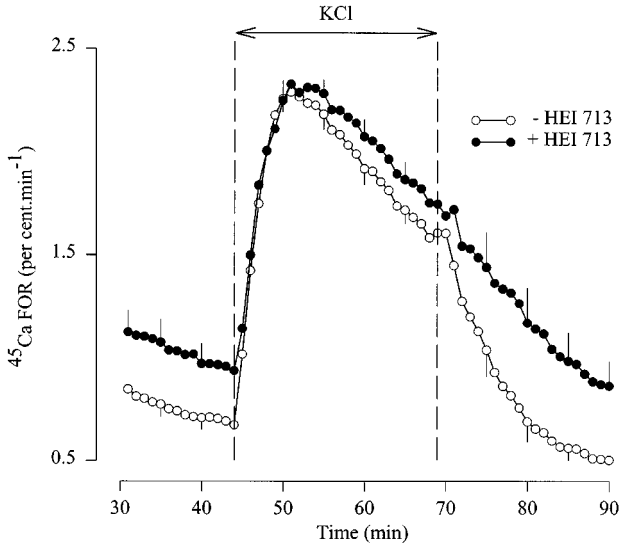


Figure 6 Effect of a rise in the extracellular concentration of K^+ from 5 to 50 mM on ^{45}Ca outflow from pancreatic islets perifused throughout in the absence or presence of HEI 713 (50 μM). Basal media contained 2.8 mM glucose and extracellular Ca^{2+}. Mean values ($\pm$s.e.mean) refer to six individual experiments.

media ($P>0.05$) (Figure 5). When HEI 713 (50 μM) was administered to islets perifused in the presence of 5.6 mM glucose and absence of extracellular Ca^{2+}, the drug again provoked a sustained and reversible rise in ^{45}Ca FOR (data not shown).

In the final series of experiments, we examined the effect of HEI 713 (50 μM) on the KCl-induced changes in ^{45}Ca outflow. A rise in the extracellular concentration of K^+ from 5–50 mM provoked a rapid and marked increase in ^{45}Ca FOR from pancreatic islets perifused in the presence of 2.8 mM glucose and extracellular Ca^{2+} (Figure 6). When the same experiments were repeated in the presence of HEI 713 (50 μM), the basal rate of ^{45}Ca outflow was higher ($P<0.05$). The presence of HEI 713 (50 μM) in the basal medium failed, however, to counteract the cationic response to high extracellular K^+ concentration (Figure 6).

Effect of HEI 713 on individual K_{ATP} channels

Spontaneous K_{ATP} channel activity recorded in the inside-out patch-clamp configuration was reduced by the addition of ATP (100 μM) in the bathing medium (data not shown). In the absence and presence of ATP (100 μM), calculated N.Po values were 2.2 and 1.1, respectively. In the continued presence of ATP, addition of HEI 713 (25 μM) reversed the effect of the nucleotide (N.Po = 4.3, data not shown).

Figure 7 illustrates the effect of HEI 713 and diazoxide on K_{ATP} channel activity. In the presence of ATP (500 μM) on the inside face of the β-cell membrane (N.Po = 0.46 ± 0.29), both HEI 713 (200 μM) and diazoxide (200 μM) provoked a marked increase in K_{ATP} channel openings. A quantitative analysis revealed that, under these experimental conditions, N.Po averaged 1.99 ± 0.26 after addition of HEI 713 (200 μM) and 3.12 ± 0.51 after the addition of diazoxide (200 μM) ($P>0.05$) (Figure 7b).

The role of SUR1 and Kir6.2 in mediating the actions of HEI 713 on K_{ATP} channel was examined using the human β-cell line, NES2Y. These cells lack K_{ATP} channels due to defects in SUR1 (MacFarlane *et al.*, 2000) and were transfected with Kir6.2CΔ26 cDNA; which produces Kir6.2 channel activity independently of SUR1 function (Tucker *et al.*, 1997). In these cells, ATP (500 μM) inhibited Kir6.2 channel activity ($n=7$) whereas the further addition of diazoxide (200 μM) was without effect ($n=6$). Furthermore, addition of HEI 713 (200 μM) in the absence ($n=7$) or presence ($n=5$) of ATP (500 μM), also failed to activate Kir6.2 channel activity (data not shown).

Effect of HEI 713 on the cytosolic free Ca^{2+} concentration of single rat pancreatic islet cells

The addition of HEI 713 (50 μM) to a physiological medium containing 2.8 mM glucose and extracellular Ca^{2+} provoked a small but sustained increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Figure 8, upper panel). Similar experiments were performed in the absence of extracellular Ca^{2+}. Under this experimental condition, HEI 713 (50 μM) still increased the $[Ca^{2+}]_i$ (data not shown). This enhancing effect of HEI 713 on $[Ca^{2+}]_i$ was slowly reversible on removal of the drug (data not shown).

A rise in the extracellular glucose concentration from 2.8 to 20.0 mM provoked, after a small initial decrease, a pronounced biphasic increase in $[Ca^{2+}]_i$ (Figure 8, lower panel). The subsequent addition of HEI 713 (50 μM) reduced the glucose-induced rise in the cytosolic free Ca^{2+} concentration (Figure 8, lower panel). The inhibitory effect of the drug was sustained and rapidly reversible.

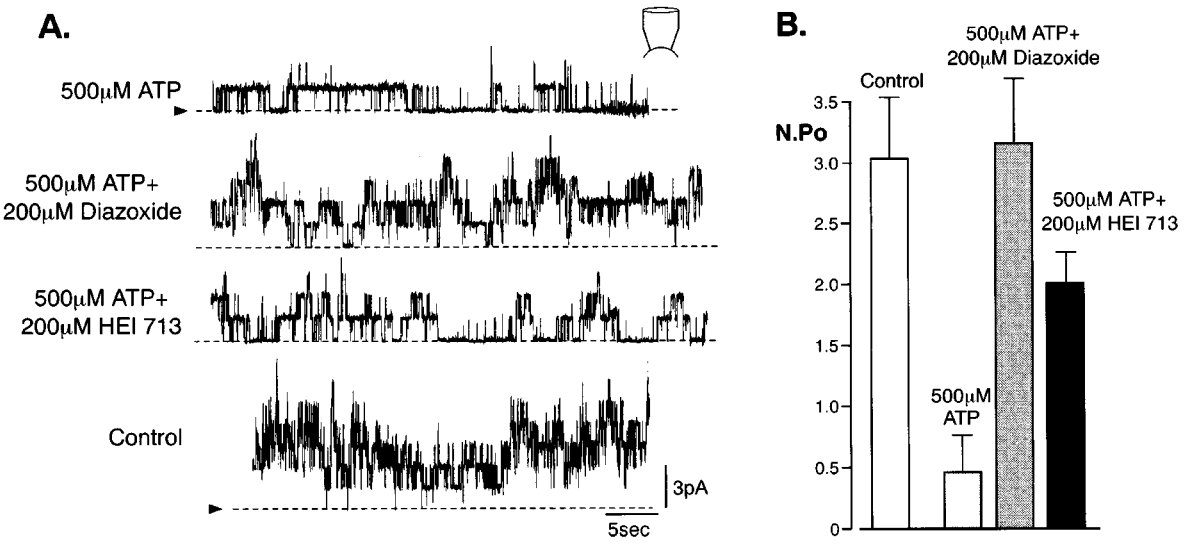


Figure 7 Effect of HEI 713 and diazoxide on K_{ATP} channels channels. (A upper and lower panels): Channel activity in the presence and in the absence of ATP in the bathing medium. Middle panels: Effects of diazoxide or HEI 713 in the continuous presence of ATP. Data were obtained from the same inside-out patch of membrane with upward deflections from the base line (dotted lines) representing outward currents. (B) Quantitative analysis of the effects of diazoxide and HEI 713 on K_{ATP} channel activity. Mean values (±s.e.mean) refer to four individual experiments.

Effect of HEI 712 and HEI 713 on the contractile activity of rat aortic rings

In rat aortic rings exposed to 30 mM K^+, the cumulative application of HEI 713 or diazoxide (10^{-7}–3.10^{-4} M) induced concentration-dependent relaxations. According to the ED_{50} values (Table 1), HEI 713 was roughly half as potent than diazoxide at reducing the vascular tone. By comparison, HEI 712, the 6-fluoroquinolinonic compound, was poorly active on aortic muscle tension (Table 1).

Figure 9 clearly indicates that the vasorelaxant effect of HEI 713 on K^+(30 mM)-induced contractile activity was counteracted by glibenclamide. ED_{50} amounted to 42.1±2.5 μM ($n=6$) in the absence, 111.2±17.3 μM ($n=5$) ($P<0.01$) in the presence of 1 μM and 164.6±32.3 μM ($n=4$) ($P<0.01$) in the presence of 10 μM glibenclamide in the physiological medium. The presence of glibenclamide (10^{-6}, 10^{-5} M) in the medium did not affect the baseline tension or the contractile responses to KCl (30 mM).

In the next series of experiments, contractile activity was elicited by high concentrations of extracellular K^+ (80 mM). Under such experimental conditions, the vasorelaxant properties of HEI 713 were markedly reduced. The ED_{50} value for HEI 713 on 80 mM K^+-induced contraction averaged 160.7±24.5 μM ($n=5$).

Effect of HEI 713 on ^{86}Rb outflow from rat aortic rings perifused in the presence and absence of glibenclamide

HEI 713 (100 μM) provoked a rapid, sustained and rapidly reversible increase in the rate of ^{86}Rb outflow from prelabelled and perifused rat aortic rings (Figure 10). The capacity of HEI 713 to stimulate ^{86}Rb outflow was almost completely abolished when glibenclamide (10 μM) was added to the perifusate (Figure 10). Indeed, the increment in ^{86}Rb FOR averaged 1.29±0.08% min^{-1} ($n=6$) in the absence and

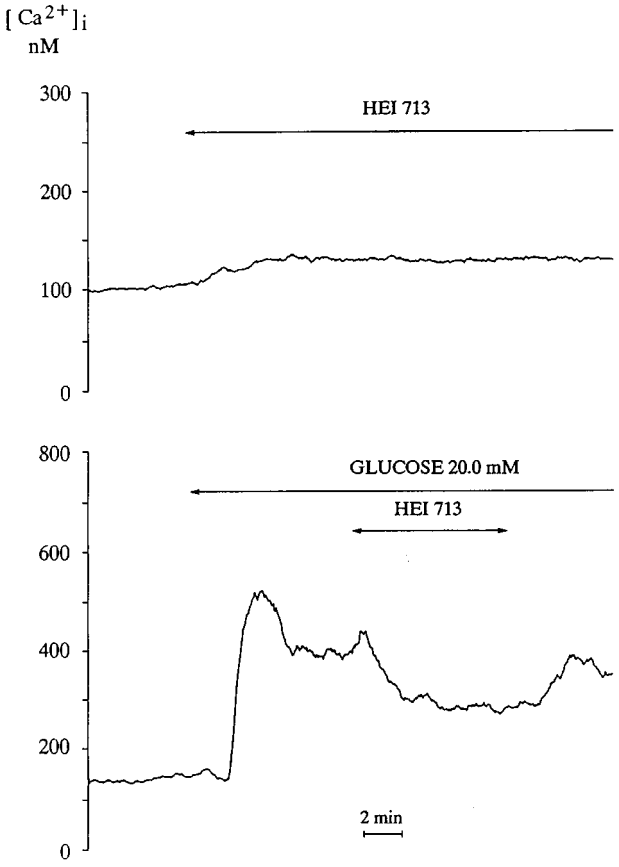


Figure 8 (Upper panel) Effect of HEI 713 (50 μM) on the $[Ca^{2+}]_i$ of a single pancreatic islet cell perifused in the presence of 2.8 mM glucose. (Lower panel) Effect of HEI 713 (50 μM) on glucose-induced increase in $[Ca^{2+}]_i$. Basal media contained extracellular Ca^{2+}. Each graph is a representative experiment conducted on a single cell.

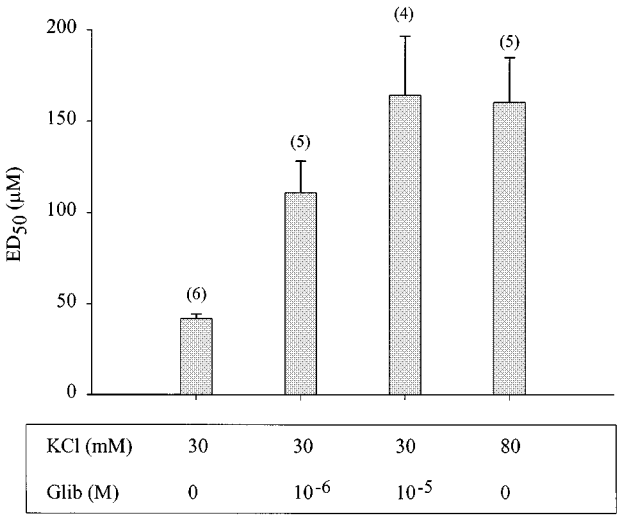


Figure 9 Effect of HEI 713 on the mechanical activity of rat aortic rings. Concentration-response curves were carried out after the addition of 30 or 80 mM K^+ and in absence or presence of glibenclamide (1 and 10 μM). ED_{50} is the HEI 713 concentration eliciting 50% relaxation of the KCl-induced concentration. The upper edges of bars represent the means whilst the line at the top of bars corresponds to the s.e.mean. Figures in parentheses are numbers of individual experiments.

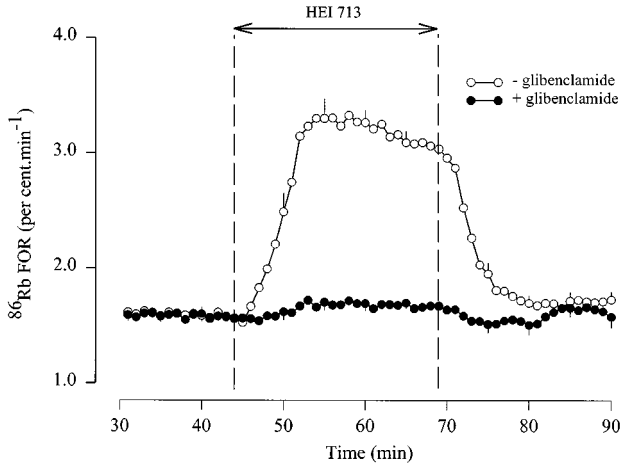


Figure 10 Effect of HEI 713 (100 μM) on ^{86}Rb outflow from aortic rings perifused throughout in the absence or presence of glibenclamide (10 μM). Basal media contained 30 mM K^+ and extracellular Ca^{2+}. Mean values (±s.e.mean) refer to six individual experiments.

$0.07 \pm 0.02\%$ min^{-1} ($n = 6$) in the presence of glibenclamide in the basal medium ($P < 0.001$).

Lipophilicity of diazoxide, HEI 712 and HEI 713

In order to compare the lipophilicity of diazoxide with that of the two quinolinonic compounds, we determined the log P′ value (logarithm of the partition coefficient between 1-octanol and a phosphate buffer at pH 7.40) of each drug by means of the shake-flask method (Cloux *et al*., 1988) (Table 1). The log P′ value for diazoxide and HEI 712 was found to be 1.21 and 1.29, respectively. We were unable to establish with an optimal precision the log P′ value of HEI 713, but a log P′ value higher than 1.5 can be predicted. Since the replacement of a fluorine atom on an aromatic ring by a chlorine atom should lead to an increased log P′ value of 0.5–0.6 units (based on the respective Hansch π values of the two substituents) (Hansch *et al*., 1973), a theoretical log P′ value around 1.8 might be attributed to HEI 713.

Discussion

The compound HEI 713 is structurally related to diazoxide but with the SO_2-N = moiety isosterically replaced by a CO-CH = fragment (Figure 1). It is now well established that the preferential conformation adopted by diazoxide in the solid state (Bandoli & Nicolini, 1977) as well as in solution (Jakobsen & Treppendahl, 1979) is the 4*H*-tautomeric form (Figure 1). Since HEI 713 may be viewed as an isosteric analogue of diazoxide, the drug should preferentially adopt the quinolin-4(1*H*)-one rather than the 4-hydroxyquinoline tautomeric conformation in solution (Katritzky, 1985).

The presence of a N-H group in the 4-position of diazoxide was found to be critical for biological activity and optimal drug-receptor interactions (de Tullio *et al*., 1996). Thus, the quinolin-4(1*H*)-one form of HEI 713 and its fluorinated analogue HEI 712, but not the 4-hydroxyquinoline form of the two drugs, can also be expected to represent the preferred conformation for optimal binding site interactions.

The biological activity of both quinolinonic compounds was tested on insulin secreting cells and on vascular smooth muscle cells. HEI 712 and HEI 713 affected the pancreatic and the vascular tissues but the fluorinated derivative (HEI 712) was much less potent than the chlorinated derivative (HEI 713) on both insulin release from pancreatic islets and smooth muscle contractile activity from aortic rings. Such a feature could be explained, at least in part, by the fact that the presence of a fluorine atom, rather than a chlorine atom, at the 6-position should have a negative impact on the electronic distribution and on lipophilicity. A chlorine atom at the 6-position could allow a better interaction of the molecule with a putative hydrophobic pocket located at the binding site.

HEI 713 was as potent as diazoxide at inhibiting insulin release from rat pancreatic islets but less potent than diazoxide at evoking vasodilator activity. According to the ED_{50} (contractile activity in aortic rings)/IC_{50} (insulin release from pancreatic islets) ratio, the quinolinone derivative appeared to be 2 fold more selective for the insulin secreting cells than diazoxide; the latter compound exhibiting no apparent selectivity.

Additional experiments suggest that the mechanism of HEI 713-induced inhibition of insulin release may be the result of changes in transmembrane ionic permeability. HEI 713 was shown to provoke a fast, sustained and rapidly reversible rise in ^{86}Rb outflow (^{42}K substitute) from prelabelled and perifused rat pancreatic islets. This finding can be interpreted as the result of an increase in membrane K^+ permeability (Malaisse *et al*., 1978; Henquin *et al*., 1992; Lebrun *et al*., 1992; 1996).

Our observations suggest that the stimulatory effect of HEI 713 on ^{86}Rb outflow could reflect the activation of ATP-sensitive K^+ (K_{ATP}) channels, since the ionic response of the pancreatic islets was sensitive to the hypoglycaemic sulpho-

nylurea glibenclamide, a K_{ATP} channel blocker (Ashcroft & Rorsman, 1989; Malaisse & Lebrun, 1990). When glibenclamide was added to the basal medium, the capacity of HEI 713 to stimulate ^{86}Rb outflow was strongly reduced. In addition, the absence of extracellular Ca^{2+} failed to reduce the stimulatory effect of HEI 713 on ^{86}Rb FOR. This observation implies that the drug mainly acts upon a Ca^{2+}-insensitive modality of ^{86}Rb extrusion (Lebrun *et al*., 1992; Antoine *et al*., 1993). Further evidence supporting an effect of HEI 713 on K_{ATP} channels comes from patch-clamp recordings. In the inside-out patch configuration, the compound was shown to increase the open state probability of K_{ATP} channels. Under identical experimental conditions, the K_{ATP} channel opener diazoxide also increased channel activity. Furthermore, these studies indicate that SUR1 mediates the effect of both the quinolinone derivative and diazoxide. In cells lacking SUR1 but expressing the ATP-sensitive Kir6.2CΔ26 channels, both HEI 713 and diazoxide failed to modify the channel open state probability.

Opening of K^+ channels would hyperpolarize the β-cell membrane and, in turn, inhibit the voltage-sensitive Ca^{2+} channels, reduce the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and, ultimately, impair the insulin secretory process. In agreement with this physiological sequence, HEI 713 was shown to inhibit both ^{45}Ca outflow and insulin release from pancreatic islets exposed to a medium containing 16.7 mM glucose and extracellular Ca^{2+}. This inhibitory effect of HEI 713 on ^{45}Ca outflow can be interpreted as the result of a reduction in $^{40}Ca^{2+}$ entry into the islets cells (Lebrun *et al*., 1982; 1996; Antoine *et al*., 1993). In agreement with this, the decrease in ^{45}Ca outflow mediated by the quinolinonic compound did not occur when the islets were perifused in the absence of extracellular Ca^{2+}. Furthermore, calcium fluorimetry experiments revealed that the drug was able to counteract the glucose-induced increase in $[Ca^{2+}]_i$. In summary, these data suggest that the inhibitory effect of HEI 713 on insulin release results from K_{ATP} channel activation, leading to a decrease in Ca^{2+} entry and $[Ca^{2+}]_i$. The failure of HEI 713 to counteract the increase in ^{45}Ca outflow provoked by a rise in the extracellular K^+ concentration further indicates that the primary action of the drug is to raise the K^+ permeability of the pancreatic β-cells. Indeed, the ^{45}Ca response to a high K^+ concentration, which is mediated by the opening of voltage-sensitive Ca^{2+} channels and inhibited by drugs acting at the Ca^{2+} channel level, is known to be unaffected by K^+ channel openers (Lebrun *et al*., 1982; 1989; 2000; Henquin *et al*., 1992).

Several observations also indicate that the vasorelaxant effect of HEI 713 on rat aortic rings mainly results from the activation of K_{ATP} channels.

The effect of HEI 713 on K^+ (30 mM)-induced contraction was sensitive to glibenclamide. Previous studies have revealed the ability of glibenclamide to reduce the myorelaxant effect of K_{ATP} channel openers (Quast & Baumlin, 1988; Lebrun *et al*., 1990; Antoine *et al*., 1992). Secondly, HEI 713 increased ^{86}Rb outflow from prelabelled and perifused rat aortic rings; suggesting that the drug provoked a rise in the membrane K^+ permeability (Quast & Baumlin, 1988, Lebrun *et al*., 1990). The capacity of HEI 713 to stimulate ^{86}Rb outflow was reduced when glibenclamide was added to the perifusate. Finally, the myorelaxant effect of HEI 713 was markedly decreased in aortic rings exposed to K^+ 80 mM. In vascular smooth muscle cells, like in a variety of other cell types, K_{ATP} channel openers are capable of inhibiting the contractile activity evoked by a 'low' but not a 'high' extracellular K^+ concentration (Lebrun *et al*., 1990; Edwards & Weston, 1993; Magnon *et al*., 1998). Taken as a whole, these data suggest that HEI 713 also activates K_{ATP} channels in vascular smooth muscle. Although HEI 713 shares vasodilator properties with diazoxide, the quinolinone derivative was significantly less potent than diazoxide in relaxing vascular smooth muscle. Incidentally, the remaining slight vasorelaxant effect of HEI 713 on aortic rings exposed to K^+ 80 mM could suggest the involvement of additional mechanism(s) not linked to K^+ channel opening (Quast, 1993).

In addition to effects on the plasma membrane K_{ATP} channels, HEI 713 also appears to interact, at least to a small extent, with an intracellular target. In both cell types, the compound elicited a glibenclamide-resistant modality of ^{86}Rb extrusion. Whether or not the physiological medium contained extracellular Ca^{2+}, the drug provoked an increase in ^{45}Ca FOR and $[Ca^{2+}]_i$ from pancreatic islets or islets cells perifused at non-insulinotropic glucose concentrations. These findings suggest that HEI 713 might promote an intracellular translocation of Ca^{2+} (Antoine *et al*., 1991) which, in turn, could activate a Ca^{2+}-sensitive and glibenclamide-resistant modality of ^{86}Rb outflow. The high lipophilicity of HEI 713 suggests that the compound could penetrate the plasma membrane and interfere with some intracellular binding sites such as the mitochondrial K_{ATP} channels. Indeed, previous data revealed that the activation of K_{ATP} channels located on the inner membrane of mitochondria elicited a mitochondrial Ca^{2+} release (Szewczyk & Marban, 1999).

In conclusion, we have designed an original quinolinone derivative which activates plasma membrane K_{ATP} channels. The compound (HEI 713) inhibits glucose-induced insulin release and exhibits some vasorelaxant properties. However, compared with diazoxide, which is devoid of tissue selectivity (pancreatic islets vs aortic rings), HEI 713 was slightly more potent on the endocrine than on the vascular tissue.

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