A potent diazoxide analogue activating ATP-sensitive K⁺ channels and inhibiting insulin release

P. Lebrun¹, P. Arkhammar², M.-H. Antoine¹, Q.-A. Nguyen¹, J. Bondo Hansen³, B. Pirotte⁴

¹ Laboratory of Pharmacology, Faculty of Medicine, Free University of Brussels, Brussels, Belgium

² Bioimage Novo Nordisk, Soborg, Denmark

³ Health Care Discovery, Novo Nordisk, Malov, Denmark

⁴ Department of Medicinal Chemistry, Institute of Pharmacy, University of Liège, Liège, Belgium

Abstract

Aims/hypothesis. To characterise the effects of BPDZ 73 (7-chloro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide), a newly synthesised diazoxide analogue, on insulin secretory cells.

Methods. Measurements of ⁸⁶Rb, ⁴⁵Ca outflow, membrane potential, [Ca²⁺]_i, insulin release in secretory cells as well as measurements of smooth muscle contractile activity and glycaemia were carried out.

Results. The analogue BPDZ 73 induced a dose-dependent decrease in insulin output. The IC₅₀ value averaged 0.73 ± 0.05 µmol/l. The drug increased the rate of ⁸⁶Rb (⁴²K substitute) outflow from perfused rat pancreatic islets. This effect was inhibited by glibenclamide, a K_{ATP} channel blocker. Measurements of DiBAC₄(3) fluorescence further indicated that BPDZ 73 hyperpolarised the insulin secreting cells. It also decreased ⁴⁵Ca outflow from pancreatic islets perfused throughout in the presence of 16.7 mmol/l

glucose and extracellular Ca²⁺. By contrast, the drug did not affect the increase in ⁴⁵Ca outflow mediated by K⁺ depolarisation. In single beta cells, BPDZ 73 inhibited the glucose-induced but not the K⁺-induced rise in [Ca²⁺]_i. Moreover, in Wistar rats, i.p. injection of BPDZ 73 provoked a considerable increase in blood glucose concentration whereas diazoxide induced a modest rise in glycaemia. Lastly, the vasorelaxant properties of BPDZ 73 were slightly less pronounced than those of diazoxide.

Conclusion/interpretation. The inhibitory effect of BPDZ 73 on the insulin-releasing process results from the activation of K_{ATP} channels with subsequent decrease in Ca²⁺ inflow and [Ca²⁺]_i. The drug seems to be a K_{ATP} channel opener, more potent and more selective than diazoxide for insulin secreting cells. [Diabetologia (2000) 43: 723–732]

Keywords Diazoxide, ATP sensitive K⁺ channel, rat pancreatic islets, insulin release.

Diazoxide, a drug chemically related to the thiazide diuretics but devoid of diuretic activity, was first marketed more than 25 years ago for parenteral therapy of hypertensive emergencies [1]. The compound, which inhibits insulin secretion, is also used to treat

various forms of hypoglycaemia [2]. More recently, treatment with diazoxide has been shown to prevent Type I (insulin-dependent) diabetes mellitus in young diabetes-susceptible *BB* rats [3]. Diazoxide has also been shown to improve insulin sensitivity, glucose tolerance and to decrease weight gain in obese Zucker rats [4]. Moreover, the drug was reported to prevent the development of obesity and insulin resistance in a rat model of naturally occurring obese diabetes [5]. Clinical studies further showed that short-term diazoxide treatment improved insulin secretion in diabetic subjects [6], preserved endogenous insulin production in patients with newly manifested autoimmune diabetes [7] and reduced body fat in hyperinsulinaemic obese adults [8]. Thus, all these investigations

Received: 25 November 1999 and in revised form: 21 February 2000

Corresponding author: P. Lebrun, Laboratory of Pharmacology, Faculty of Medicine (CP 617), Université Libre de Bruxelles, Route de Lennik 808, B-1070 Brussels, Belgium

Abbreviations: FOR, Fractional outflow rate; K_{ATP} channels, ATP-sensitive K⁺ channels; BPDZ 73, 7-chloro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide; ED₅₀, efficacy dose 50 %.

Fig. 1. Chemical structure of diazoxide and BPDZ 73

suggest that modifications in endogenous insulin secretion rate, as induced by diazoxide, can alleviate or prevent several diseased states involving altered endocrine pancreatic function.

A pharmacological route to interfere with the insulin secretory process is to develop compounds acting at the level of the ATP-sensitive K^+ (K_{ATP}) channel. These channels, heteromultimers composed of sulphonylurea receptor (SUR1) and Kir6.2 subunits [9], equip the beta-cell plasma membrane and couple the cellular metabolism to electrical activity. The closing of beta-cell K_{ATP} channels initiates membrane depolarisation and thereby eventually leads to exocytosis of secretory granules whereas the activation of K_{ATP} channels promotes membrane hyperpolarisation and decreases insulin release [10, 11].

According to their tissue localisation and their SUR subunit type, the sensitivity of K_{ATP} channels to pharmacological agents vary widely [9, 11, 12]. In the search for new K_{ATP} channel modulators with fewer side effects than diazoxide [1, 7], we recently synthesised 7-chloro-3-isopropylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (BPDZ 73). This compound corresponds to a diazoxide analogue having the methyl side chain in the 3-position of the heterocycle replaced by an isopropylamino group (Fig. 1). The present study aims at evaluating the *in vivo* and *in vitro* effect of BPDZ 73. Because the drug was found to be much more potent than diazoxide at increasing the blood glucose concentration and at reducing the glucose-induced insulin release from rat pancreatic islets, additional experiments were conducted to characterise the selectivity and the mechanism of action of BPDZ 73.

Materials and methods

Measurements of insulin release from incubated pancreatic islets. Experiments were done with pancreatic islets isolated by the collagenase method from fed albino rats. The laboratory animal care was approved by the ethics committee of the FREE University of Brussels.

Groups of ten islets, each derived from the same batch of islets, were preincubated for 30 min at 37 °C in 1 ml of a bicarbonate-buffered solution (in mmol/l: 115 NaCl, 5 KCl, 2.56 CaCl₂, 1 MgCl₂, 24 NaHCO₃) supplemented with 2.8 mmol/l

glucose, 0.5 % (w/v) dialysed albumin (fraction V) and equilibrated with a mixture of O₂ (95 %) and CO₂ (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 mmol/l glucose and, in addition, increasing concentrations of BPDZ 73. Individual experiments were carried out as quadruplicate measurements with the values corresponding to the number of samples pooled. Due to the islet-related variations, insulin release was expressed as a percentage of the value recorded in control experiments (100 %), i.e. in the absence of drug and presence of 16.7 mmol/l glucose. The release of insulin was measured radioimmunologically as reported previously [13].

Measurements of ⁸⁶Rb, ⁴⁵Ca outflow and insulin release from perfused pancreatic islets. Experiments were done with pancreatic islets isolated by the collagenase method from fed albino rats.

The methods used to measure ⁸⁶Rb (⁴²K substitute) outflow, ⁴⁵Ca outflow and insulin release from perfused islets have been described previously [14–17]. Groups of 100 islets were incubated for 60 min in a bicarbonate-buffered medium (in mmol/l: NaCl 115, KCl 5, CaCl₂ 2.56, MgCl₂ 1, NaHCO₃ 24) containing 16.7 mmol/l glucose and either ⁸⁶Rb ion (0.15–0.25 mmol/l; 1.85 10⁶ Bq/ml) or ⁴⁵Ca ion (0.02–0.04 mmol/l; 3.70 10⁶ Bq/ml). After incubation, the islets were washed four times with a non-radioactive medium and then placed in a perfusion chamber. The perfusate was delivered at a constant rate (1.0 ml/min). From the 31st to the 90th min of perfusion, 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 (last tube never below 250 cpm) and the remainder was stored at –20 °C for insulin radioimmunoassay [13]. At the end of the perfusion, the radioactive content of the islets was also determined. The outflow of ⁸⁶Rb or ⁴⁵Ca ion (cpm/min) was expressed as a fractional outflow rate (% of instantaneous islet content/min; FOR). The validity of ⁸⁶Rb as a tracer for the study of K^+ handling in pancreatic islets has been assessed previously [18].

Measurements of DiBAC₄(3) fluorescence from β -TC3 cells. Insulin-producing β -TC3 cells, a glucose-responsive beta-cell line [19], were cultured to about 80 % confluency in black-walled microtitre plates [96-well format, tissue treated, View-Plate (Packard, Meriden, Conn., USA)]. The β -TC3 cells were maintained in DMEM containing, in addition, glutamax-1 (L-alanyl-L-glutamine 0.862 mg/ml), sodium pyruvate (110 mg/ml), pyridoxine (4 mg/ml), glucose (25 mmol/l), non-essential amino acids, fetal calf serum (15 %), penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Life Technologies, Merelbeke, Belgium) [19]. After washing and pre-incubation at 37 °C in a modified Krebs-Ringer-HEPES buffer (in mmol/l: NaCl 140, KCl 3.6, NaH₂PO₄ 0.5, MgSO₄ 0.5, NaHCO₃ 2.0, CaCl₂ 1.5, D-glucose 10 and HEPES 10; pH 7.4), the cells were incubated in the same buffer with the addition of 4 μ mol/l of the membrane-potential sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) [20] (Molecular Probes, Eugene, Ore., USA). The plates were then placed on a programmable stage fitted to a Nikon TMD microscope equipped with a 20 $\times$ NA 0.5 Fluor objective. The cells in the microtitre plate were first allowed to equilibrate with the added probe. Thereafter, the cells in the field of view from one well in the plate were excited at 470 $\pm$ 6 nm at 1 Hz for 20 s. During this time, a sample of BPDZ 73 or diazoxide was added to the well as a 2 $\times$ concentrated solution in buffer with DiBAC₄(3). Emitted fluorescence from the cells in the well was measured and averaged for the time 3 to 5 s immediately after addition. By an automated procedure, the fluorescence from the same

field of cells in the well was again measured 45 min later. At this time, no addition was made but the average signal during 3 s of the total 20 s recording time was extracted. This procedure was repeated for each of the 96 wells of the microtitre plate in succession. For each compound and concentration, a total of six to eight individual wells were used to construct the data sets (Fig. 4). Total time for the measurement of one plate was 90 min but each well was incubated with compound for 45 min. We used Microsoft Excel and MacCurveFit 1.4 (Micro-soft, Redmond, Wash., USA) to process the data which are expressed as the change in DiBAC₄(3) fluorescence during the 45 min of incubation. Because the number of cells in the field of view in the microscope varied from well to well by a factor of 2–3, the changes in fluorescence were normalised individually to the initial reading for each well.

Measurements of fura-2 fluorescence from single rat pancreatic islet cells. Pancreatic islets were disrupted in a Ca²⁺-deprived medium and then centrifuged through an albumin solution 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) supplemented with 10% (v/v) newborn calf serum and containing glutamine (2.3 mmol/l), glucose (16.7 mmol/l), penicillin G (100 U/ml) and streptomycin (100 µg/ml). The cells were then incubated with fura-2 AM (2 µmol/l) (Molecular Probes) for 1 h and, after washing, 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 perfuse the cells contained (in mmol/l) NaCl 115, KCl 5, CaCl₂ 2.56, MgCl₂ 1, NaHCO₃ 24, glucose 2.8 and was gassed with O₂ (95%):CO₂ (5%). Fura-2 fluorescence of single-loaded cells was measured by use of dual-excitation microfluorimetry with a Spex photometric system (Optilas, Alphen aan den Rijn, Holland). The excitation wavelengths (340 nm and 380 nm) were alternated at the frequency of 1 Hz. The emission wavelength was set at 510 nm. We calculated [Ca²⁺]_i as described previously [17]. Individual experiments were repeated at least four times, on different cell populations.

Measurements of mechanical activity of rat aortic rings. Experiments were conducted as described previously [21]. Aortas were removed from fed albino rats and cut into transverse rings (3–4 mmol/l long). The endothelium was removed by rubbing the intimal surface with forceps. The segments were suspended under 1-g tension by means of steel hooks in an organ bath containing a bicarbonate-buffered solution of the following composition (in mmol/l): NaCl 118, KCl 4.7, CaCl₂ 2.5, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 5. The solutions were maintained at 37°C and bubbled continuously with a mixture of O₂ (95%) and CO₂ (5%). The isometric contractions were measured with a grass-force displacement transducer. After 60 min of equilibration, the rings were exposed to 30 mmol/l KCl. When the tension had stabilised, increasing concentrations of either diazoxide or BPDZ 73 were added to the organ bath until maximum relaxation.

Measurements of glycaemia. Adult albino rats weighing 300–350 g were allowed to settle in for at least 3 days in the laboratory before use. They had free access to water and received a standard pellet diet. Conscious rats were placed for 60 min before blood sampling and throughout the duration of the study in a small cabinet. Drugs were dissolved in a sterile physiological sodium salt solution (NaCl 0.9%) supplemented with NaOH. At zero time, 20 mg/kg of diazoxide or BPDZ 73 were injected intraperitoneally (i.p.). Control animals received an equivalent volume of NaCl 0.9% supplemented with NaOH;

5 ml · kg⁻¹ body weight. Blood was taken from the tail at time –5 min, 30 min, 60 min, 90 min, 120 min and 180 min. Blood glucose concentration was measured, in duplicate, using a reagent strip in combination with a glucometer (Accutrend, Boehringer-Mannheim, Mannheim, Germany).

Drugs. In some experiments, extracellular Ca²⁺ was eliminated by the omission of CaCl₂ from the physiological medium and the addition of 0.5 mmol/l ethylene glycol-bis (β-aminoethyl ether)N,N'-tetra-acetic acid (EGTA; Sigma-Aldrich, Bornem, Belgium). According to the experiments, the media were enriched with glucose (Merck, Darmstadt, Germany), glibenclamide (Hoechst Roussel, Brussels, Belgium), diazoxide (Sigma-Aldrich), verapamil hydrochloride (Knoll, Ludwigshafen, Germany) or BPDZ 73.

The BPDZ 73 was synthesised at the Department of Medicinal Chemistry, University of Liège, Belgium. The IR spectrum was recorded as a KBr pellet on a Perkin-Elmer 1750FT-spectrophotometer (Perkin Elmer, Beaconfield, UK). The proton NMR spectrum was taken on a Bruker AW-80 (80 MHz) instrument (Bruker, Karlsruhe, Germany). The compound was obtained as described previously for structural analogues [22, 23] using 7-chloro-3-methylsulphonyl-4H-1,2,4-benzothiadiazine 1,1-dioxide [24] and isopropylamine as starting materials; m.p. 267–269°C (litt.: 264–267°C [25]); IR (KBr): 3292 (N-H), 3173, 3076 (C-H arom.), 2990, 2975 (C-H aliph.), 1634, 1569, 1481 (C = C, N-H), 1241, 1177, 1151, 1120 (S = O); NMR (DMSO-d₆, HMDS, δ ppm): 1.10 (d, 6H, CH(CH₃)₂), 3.85 (m, 1H, CH(CH₃)₂), 6.95 (b, 1H, N-H exocyclic), 7.10 (d, 1H, 5-H), 7.50 (d, 1H, 6-H), 7.55 (s, 1H, 8-H), 10.35 (bs, 1H, N-H intracyclic).

For the in vitro experiments, glibenclamide, diazoxide and BPDZ 73 were dissolved in dimethylsulphoxide which was added to both control and test media. At the final concentrations used, dimethylsulphoxide fails to affect islet function and smooth muscle contractility [17, 21, 26]. When high concentrations of extracellular K⁺ were used, the concentration of extracellular NaCl was lowered to keep osmolarity constant.

Calculations. Results are expressed as the means (± SEM). The magnitude of the increase in ⁸⁶Rb and ⁴⁵Ca outflow was estimated in each individual experiment from the integrated outflow of ⁸⁶Rb or ⁴⁵Ca observed during stimulation (45th–68th min) after correction for basal value (40th–44th min). The inhibitory effect of BPDZ 73 on ⁴⁵Ca outflow and insulin release was taken as the difference between the mean value for ⁴⁵Ca outflow or insulin output recorded in each individual experiment between the 40–44th and 60–68th min of perfusion. The IC₅₀ value for insulin release (concentration giving a 50% reduction of the secretory response to 16.7 mmol/l glucose) was assessed graphically. For the rat aortic rings, the relaxation responses were expressed as the percentage of the contractile responses to KCl. The efficacy dose 50% (ED₅₀) values were graphically assessed for each dose response curve as the concentration evoking 50% inhibition of the sustained contraction induced by KCl. The statistical significance of differences between mean data was assessed by use of Student's *t* test or by analysis of variance followed for multiple comparisons by a Scheffe test procedure. A *p* value less than 0.05 was considered significant.

Results

Chemical synthesis of BPDZ 73. The BPDZ 73 (Fig. 1) was obtained from the reaction of an excess of isopropylamine with 7-chloro-3-methylsulphonyl-

Fig. 2. Effect of increasing concentrations of BPDZ 73 on insulin secretion from pancreatic islets incubated in the presence of 16.7 mmol/l glucose. Insulin release (mean values $\pm$ SEM) was expressed as a per cent of the value recorded in control experiments (100%; no added drug and presence of 16.7 mmol/l glucose). The upper edges of the open bars represent the means and the cross-hatching at top of bars corresponds to the SE. Figures in parentheses refer to number of samples

4*H*-1,2,4-benzothiadiazine 1,1-dioxide, a useful synthetic intermediate in the access way to 3-alkylamino-substituted benzothiadiazine 1,1-dioxides [22, 23]. The synthesis of the 3-methylsulphanyl-substitut-

ed intermediate was done in three steps starting from 4-chloroaniline and following methods described previously [24, 27].

Effects of BPDZ 73 and diazoxide on insulin release from glucose-stimulated rat pancreatic islets. The addition of increasing concentrations of BPDZ 73 provoked a dose-dependent decrease in insulin output from rat pancreatic islets incubated in the presence of 16.7 mmol/l glucose (Fig. 2). After the addition of 0.1 μ mol/l, 1 μ mol/l and 10 μ mol/l BPDZ 73 to the incubation medium, the insulin release represented $90.4 \pm 3.5\%$ (NS); $36.2 \pm 2.4\%$ ($p < 0.05$); and $4.9 \pm 0.4\%$ ($p < 0.05$) of the control value, respectively. The IC_{50} value averaged 0.73 ± 0.05 μ mol/l.

Previous investigations indicated that, under the same experimental conditions, the IC_{50} value for the inhibitory effect of diazoxide on insulin release amounted to 24.8 ± 3.4 μ mol/l [17].

Effects of BPDZ 73 on ^{86}Rb outflow from perifused rat pancreatic islets. In the presence of 5.6 mmol/l glucose in the basal medium, the addition of BPDZ 73

Fig. 3. **A** Effect of 1 μ mol/l ($\circ$) and 10 μ mol/l ($\bullet$) BPDZ 73 on ^{86}Rb outflow from perifused pancreatic islets. **B** Effect of 50 μ mol/l BPDZ 73 on ^{86}Rb outflow from pancreatic islets perifused throughout in the absence ($\bullet$) or presence ($\circ$) of 10 μ mol/l glibenclamide. Basal media contained 5.6 mmol/l glucose and extracellular Ca^{2+} . Mean values ($\pm$ SEM) refer to 4–5 individual experiments

Fig. 4. Changes in DiBAC₄(3) fluorescence from β -TC3 cells, in the presence of 10 mmol/l D-glucose, during 45 min incubation with different concentrations of BPDZ 73 (●) and diazoxide (○). Each point is the average of measurements from six (BPDZ 73) or eight (diazoxide) individual wells. Because the number of cells contributing to the basal signal was different from well to well, the responses, seen as changes (reductions) in fluorescence, were normalised to the initial reading for each well and given as mean $\pm$ SEM. The smooth curves were computed using a four-parameter non-linear curve-fitting algorithm reduced to three parameters by fixing the Hill slope to -1.5 for both curves. During the fitting procedure, the data points were weighted inversely to their respective SEM values. The predicted bottom level for the diazoxide curve was -0.42 using this approach and seems to be reasonable when compared with the maximum effect of BPDZ 73. Measurements above $300 \mu\text{mol/l}$ diazoxide were avoided due to solubility problems with the compound

provoked a concentration-dependent increase in ^{86}Rb outflow from pre-labelled and perfused rat pancreatic islets (Fig. 3). The integrated outflow of ^{86}Rb measured during exposure to the drug averaged $0.10 \pm 0.05 \text{ \%}/\text{min}$ after addition of $1 \mu\text{mol/l}$; $1.42 \pm 0.13 \text{ \%}/\text{min}$ after addition of $10 \mu\text{mol/l}$ and $2.43 \pm 0.23 \text{ \%}/\text{min}$ after the addition of $50 \mu\text{mol/l}$ BPDZ 73, respectively ($p < 0.05$ in each case). The withdrawal of the drug from the perfusate was accompanied by a decrease in ^{86}Rb FOR.

When the physiological medium was enriched with the hypoglycaemic sulphonylurea glibenclamide ($10 \mu\text{mol/l}$); the stimulatory effect of $1 \mu\text{mol/l}$ (data not shown), $10 \mu\text{mol/l}$ (data not shown) and $50 \mu\text{mol/l}$ BPDZ 73 (Fig. 3) was completely abolished.

In islets exposed throughout to 16.7 mmol/l instead of 5.6 mmol/l glucose, $10 \mu\text{mol/l}$ BPDZ 73 again elicited a rapid, sustained and reversible increase in

the rate of ^{86}Rb outflow (data not shown). Under these experimental conditions the stimulatory effect of BPDZ 73 ($10 \mu\text{mol/l}$) only represented, however, $44.8 \pm 9.5 \text{ \%}$ of that recorded in the presence of 5.6 mmol/l glucose in the perfusate ($p < 0.05$).

Effects of BPDZ 73 and diazoxide on β -TC3 membrane potential. Qualitative changes in membrane potential were assessed in the insulin-producing cell line, β -TC3, by measuring DiBAC₄(3) fluorescence (Fig. 4). The addition of BPDZ 73 was found to provoke a concentration-dependent hyperpolarisation of the cells incubated in the presence of 10 mmol/l glucose.

Under the same experimental conditions, diazoxide also hyperpolarised the cells. Diazoxide was, however, clearly less potent than BPDZ 73 at increasing membrane potential. Thus, diazoxide had an EC_{50} value of $77.4 \pm 4.3 \mu\text{mol/l}$ and BPDZ 73 an EC_{50} value of $2.2 \pm 0.6 \mu\text{mol/l}$.

Additional experiments conducted on discrete mouse beta cells in the context of a whole pancreatic islet produced ED_{50} values for the two compounds in the same range as those found in β -TC3 cells. The ED_{50} value for BPDZ 73 was found to be about $1 \mu\text{mol/l}$ in four experiments and the value for diazoxide was about $90 \mu\text{mol/l}$ in two experiments (data not shown).

Effects of BPDZ 73 on ^{45}Ca outflow and insulin release from perfused rat pancreatic islets. When the physiological medium contained 16.7 mmol/l glucose and extracellular Ca^{2+} , the addition of BPDZ 73 provoked an immediate and pronounced inhibition of ^{45}Ca outflow (Fig. 5). The inhibitory effect of the drug was reversible and concentration-dependent. Thus, the paired difference in ^{45}Ca FOR before (min 40–44) and during (min 60–68) exposure to $1 \mu\text{mol/l}$ and $10 \mu\text{mol/l}$ BPDZ 73 averaged $0.68 \pm 0.07 \text{ \%}/\text{min}$ and $0.87 \pm 0.04 \text{ \%}/\text{min}$, respectively ($p < 0.05$). The withdrawal of the drug from the perfusing medium was accompanied by an increase in ^{45}Ca outflow. The latter increase was more rapid after the removal of the lower drug concentration (Fig. 5).

To further investigate the effects of BPDZ 73 on ^{45}Ca movements, similar experiments were done in the presence of 16.7 mmol/l glucose but absence of extracellular Ca^{2+} . In islets exposed to Ca^{2+} -depleted media, the basal rate of ^{45}Ca outflow (min 40–44) was lower ($0.69 \pm 0.02 \text{ \%}/\text{min}$, $n = 8$, $p < 0.05$) and the addition of either $1 \mu\text{mol/l}$ or $10 \mu\text{mol/l}$ BPDZ 73 did not affect ^{45}Ca FOR (data not shown). The addition of $1 \mu\text{mol/l}$ BPDZ 73 also failed to modify the rate of ^{45}Ca outflow from islets perfused in the presence of a Ca^{2+} -free solution containing 5.6 or 8.3 mmol/l glucose throughout ($n = 4$ in each case; data not shown).

Fig. 5 A, B. Effect of 1 $\mu\text{mol/l}$ (○) and 10 $\mu\text{mol/l}$ (●) BPDZ 73 on ^{45}Ca outflow (A) and insulin release (B) from pancreatic islets perfused throughout in the presence of 16.7 mmol/l glucose. Basal media contained extracellular Ca^{2+} . Mean values ($\pm$ SEM) refer to six individual experiments

Increasing concentrations of BPDZ 73 provoked an immediate, sustained and concentration-dependent inhibition of insulin output from islets exposed to 16.7 mmol/l glucose and extracellular Ca^{2+} (Fig. 5). During exposure to 1 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$ BPDZ 73 (min 60–68), the release of insulin represented, respectively, $40.43 \pm 1.19\%$ and $10.63 \pm 1.63\%$ of that recorded before treatment with the drug (min 40–44) ($p < 0.05$). The removal of 1 $\mu\text{mol/l}$ BPDZ 73 was followed by a rapid increase and the omission of 10 $\mu\text{mol/l}$ BPDZ 73 resulted in a delayed increase in insulin output (Fig. 5).

In another series of experiments, we characterised the effects of BPDZ 73 on the secretory response induced by a sudden increase in the extracellular glucose concentration. A rise in the glucose concentration from 5.6 to 16.7 mmol/l provoked a rapid and sustained insulin output (Fig. 6). When the same experiment was repeated in the presence of 1 $\mu\text{mol/l}$ BPDZ 73 throughout, the basal rate of insulin release (min 40–44) was unaffected ($p > 0.05$) but the secretagogue action of 16.7 mmol/l glucose was almost completely abolished (Fig. 6). The integrated output of insulin measured during exposure to 16.7 mmol/l

Fig. 6. Effect of a rise in the extracellular glucose concentration from 5.6 to 16.7 mmol/l on insulin release from pancreatic islets perfused throughout in the absence (○) or presence (●) of 1 $\mu\text{mol/l}$ BPDZ 73. Basal media contained extracellular Ca^{2+} . Mean values ($\pm$ SEM) refer to four individual experiments

glucose and corrected for basal values (min 40–44), averaged $0.82 \pm 0.11 \mu\text{U} \cdot \text{min}^{-1} \cdot \text{islet}^{-1}$ in the absence and $0.13 \pm 0.02 \mu\text{U} \cdot \text{min}^{-1} \cdot \text{islet}^{-1}$ in the presence of 1 $\mu\text{mol/l}$ BPDZ 73, respectively ($p < 0.05$).

Effects of BPDZ 73 and verapamil on KCl -induced changes in ^{45}Ca outflow from perfused rat pancreatic islets. In the absence of glucose and presence of Ca^{2+} in the medium, a sudden rise in the extracellular concentration of K^{+} provoked an immediate and pronounced increase in ^{45}Ca outflow from perfused islets (Fig. 7). When the same experiment was repeated in the presence of BPDZ 73 (10 $\mu\text{mol/l}$ and 50 $\mu\text{mol/l}$) in the perfusate, the basal rate of ^{45}Ca outflow was unaffected ($p > 0.05$) (Fig. 7). Moreover, BPDZ 73 did not affect the cationic response to 50 mmol/l K^{+} (Fig. 7). Indeed, the integrated outflow of ^{45}Ca observed during exposure to 50 mmol/l K^{+} averaged $0.96 \pm 0.05\%/ \text{min}$ in the absence, $0.98 \pm 0.08\%/ \text{min}$ in the presence of 10 $\mu\text{mol/l}$ BPDZ 73 and $0.85 \pm 0.09\%/ \text{min}$ in the presence of 50 $\mu\text{mol/l}$ BPDZ 73, respectively ($p > 0.05$ in each case).

For comparison, we characterised the effect of the Ca^{2+} channel blocker verapamil [28] on the cationic response to high extracellular K^{+} (Fig. 7). The presence of verapamil (10 or 50 $\mu\text{mol/l}$) in the perfusing medium provoked a concentration-dependent de-

Fig. 7. **A** Effect of 10 $\mu\text{mol/l}$ (●) and 50 $\mu\text{mol/l}$ (▲) BPDZ 73 on KCl (50 mmol/l; ○)-induced increase in ⁴⁵Ca outflow. **B** Effect of 10 $\mu\text{mol/l}$ (●) and 50 $\mu\text{mol/l}$ (▲) verapamil on KCl (50 mmol/l; ○)-induced increase in ⁴⁵Ca outflow. Basal media contained extracellular Ca^{2+} . Mean values ($\pm$ SEM) refer to 4–12 individual experiments

crease in both the basal ⁴⁵Ca outflow (min 10–14) and the K⁺ response. Thus, ⁴⁵Ca outflow before the rise in extracellular K⁺ concentration averaged 0.89 ± 0.04 %/min in the absence, 0.81 ± 0.04 %/min in the presence of 10 $\mu\text{mol/l}$ (NS) and 0.69 ± 0.06 %/min in the presence of 50 $\mu\text{mol/l}$ verapamil ($p < 0.05$), respectively. The magnitude of the increment in ⁴⁵Ca outflow evoked by K⁺ averaged 1.11 ± 0.07 %/min in the absence and 0.60 ± 0.06 %/min in the presence of 10 $\mu\text{mol/l}$ verapamil ($p < 0.05$). In islets exposed throughout to 50 $\mu\text{mol/l}$ verapamil, the stimulatory effect of K⁺ was short-lasting and greatly reduced.

Effects of BPDZ 73 on the cytosolic free Ca^{2+} concentration of single rat pancreatic islet cells. Raising the extracellular glucose concentration from 2.8 to 16.7 mmol/l provoked an initial decrease followed rapidly by a pronounced and sustained rise in $[\text{Ca}^{2+}]_i$ (Fig. 8). The addition of BPDZ 73 (1 $\mu\text{mol/l}$) during the 16.7 mmol/l glucose stimulation reduced the cytosolic free Ca^{2+} concentration (Fig. 8). The inhibitory effect of BPDZ 73 was rapid, pronounced and reversible. Similar results were recorded with 10 $\mu\text{mol/l}$ BPDZ 73, except that the inhibitory effect of the compound was less rapidly reversible (data not shown).

By contrast, BPDZ 73 (1 $\mu\text{mol/l}$) did not affect the rise in $[\text{Ca}^{2+}]_i$ induced by 50 mmol/l K⁺ (Fig. 8).

Effects of diazoxide and BPDZ 73 on the contractile activity of rat aortic rings. In rat aortic rings, a rise in the extracellular concentration of K⁺ provoked an increase in muscle tension which, after a few minutes, reached a plateau phase.

When a sustained contraction was obtained, the cumulative application of diazoxide or BPDZ 73 to the bathing medium induced concentration-dependent relaxations (Fig. 9). The relaxant potency of diazoxide, as calculated from the EC₅₀ value, was, however, higher than that of BPDZ 73 ($p < 0.05$). Thus, the EC₅₀ value for diazoxide amounted to 22.0 ± 2.6 $\mu\text{mol/l}$ whereas the EC₅₀ value for BPDZ 73 amounted to 36.3 ± 2.2 $\mu\text{mol/l}$.

Effects of BPDZ 73 and diazoxide on blood glucose concentration. In control rats receiving the vehicle, there was a continuous and small decrease in blood glucose concentration (Fig. 10). The glycaemia averaged 145.0 ± 4.5 mg/100 ml at time –5 min and 126.3 ± 6.3 mg/100 ml at the 180th min.

Treatment with the solution of diazoxide provoked a modest increase in blood glucose concentration peaking at the 60th min at 206.1 ± 7.60 mg/100 ml ($p < 0.05$) (Fig. 10). At the 180th min, the blood glucose concentration was no more significantly different from its initial value (–5 min) ($p > 0.05$).

The changes in blood glucose resulting from treatment with BPDZ 73 were much more pronounced than those induced by diazoxide (Fig. 10). The rise in blood glucose concentration was more rapid, pronounced and sustained. The peak value was recorded at the 120th min and amounted to 348.5 ± 35.0 mg/100 ml ($p < 0.05$). Thereafter, a slow decrease was noticed, eventually reaching at the 180th min a value of 245.0 ± 21.3 mg/100 ml. This concentration was higher than that recorded at time –5 min and at the 180th min in control experiments or after diazoxide injection ($p < 0.05$ in each case).

Fig. 8. **A** Effect of 16.7 mmol/l glucose on $[Ca^{2+}]_i$. **B** Effect of 1 μ mol/l BPDZ 73 on glucose (16.7 mmol/l)-induced increase in $[Ca^{2+}]_i$. **C** Effect of 1 μ mol/l BPDZ 73 on KCl (50 mmol/l)-induced increase in $[Ca^{2+}]_i$. Basal media contained 2.8 mmol/l glucose and extracellular Ca^{2+} . Each graph is a representative experiment conducted on a single cell

Fig. 9. Concentration-response curves for the inhibitory effect of diazoxide (○) and BPDZ 73 (●) on precontracted rat aortic rings. Mean values ($\pm$ SEM) refer to six individual experiments

Fig. 10. Time course for the changes in blood glucose concentration in fed rats injected intraperitoneally at time zero with BPDZ 73 (●), diazoxide (Δ) or the vehicle (○). Mean values ($\pm$ SEM) refer to eight to ten individual experiments

Discussion

In an attempt to develop original K_{ATP} channel modulators interfering with the insulin secretory process, we recently synthesised numerous diazoxide derivatives. Among the different compounds, BPDZ 73 proved to be an effective inhibitor of insulin secre-

tion. The present data clearly show that in vitro BPDZ 73 provoked a concentration-dependent inhibition of insulin release from rat pancreatic islets incubated in the presence of insulinotropic glucose concentrations. Moreover, and according to the IC_{50} values, BPDZ 73 seemed to be about 35 times more potent than diazoxide at reducing the glucose-induced insulin release. This higher capacity of BPDZ 73 to inhibit insulin output was confirmed by our in vivo investigations indicating that the drug provoked a pronounced increase in blood glucose concentration whereas diazoxide induced a modest, less-sustained rise in glycaemia.

Different experimental approaches showed that the inhibitory effect of BPDZ 73 on the insulin-releasing process resulted from a decrease in Ca^{2+} entry following K_{ATP} channel activation.

Radioisotopic experiments conducted on pre-labelled and perfused rat pancreatic islets, an experimental procedure which has the potential advantage of preserving the complex intercellular interactions [11], showed that BPDZ 73 elicited a concentration-dependent increase in ^{86}Rb outflow. This finding indirectly indicates that the drug provokes an increase in membrane K^+ permeability [11, 17, 29].

The ^{86}Rb efflux techniques also provide information about the type of K^+ channel affected by BPDZ 73. Thus, the BPDZ 73-induced rise in ^{86}Rb outflow was abolished by glibenclamide, a hypoglycaemic sulphonylurea known to close the K_{ATP} channel [10, 30]. The increase in ^{86}Rb FOR was, however, more pronounced in the presence of 5.6 mmol/l than in the presence of 16.7 mmol/l glucose in the physiological medium, a feature reminiscent of the effects of different K_{ATP} channel openers on ^{86}Rb outflow from pancreatic islets [11, 17, 29]. These data, as well as the close similarities between the effects of BPDZ 73 and diazoxide on ^{86}Rb -loaded pancreatic islets [16, 29] suggest that BPDZ 73 activates K_{ATP} channels in islet cells.

The opening of K_{ATP} channels, which regulate membrane potential and control-cell excitability, might be expected to hyperpolarise the beta-cell membrane. In agreement with such a view, measurements of DiBAC₄(3) fluorescence showed that BPDZ 73 induced a concentration-dependent hyperpolarisation of the membrane potential.

It must be emphasised that, whatever the experimental protocol used, BPDZ 73 was about 35 times more potent than diazoxide at hyperpolarising the plasma membrane from β -TC3 cells and at reducing insulin output from whole pancreatic islets.

An activation of K_{ATP} channels responsible for membrane repolarisation might restrict the opening of the voltage-sensitive Ca^{2+} channels equipping the beta-cell membrane. This should lead to a decrease in Ca^{2+} entry, reduction in $[Ca^{2+}]_i$ and, ultimately, to an inhibition of insulin secretion. This scheme is sup-

ported by three lines of evidence. Firstly, BPDZ 73 was shown to provoke a concentration-dependent reduction in ^{45}Ca outflow from pancreatic islets perfused in the presence of 16.7 mmol/l glucose and extracellular Ca^{2+} . Under such experimental conditions, namely in islets exposed continuously to insulinotropic concentrations of glucose and Ca^{2+} , a decrease in ^{45}Ca FOR is known to reflect a reduction in $^{40}Ca^{2+}$ entry through voltage-sensitive Ca^{2+} channels [14, 31]. The lack of effect of BPDZ 73 on ^{45}Ca outflow from islets exposed to Ca^{2+} -free media strengthens this interpretation and further indicates that the drug does not provoke an intracellular Ca^{2+} redistribution [15, 32]. Secondly, measurements of fura-2 fluorescence clearly showed the capacity of BPDZ 73 to counteract the glucose-induced increase in $[Ca^{2+}]_i$. Thirdly, the simultaneous measurement of insulin release from pancreatic islets perfused in the presence of 16.7 mmol/l glucose and extracellular Ca^{2+} had a time course parallel to that of the ^{45}Ca FOR response.

All these findings provide direct support for the view that, following K_{ATP} channel activation, the reduction in Ca^{2+} entry mediates the inhibitory effect of BPDZ 73 on the insulin releasing process.

To further the understanding of the cascade of events leading to inhibition of insulin release, we compared the effects of BPDZ 73 and verapamil on the cationic responses to high K^+ concentrations. A rise in the extracellular K^+ concentration depolarises the beta-cell membrane, opens the voltage-sensitive Ca^{2+} channels and increases Ca^{2+} inflow. In ^{45}Ca -loaded pancreatic islets, the increment in ^{45}Ca FOR evoked by high K^+ reflects a sustained stimulation of isotopic exchange between influent $^{40}Ca^{2+}$ and effluent ^{45}Ca [14]. This increase in ^{45}Ca outflow was clearly sensitive, in a concentration-related manner, to the Ca^{2+} channel blocker verapamil [28] but was resistant to BPDZ 73. Moreover, BPDZ 73 also failed to affect the increase in $[Ca^{2+}]_i$ elicited by a high K^+ concentration. These results indicate that BPDZ 73 does not directly interact at the level of the voltage-sensitive Ca^{2+} channels and confirm our hypothesis that the primary action of the compound is to raise the K^+ permeability of the beta-cell plasma membrane.

Note that, under the different in vitro experimental conditions, the effects of BPDZ 73 were always clearly reversible. Such a feature implies that the responses to the drug were not caused by any damage to insulin-secreting cells.

Lastly, measurements of smooth muscle contractile activity in rat aortic rings showed that BPDZ 73 was slightly less potent than diazoxide at inhibiting the mechanical responses evoked by K^+ depolarisation. According to the EC_{50} (contractile activity in aortic rings): IC_{50} (insulin release from pancreatic islets) ratio, BPDZ 73 seemed to be more selective for the insulin-secreting cells than diazoxide.

The data presented here indicate that in insulin-secreting cells the compound BPDZ 73 activates K_{ATP} channels and hyperpolarises the plasma membrane. The activation of K_{ATP} channels responsible for repolarisation restricts the opening of voltage-sensitive Ca^{2+} channels, decreases Ca^{2+} inflow, reduces the cytosolic Ca^{2+} concentration and, ultimately, inhibits the insulin secretory rate. We describe a new K_{ATP} channel opener that is 35 times more potent than diazoxide on insulin-secreting cells. Moreover, and by contrast to diazoxide, this new compound seemed to have some selectivity for the pancreatic endocrine tissue compared with the vascular smooth muscle tissue. The *in vitro* and *in vivo* effectiveness of BPDZ 73 calls for further pharmacomodulation of 7-halogeno-substituted benzothiadiazine dioxides to identify new chemical entities with a powerful, and hopefully selective, biological activity on pancreatic beta cells. Such compounds could be expected to be valuable drugs in different therapeutical applications including the treatment of hyperinsulinaemia-induced hypoglycaemia in adults and children and the preservation of beta-cell function in autoimmune diabetes.

Acknowledgements. The authors are indebted to J. Sergiooris and M. Hermann for technical assistance, to P. Surardt for secretarial help and to Dr K. Bokvist for comments. Q.-A. Nguyen was supported by a grant-in-aid from the Free University of Brussels. P. Lebrun is Research Director and B. Pirotte is Research Associate of the National Fund for Scientific Research (Belgium).

References

- Koch-Weser J (1976) Diazoxide. *N Engl J Med* 294: 1271–1274
- Meissner T, Brune W, Mayatepek E (1997) Persistent hyperinsulinaemic hypoglycaemia of infancy: therapy, clinical outcome and mutational analysis. *Eur J Pediatr* 156: 754–757
- Vlahos WD, Seemayer TA, Yale JF (1991) Diabetes prevention in BB rats by inhibition of endogenous insulin secretion. *Metabolism* 40: 825–829
- Alemzadeh R, Slonim AE, Zdanowicz ML, Maturo J (1993) Modification of insulin resistance by diazoxide in obese Zucker rats. *Endocrinology* 133: 705–712
- Aizawa T, Taguchi N, Sato Y et al. (1995) Prophylaxis of genetically determined diabetes by diazoxide: a study in a rat model of naturally occurring obese diabetes. *J Pharmacol Exp Ther* 275: 194–199
- Greenwood RH, Mahler RF, Hales CN (1976) Improvement in insulin secretion in diabetes after diazoxide. *Lancet* 28: 444–447
- Björk E, Berne C, Kämpe O, Wibell L, Oskarsson P, Karlsson FA (1996) Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes. *Diabetes* 45: 1427–1430
- Alemzadeh R, Langley G, Upchurch L, Smith P, Slonim AE (1998) Beneficial effect of diazoxide in obese hyperinsulinemic adults. *J Clin Endocrinol Metab* 83: 1911–1915
- Babenko AP, Aguilar-Bryan L, Bryan J (1998) A view of SUR/K_{IR} 6.X, K_{ATP} channels. *Annu Rev Physiol* 60: 667–687
- Ashcroft FM, Rorsman P (1989) Electrophysiology of the pancreatic B-cell. *Prog Biophys Mol Biol* 54: 87–143
- Lebrun P, Antoine M-H, Herchuelz A (1992) Minireview: K^+ channel openers and insulin release. *Life Sci* 51: 795–806
- Ashcroft FM, Gribble FM (1998) Correlating structure and function in ATP-sensitive K^+ channels. *Trends Neurosci* 21: 288–294
- Leclercq-Meyer V, Marchand J, Woussen-Colle MC, Giroix MH, Malaisse WJ (1985) Multiple effects of leucine on glucagon, insulin and somatostatin secretion from the perfused rat pancreas. *Endocrinology* 116: 1168–1174
- Lebrun P, Malaisse WJ, Herchuelz A (1982a) Evidence for two distinct modalities of Ca^{2+} influx into pancreatic B cell. *Am J Physiol* 242: E59–E66
- Lebrun P, Malaisse WJ, Herchuelz A (1982b) Nutrient-induced intracellular calcium movement in rat pancreatic B cell. *Am J Physiol* 243: E196–E205
- Lebrun P, Devreux V, Hermann M, Herchuelz A (1989) Similarities between the effects of pinacidil and diazoxide on ionic and secretory events in rat pancreatic islets. *J Pharmacol Exp Ther* 250: 1011–1018
- Lebrun P, Antoine M-H, Ouedraogo R et al. (1996) Activation of ATP-dependent K^+ channels and inhibition of insulin release: effect of BPDZ 62. *J Pharmacol Exp Ther* 277: 156–162
- Malaisse WJ, Boschero AC, Kawazu S, Hutton JC (1978) The stimulus secretion coupling of glucose-induced insulin release. XXVII. Effect of glucose on K^+ fluxes in isolated islets. *Pflügers Arch* 373: 237–242
- Efrat S, Linde S, Kofod H et al. (1988) Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci USA* 85: 9037–9041
- Epps DE, Wolfe ML, Groppi V (1994) Characterization of the steady state and the dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) in model systems and cells. *Chem Phys Lipids* 69: 137–150
- Antoine MH, Berkenboom G, Fang ZY, Fontaine J, Herchuelz A, Lebrun P (1992) Mechanical and ionic response of rat aorta to diazoxide. *Eur J Pharmacol* 216: 299–306
- de Tullio P, Pirotte B, Lebrun P et al. (1996) 3- and 4-substituted 4H-pyrido[4,3-e]-1,2,4-thiadiazine 1,1-dioxides as potassium channel openers: synthesis, pharmacological evaluation, and structure-activity relationships. *J Med Chem* 39: 937–948
- Pirotte B, de Tullio P, Lebrun P et al. (1993) 3-(Alkylamino)-4H-pyrido[4,3-e]-1,2,4-thiadiazine 1,1-dioxides as powerful inhibitors of insulin release from rat pancreatic B-cells: a new class of potassium channel openers? *J Med Chem* 36: 3211–3213
- Di Bella M, Rinaldi M, Fabio U, Manicardi G (1973) Azione antimicrobica di derivati dell' 1,2,4-benzotiadiazin-1,1-diossido. *Farmaco* 28: 411–417
- Raffa L, Di Bella M, Ferrari P, Rinaldi M (1974) Azione cardiovascolare di derivati dell' 1,2,4-benzotiadiazin-1,1-diossido. *Farmaco* 29: 411–423
- Lebrun P, Atwater I (1985) Effects of the calcium channel agonist, BAY K 8644, on electrical activity in mouse pancreatic B-cells. *Biophys J* 48: 919–930
- Girard Y, Atkinson JG, Rokach J (1979) A new synthesis of 1, 2, 4-benzothiadiazines and a selective preparation of o-aminobenzenesulphonamides. *J Chem Soc [Perkin 1]*: 1043–1047
- Godfraind T, Miller R, Wibo M (1986) Calcium antagonism and calcium entry blockade. *Pharmacol Rev* 38: 321–416
- Henquin JC, Debuyser A, Drews G, Plant TD (1992) Regulation of K^+ permeability and membrane potential in insulin secreting cells. In: Flatt PR (ed) *Nutrient regulation of insulin secretion*. Portland Press, London, pp 173–191
- Malaisse WJ, Lebrun P (1990) Mechanisms of sulfonylurea-induced insulin release. *Diabetes Care* 13[Suppl 3]:9–17
- Plasman P-O, Hermann M, Herchuelz A, Lebrun P (1990) Sensitivity to Cd^{2+} but resistance to Ni^{2+} of Ca^{2+} inflow into rat pancreatic islets. *Am J Physiol* 258: E529–E533
- Antoine M-H, Ouedraogo R, Sergiooris J, Hermann M, Herchuelz A, Lebrun P (1996) Hydroxylamine, a nitric oxide donor, inhibits insulin release and activates K^+ channels. *Eur J Pharmacol* 313: 229–235