Effect of the hypoglycaemic drug (–)-AZ-DF-265 on ATP-sensitive potassium channels in rat pancreatic β -cells

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1 It has previously been shown that the hypoglycaemic drug (–)-AZ-DF-265 stimulates insulin release from mouse islets; in the presence of 3 mM glucose it also inhibits ⁸⁶Rb-efflux from ⁸⁶Rb-loaded islets. Based on these data we tested the hypothesis that (–)-AZ-DF-265 inhibits ATP-sensitive potassium channels.

2 We voltage-clamped the plasma membrane of single rat pancreatic β -cells in the whole-cell configuration and measured the current flowing through ATP-sensitive potassium channels. (–)-AZ-DF-265 was applied to the outside of the cell; it inhibited the current half-maximally at a concentration of 1.2 ± 0.2 nM with a Hill coefficient of 0.7 ± 0.1 . The inhibition was reversible on washing.

3 In intact RINm5F cells, the sulphonylurea [³H]-glibenclamide bound with an affinity of 0.24 ± 0.01 nM and a Hill coefficient of 2.1 ± 0.4 . Treatment with (–)-AZ-DF-265 led to the displacement of [³H]-glibenclamide; this effect was half-maximal at 8.6 ± 1.7 nM and displayed a Hill coefficient of 0.53 ± 0.01 . Meglitinide and tolbutamide, which represent, respectively, the benzamido and sulphonylurea moieties of glibenclamide, showed Hill coefficients of 0.8 ± 0.1 and 0.9 ± 0.1 , respectively.

4 At pH 7.4 and with phosphate/borate buffer, (–)-AZ-DF-265 had an apparent octanol/water partition coefficient of about 53, which is intermediate between the partition coefficients of glibenclamide and glipizide. Nevertheless, (–)-AZ-DF-265 bound only to a minor degree to glass and plastic.

5 In conclusion, (–)-AZ-DF-265 inhibits ATP-sensitive potassium channels and displaces [³H]-glibenclamide from the sulphonylurea receptor.

Keywords: AZ-DF-265; pancreatic β cells; RINm5F cells; ATP-sensitive potassium channel; glibenclamide; meglitinide; tolbutamide; sulphonylurea receptor

Introduction

Several new hypoglycaemic drugs have been developed in efforts to circumvent the problem of a gradual loss of responsiveness which is common with type II diabetic patients who use sulphonylurea hypoglycaemic drugs (Jackson & Bressler, 1981; Asmal & Marble, 1984; Henquin, 1990). The mechanism of action of these new drugs remains to be explained.

Stimulus-secretion coupling in pancreatic β -cells is thought to proceed as follows: an elevated concentration of glucose leads to a more pronounced inhibition of adenosine 5'-triphosphate (ATP)-sensitive potassium channels, which in turn leads to a depolarization of the plasma membrane, the opening of voltage-sensitive calcium channels, and the activation by increased intracellular calcium of the exocytotic machinery which moves secretory granules containing insulin to the plasma membrane surface (Prentki & Matschinsky, 1987; Ashcroft, 1988; Cook *et al.*, 1988).

Sulphonylureas inhibit ATP-sensitive potassium channels both in intact cells and in excised membrane patches (Sturgess *et al.*, 1985; Trube *et al.*, 1986). It is not known whether the sulphonylurea binding site is on the channel itself or on a protein that affects channel activity. We have shown previously that the non-sulphonylurea drug, linogiride, also leads to the inhibition of ATP-sensitive potassium channels (Ronner *et al.*, 1991). Garrino & Henquin (1988) reported that another new drug, (–)-AZ-DF-265 (4-[N-(α -phenyl-2-piperidino-benzyl) carbamoyl] methyl benzoic acid) stimulates insulin release and electrical activity, and also inhibits ⁸⁶Rb-efflux from ⁸⁶Rb-preloaded mouse islets in the presence of 3 mM glucose. Since the substituted carbamoyl group of

(–)-AZ-DF-265 may be homologous with the sulphonylurea group, we tested the hypotheses that (–)-AZ-DF-265 inhibits ATP-sensitive K⁺-channels and displaces [³H]-glibenclamide from its binding site.

Methods

Animals and isolation of islets

Pathogen-free male Wistar rats were obtained from Charles River Laboratories, Inc. (Kingston, N.Y., U.S.A.). Islets were isolated in sterile fashion by a procedure which was modified from that of Lacy & Kostianovsky (1967). After hand-picking, the islets were further freed of exocrine tissue by centrifugation in a discontinuous Ficoll gradient (Lindall *et al.*, 1969). One hundred islets were then dissociated into single cells with trypsin in the absence of calcium, but with the addition of DNase, and the cells purified and washed as described by Pipeleers *et al.* (1985). The cells were then allowed to attach to poly-D-lysine-coated glass cover-slips and maintained for 1–2 days in Eagle's minimum essential medium (MEM) containing glucose (10 mM), penicillin (67,000 u l^{–1}), streptomycin (67 mg l^{–1}), and amphotericin (1.7 mg l^{–1}). A detailed account of these procedures has been published (Ronner *et al.*, 1991).

Electrophysiological experiments

The cells were placed in a thermostated chamber containing the following medium (mM): KCl 5.6, NaCl 140, MgCl₂ 1.2, CaCl₂ 2.6, HEPES-KOH 10, pH 7.4. The chamber had an inside diameter of 22 mm and was filled with 0.3–1 ml medium. The chamber was perfused at a flow rate (driven by a peristaltic pump) of 2 to 3 times the chamber volume per

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min. The concentration of drug in the chamber was calculated from the equation $d = D(e^{t/V} - 1)/e^{t/V}$, whereby d denotes the average concentration of (–)-AZ-DF-265 in the chamber, D is the concentration of (–)-AZ-DF-265 in the incoming perfusion medium, e is the basis of the natural logarithm (i.e., 2.718), t is the time since switching to the new solution, f is the perfusate flow-rate, and V is the chamber volume. The traces (continuous lines) in Figure 1 are the result of these calculations. Control experiments with bromphenol blue showed that new medium distributed homogeneously over the entire volume of the chamber within the expected time. In addition, perfusion of cells with twice the normal concentration of K^+ led to a drop in holding current following the predicted, calculated time-course. All experiments were performed at 21–23°C. The following materials were in contact with (–)-AZ-DF-265: borosilicate glass, plastic pipette tips, PVC tubing, and in some instances silicone rubber tubing.

Electrodes were pulled from Corning 7052 glass (i.d. 1.15 mm, o.d. 1.75 mm; Garner Glass Co., Claremont, CA, U.S.A.), coated with Sylgard 184 (Dow Corning, Midland, MI, U.S.A.), and fire-polished to tip openings with 'bubble numbers' of 5.0–5.2 (Corey & Stevens, 1983). The electrodes were filled with the following solution (mM): K^+ 157 (as KCl 125), $MgCl_2$ 1, $CaCl_2$ 2, EGTA 10, and HEPES-KOH 5, pH 7.15 (free $[Ca^{2+}] = \sim 53$ nM); equal concentrations of ATP and additional $MgCl_2$ were added as noted for individual experiments.

β -Cells were selected for their large size (Pipeleers & Pipeleers-Marichal, 1981; our own immunocytochemical experiments, not shown); we chose cells which had an apparent diameter of 15–17 μ m when observed in phase contrast. The plasma membrane potential was held constant at –70 mV in the whole-cell configuration. Alternating hyperpolarizing and depolarizing pulses of 10 mV amplitude and 200 ms duration were applied every 2 s (Trube *et al.*, 1986). The membrane current was filtered through a low-pass eight-pole Bessel filter set to a corner frequency of 1,000 Hz (–3 dB). The current was then digitized at a rate of 500 Hz and recorded onto the hard-disc of a computer with 'pCLAMP' software (versions 5.0 and 5.5; Axon Instruments, Foster City, CA, U.S.A.). This recording set-up has been described in greater detail in an earlier publication (Ronner *et al.*, 1991). Average current excursions from the holding current in response to the voltage pulses were calculated from the average integrated response to sets of 10 stimuli, as described previously (Ronner *et al.*, 1991). Outward currents were given a positive sign. Current responses in the negative direction are not shown in the semi-logarithmic plots; within 5% error they had the same magnitude as the positive current deflections. Whenever the holding current (at –70 mV) was more negative than –10 pA, the seal between the cell and the electrode was assumed to have deteriorated and recordings were terminated. Even at a more negative membrane potential of –80 mV the current was always more positive than –50 pA.

(–)-AZ-DF-265 was dissolved in 30 mM NaOH to a concentration of 2 mM. This stock solution was stored in separate aliquots at –20°C. Final concentrations in the bath medium of 1–1,000 nM were obtained by appropriate dilution of the stock solution.

Octanol water partition experiments

The aqueous phase consisted of 50 mM boric acid and 50 mM mono- or dipotassium phosphate titrated to pH 7–9 with KOH. (–)-AZ-DF-265 was added to the aqueous phase to final concentrations of 46–182 μ M. We accounted for the small change in pH (i.e., 0.09–0.13) caused by the addition of (–)-AZ-DF-265, which had been dissolved in 30 mM NaOH. The aqueous phase and 1-octanol, at a ratio of 5:1, were vortexed vigorously. After centrifugation (15 min, 450 g) the absorbance of the lower, aqueous phase was deter-

mined at 240 nm; for this measurement aqueous phases of pH 8 to 9 were diluted 2 to 8 fold with pure phosphate-borate buffer of the same pH. The extinction coefficient of (–)-AZ-DF-265 was determined by adding 25–37 μ M (–)-AZ-DF-265 to octanol-pretreated buffer of the appropriate dilution. Over the pH range 7–9, the weighted mean extinction coefficient was $16,400 \pm 500$ (means $\pm$ s.e.mean, 13 data pairs). The concentration of (–)-AZ-DF-265 in the octanol phase was calculated from the difference in concentration in the aqueous phase before and after equilibration with octanol; thereby, we took into account that octanol is soluble in water to 4.5 mM and that water is soluble in octanol to 2.3 M (Leo *et al.*, 1971).

[³H]-glibenclamide binding to intact RINm5F cells

RINm5F cells were grown to confluence in tissue culture medium RPMI 1640 (Moore *et al.*, 1967) supplemented with 10% (vol/vol) foetal calf serum, 100,000 u l^{–1} penicillin, and 100 mg l^{–1} streptomycin. The cells were scraped with a plastic policeman into medium of the same composition as the one used in electrophysiological experiments except that it also contained 10 mM glucose (i.e. (mM): KCl 5.6, NaCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6, glucose 10, HEPES-KOH 10, pH 7.4). The cells were sedimented and resuspended. Samples of $2\text{--}5 \times 10^6$ cells in 1 ml final volume were incubated at room temperature for 90 min in plastic Eppendorf centrifuge tubes in the presence of 0.33 nM [³H]-glibenclamide (about 2,400 c.p.m. ml^{–1}; 8.3 Ci mmol^{–1}; manufactured by Chemsyn Science Laboratories, Lenexa, Kansas, U.S.A.) and various concentrations of non-radioactive glibenclamide, (–)-AZ-DF-265, meglitimide or tolbutamide. Coating of the plastic tubes with Sigmacote (Sigma) was necessary to reduce non-specific binding of [³H]-glibenclamide from about 150 c.p.m. to less than 3 c.p.m. The tubes were vortexed briefly every 20 min to counteract the settling of the cells. At the end of the incubation, the cells were sedimented by centrifugation for 2 s at 15,800 g in a Beckman microfuge E. The pellets were resuspended in 0.8 ml of drug-free saline incubation medium and resedimented within less than 30 s. The final pellets were suspended in 0.4 ml incubation medium. The tubes with the cell suspension were then placed in scintillation vials containing 10 ml EcoLite (+) scintillation fluid (ICN Biomedicals, Costa Mesa, CA, U.S.A.). Radioactivity was determined by counting for 10–60 min; the counting efficiency was about 28%.

Reagents

Trypsin and DNase were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). (–)-AZ-DF-265 (i.e., 4-[N-(α -phenyl-2-piperidino-benzyl) carbamoyl] methyl] benzoic acid) of 99.7% enantiomeric purity was a generous gift of the Dr Karl Thomae GmbH (Biberach an der Riss, Germany). Glipizide was a gift of Pfizer (Groton, CT, U.S.A.) and meglitinide of Hoechst AG (Frankfurt am Main, Germany). Inorganic salts and 1-octanol were of the 'analyzed' grade and were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Eagle's MEM, RPMI 1640 culture medium, antibiotics, and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis and curve fitting

Current responses to the 10 mV pulses were plotted semi-logarithmically against time. Based on the decline of the current amplitude in an individual experiment and in control experiments carried out on the same day without application of (–)-AZ-DF-265, estimates of channel run-down were made. The average of these two values was then used as the probable rate of current decline (run-down) observed in an individual experiment. The maximal inhibition of the current response by (–)-AZ-DF-265 was then determined relative to

the estimate for run-down. For curve fitting, means $\pm$ s.e. means were calculated for all experiments carried out at a similar concentration of (–)-AZ-DF-265. The following curve, which describes drug-receptor interactions, was then fitted to these means:

$$I/I_c = 1 - \{a/[1 + (IC_{50}/d)^n]\} \quad (\text{Eq. 1})$$

Thereby, I , is the current measured in the presence of drug; I_c , is an estimate of the current in the absence of drug; a , is the fraction of the current that is sensitive to the drug; IC_{50} , is the concentration of the drug which produces a half-maximal effect; d , is the test concentration of the drug; and n is a measure of the concentration-dependence of the interaction between drug and target. The data were fitted to equation 1 using a Marquardt-Levenberg algorithm which is available in the graphing programme Sigmaplot (version 4.0, Jandel Scientific, Corte Madera, CA, U.S.A.). The means were given weights of $1/\text{s.e. mean}$. Errors stated for the fitted parameters are asymptotic standard deviations obtained from the fitting programme.

For each preparation of RINm5F cells we measured the displacement of [^3H]-glibenclamide by cold glibenclamide (control) and by one of the test drugs (i.e., (–)-AZ-DF-265, meglitinide, or tolbutamide). Since the displacement of [^3H]-glibenclamide by one of the test drugs depends on the affinities of both glibenclamide and the test drug, we combined the data for the displacement of [^3H]-glibenclamide by cold glibenclamide and by the test drug, and we fitted the aggregate data to the equation:

$$c = a + \{b/[1 + (IC_{50(\text{Glib})}/d)^n(1 + (I/IC_{50(\text{test})})^m)]\}(e/d) \quad (\text{Eq. 2})$$

with the Marquardt-Levenberg algorithm described above. Equation 2 is derived from equation 1 by inserting the term $1 + (I/IC_{50(\text{test})})^m$, for the competition of the test drug for the binding site of [^3H]-glibenclamide, and the correction factor e/d for the dilution of [^3H]-glibenclamide by cold glibenclamide. In equation 2, c reflects the measured amount of [^3H] in the final sample; the variable, a , is a measure of non-specific binding of [^3H]-glibenclamide; the variable, b , is a measure of the number of glibenclamide binding sites; the variable, $IC_{50(\text{Glib})}$, is the concentration of glibenclamide at which half of the binding sites are saturated; d , is the total concentration of glibenclamide (radioactive and non-radioactive); I , is the concentration of test drug; the variable, $IC_{50(\text{test})}$, is the concentration of the test drug at which half of the binding sites are saturated; e , is the concentration of [^3H]-labelled glibenclamide, and both variables n and m are Hill coefficients.

Results

We have shown previously (Ronner *et al.*, 1991) that the peak potassium current as measured from pancreatic β -cells via the whole-cell configuration is inhibited by more than 95% by increasing the concentration of ATP from 0.3 mM to 3 mM. With 0.3 mM ATP in the electrode, tolbutamide (1 mM) inhibited the current by 97%. These data are in good agreement with published data (Trube *et al.*, 1986; Ohno-Shosaku *et al.*, 1987; Ashcroft, 1988; Sturgess *et al.*, 1988; Zünkler *et al.*, 1988; Ashcroft *et al.*, 1989) and suggest that most of the current observed under our recording conditions is carried through ATP- and tolbutamide-sensitive K^+ -channels.

Figure 1 shows the effect of (–)-AZ-DF-265 on the β -cell K^+ -current. With 3 nM (–)-AZ-DF-265 (Figure 1b), the time course for the onset of the inhibition was slower than the rate of exchange of the bath solution (as calculated from the volume of the bath and the rate of perfusion; illustrated by the almost vertical line). A few minutes were thus required for (–)-AZ-DF-265 to reach equilibrium binding to its target. The wash-out of the drug and recovery of the current

response were much slower than the onset of drug action. With low concentrations of (–)-AZ-DF-265 (i.e., 1–3 nM) the current stayed at a near constant level after removal of the drug (Figure 1b). A more pronounced recovery of the current was observed with levels of (–)-AZ-DF-265 of ≥ 30 nM which produced almost complete inhibition of the current (Figure 1c).

Figure 2 shows a concentration-response curve for the inhibition of the whole-cell current by (–)-AZ-DF-265. When fitted to equation 1 (see Methods) the best fit was obtained for $IC_{50} = 1.19 \pm 0.24$ nM, $a = 0.98 \pm 0.02$, and $n = 0.72 \pm 0.11$.

In the presence of bovine serum albumin (BSA) 1 mg ml^{-1} , 30 and 100 nM (–)-AZ-DF-265 inhibited the current by

Figure 1 Effect of (–)-AZ-DF-265 on rat β -cell potassium current. Recordings from single cells in the whole-cell voltage-clamp configuration with 0.3 mM ATP in the electrode solution. Panels show average current levels in response to 10 mV de- and hyperpolarizing pulses (cell polarized to -70 mV). The chamber was perfused with bath medium (a); and medium contained 3 and 30 nM (–)-AZ-DF-265 during times indicated in panels (b) and (c), respectively. The concentration of (–)-AZ-DF-265 in the chamber was calculated mathematically (see Methods). Dashed lines show the rate of current run-down based on selected data points from individual experiments (indicated by $\bullet$). The dotted line in (b) shows the run-down observed on the same day in control experiments without the application of (–)-AZ-DF-265. The near-vertical lines show rate of exchange of bath medium.

79 ± 1 and $90 \pm 2\%$, respectively (mean $\pm$ s.e.mean; $n = 2$). When equation 1 was fitted to these means, and with n set to 0.72, the values of IC_{50} and a were 4.8 nM and 1.0, respectively. By comparing these data to the inhibition obtained in the absence of BSA one may conclude that in the presence of BSA (1 mg ml^{-1}) only about one fourth of the $(-)$ -AZ-DF-265 is free while the rest is bound to BSA.

We examined the competition of $(-)$ -AZ-DF-265 and glibenclamide for glibenclamide binding sites. To circumvent the need for large quantities of islet cells, we used intact RINm5F cells for our $[^3\text{H}]$ -glibenclamide binding experiments. We incubated the cells in the same medium as in our electrophysiological experiments, except that 10 mM glucose was added. Figure 3a shows a summary graph derived from a total of 13 such binding experiments in which each concentration of drug had been tested in duplicate; error bars illustrate the s.e.mean with the number of observations set to 4 or 5, respectively. Equation 2 was fitted to means which were given weights of $1/\text{s.e.}$ Glibenclamide and $(-)$ -AZ-DF-265 bound half-maximally at concentrations of 0.25 ± 0.02 and 8.6 ± 1.7 nM, respectively, while the associated slope factors n and m had values of 2.0 ± 0.8 and 0.53 ± 0.01 , respectively. If n was set to 1, $IC_{50}(\text{Glib})$, $IC_{50}(\text{AZDF})$, and m had values of 0.30 ± 0.06 , 8.1 ± 1.6 , and 0.51 ± 0.04 , respectively. Thus, $(-)$ -AZ-DF-265 convincingly displays negative cooperativity.

Glibenclamide may consist of two pharmacologically effective moieties built around an amide and a sulphonylurea group, respectively (groups 'C' and 'E-F' in Gutsche *et al.*, 1974; see also: Rufer & Losert, 1979; Garrino *et al.*, 1986; Henquin *et al.*, 1987). The hypoglycaemic drug, meglitinide, carries the substituted amide moiety of glibenclamide, while tolbutamide carries the sulphonylurea moiety. Thus, the presence of two moieties could be responsible for the observed positive cooperativity of glibenclamide binding. As Figure 3 (b,c) shows the Hill coefficients for tolbutamide and meglitinide were 0.9 ± 0.1 and 0.8 ± 0.1 , respectively while the Hill coefficients for glibenclamide were 3.4 ± 2.3 and 3.0 ± 2.4 , respectively, in these two sets of experiments. When data on the displacement of $[^3\text{H}]$ -glibenclamide by cold glibenclamide were pooled from 15 different experiments, equation 2 produced a Hill coefficient of 2.1 ± 0.4 (graph not shown).

Figure 2 Concentration-response curve for inhibition of β -cell potassium current by $(-)$ -AZ-DF-265. Means were fitted to equation 1 (see Methods). The number of experiments at each concentration of $(-)$ -AZ-DF-265 was as follows: 5 (0.3 nM), 4 (1 nM), 6 (3 nM), 5 (10 nM), 4 (30 nM), 1 (100 nM), 2 (300 nM). The fitted curve is drawn according to equation 1 with $a = 0.987$, $IC_{50} = 1.19$ nM, and $n = 0.72$. Data are plotted as the ratio of the current in the presence of $(-)$ -AZ-DF-265 to that in the absence of the drug (I/I_c).

Figure 3 Displacement of $[^3\text{H}]$ -glibenclamide from intact RINm5F cells by cold glibenclamide and by $(-)$ -AZ-DF-265, meglitinide, and tolbutamide. Cells were incubated in the same medium as in electrophysiological experiments, except that 10 mM glucose was added. The data were fitted to equation 2 (see Methods). (a) Displacement of $[^3\text{H}]$ -glibenclamide by cold glibenclamide ($\bullet$) and by $(-)$ -AZ-DF-265 ($\blacktriangledown$). Mean of 4 experiments. Fitted curves are drawn to $a = 0.18$, $b = 1.33$, $IC_{50}(\text{Glib}) = 0.25$ nM, $n = 2.0$, $IC_{50}(\text{AZDF}) = 8.6$ nM, and $m = 0.53$. (b) Displacement of $[^3\text{H}]$ -glibenclamide by cold glibenclamide ($\bullet$) and by meglitinide ($\blacktriangledown$). Mean of 4 experiments. Fitted curves are drawn to $a = 0.21$, $b = 1.03$, $IC_{50}(\text{Glib}) = 0.22$ nM, $n = 2.98$, $IC_{50}(\text{Megli}) = 4.5$ μM , and $m = 0.76$. (c) Displacement of $[^3\text{H}]$ -glibenclamide by cold glibenclamide ($\bullet$) and by tolbutamide ($\blacktriangledown$). Mean of 5 experiments. Fitted curves are drawn to $a = 0.19$, $b = 1.07$, $IC_{50}(\text{Glib}) = 0.24$ nM, $n = 3.36$, $IC_{50}(\text{Tolb}) = 10.9$ μM , and $m = 0.93$. In (a), (b) and (c), s.e.means shown by vertical bars.

In two experiments, RINm5F cells were incubated with 0.33 nM $[^3\text{H}]$ -glibenclamide for 40 or 60 min, after which an excess of cold glibenclamide ($0.1 \mu\text{M}$) or $(-)$ -AZ-DF-265 ($2 \mu\text{M}$) was added. Within the next 90 min, 39 or 34%, respectively, of the $[^3\text{H}]$ -glibenclamide dissociated from the cells with no significant difference between cold glibenclamide and $(-)$ -AZ-DF-265. Hence, the displacement of $[^3\text{H}]$ -glibenclamide by cold $(-)$ -AZ-DF-265 in the competition

experiments shown in Figure 3a is not due to an (–)-AZ-DF-265-induced allosteric increase of the rate of dissociation of glibenclamide.

We determined the lipid solubility of (–)-AZ-DF-265 as it influences the ease with which the drug can be used experimentally. We chose 1-octanol as a membrane-lipid-like phase since data are already available on the partitioning of sulphonylureas between water and octanol (Panten *et al.*, 1989). The data in Figure 4 show that (–)-AZ-DF-265 predominantly partitioned into the octanol phase at physiological pH. Compared to two high-affinity second generation sulphonylureas, glibenclamide and glipizide (apparent partition coefficients at pH 7.5 estimated to be 71 and 2.0, respectively, based on data of Panten *et al.*, 1989), (–)-AZ-DF-265 at pH 7.5 had an intermediate octanol/water partition coefficient of 44.

Discussion

The hypoglycaemic drug (–)-AZ-DF-265 potently inhibits the pancreatic β -cell ATP-sensitive K^+ current. The residual K^+ current after maximal block by (–)-AZ-DF-265 was similar to that observed after maximal block by tolbutamide or linogiride (Ronner *et al.*, 1991).

(–)-AZ-DF-265 was as potent in inhibiting β -cell ATP-sensitive potassium currents as the second generation sulphonylureas. Thus, 1.2 nM (–)-AZ-DF-265 inhibited the current half-maximally in our experiments. By comparison, as much as 27 nM glibenclamide was needed to inhibit the ATP-sensitive K^+ -current of CRI-G1 insulinoma cells half-maximally (Sturgess *et al.*, 1988). However, in mouse β -cells, in the presence of 0.2% (w/v) serum albumin, 4 and 6.4 nM glibenclamide and glipizide, respectively, half-maximally inhibited the ATP-sensitive K^+ -current (Zünkler *et al.*, 1988). Furthermore, taking into account that only 17 and 57% of glibenclamide and glipizide, respectively, were free, half-maximal inhibition was achieved at free concentrations of 0.7 and 3.6 nM, respectively (Panten *et al.*, 1989).

(–)-AZ-DF-265 was about 9 to 80 fold more potent in our electrophysiological assay ($IC_{50} \approx 1.2$ nM) than in the insulin secretory studies of Garrino & Henquin (1988). (IC_{50} in their study estimated by us to be about 100 and 11 nM at 3 and 10 mM glucose, respectively). The higher apparent potency in our assay compared to the secretion studies can be explained in part by the omission of serum albumin. Thus, in the presence of 0.1% (w/v) BSA, we estimate that half-maximal inhibition of the β -cell K^+ -current was achieved at about 5 nM (–)-AZ-DF-265. The effects of (–)-AZ-DF-265 on ATP-sensitive K^+ channels were only slowly reversible on wash-out of the drug. Similarly, the effects of (–)-AZ-DF-265 on insulin release from perfused mouse islets disappeared only after a 20–30 min wash-out (Garrino & Henquin, 1988).

The octanol-water partition experiments with the phosphate-borate buffer are directly comparable to those of Panten *et al.* (1989) with sulphonylureas. At pH 7.4 we observed an apparent distribution coefficient of 53. Our data with pure phosphate buffer (distribution coefficient of 97 at pH 7.4; see Figure 4) compare favourably with the distribution coefficient of 132 at pH 7.4 which was reported to us by Dr K. Noll (Dr Karl Thomae GmbH, Biberach an der Riss, Germany; personal communication, 1990). A detailed mathematical analysis of the pH-dependence of the apparent partition coefficient was not performed because the pK values of (–)-AZ-DF-265 are, as yet, unknown. We used (–)-AZ-DF-265 in nanomolar concentrations in aqueous solutions in the absence of carrier protein. (–)-AZ-DF-265 did not noticeably adsorb to plastic tubing or glass.

We demonstrate that (–)-AZ-DF-265 displaces [3 H]-glibenclamide from its binding site. The simplest explanation is that (–)-AZ-DF-265 and glibenclamide compete for the same binding sites. This view is supported by the perceived homology between the substituted carbamoyl group of (–)-

Figure 4 The octanol/water partitioning of (–)-AZ-DF-265 as a function of pH. The water phase contained either 50 mM K-borate and 50 mM K-phosphate (●) or 100 mM Na/K-phosphate (○) at the indicated pH. (–)-AZ-DF-265 was dissolved in the water phase at 46–182 μ M. The concentration remaining in the water phase after equilibration with 1-octanol was determined by measuring the absorbance at 240 nm against controls without drug. The points show mean (s.e.mean indicated by vertical bars) of 3–5 experiments at each pH.

AZ-DF-265 and the benzamido as well as possibly the sulphonylurea group of glibenclamide. In addition, (–)-AZ-DF-265 did not affect the rate of dissociation of bound [3 H]-glibenclamide. Hence, an alternative situation, in which (–)-AZ-DF-265 influences sulphonylurea binding either allosterically or via another protein, appears less probable.

Glibenclamide with its sulphonylurea and benzamido moieties may be bifunctional (Gutsche *et al.*, 1974) and bind to two closely spaced subunits of its receptor. Indeed, glibenclamide consistently showed positive cooperativity in our individual assays and also in pooled data from 15 different experiments. On the other hand, (–)-AZ-DF-265 is most likely mono-functional. (–)-AZ-DF-265 showed marked negative cooperativity ($m = 0.53 \pm 0.01$) in its displacement of [3 H]-glibenclamide. This could be due to steric hindrance between neighbouring bound (–)-AZ-DF-265 molecules. Indeed, meglitinide and tolbutamide, which represent essentially the benzamido and sulphonylurea moieties of glibenclamide respectively, had Hill coefficients of about 1.

Recently, Verspohl *et al.* (1990) showed that AG-EE-86 and AG-EE-388 displace [3 H]-glibenclamide from a particulate fraction of RINm5F cells. The AG-EE drugs differ from AZ-DF-265 in one substituent of the central carbamoyl group. Verspohl *et al.* (1990) fitted their data to a model with two types of receptors with a 2,000 fold difference in affinity. Our data did not permit us to determine the affinity of the low affinity receptors. However, visual inspection and comparison of our data with those of Verspohl suggests that low affinity binding sites were much less abundant in our preparation of intact cells than in their membrane preparation. In addition, our model which presumes exclusively high affinity glibenclamide binding sites fits our data well.

Compared to a 98.6% pure (–)-enantiomer, a 94.4% pure (+)-enantiomer of AZ-DF-265 is at least 10 fold less potent in stimulating insulin release from mouse islets (Garrino & Henquin, 1988). We have not yet studied the effect of (+)-AZ-DF-265 on the activity of ATP-sensitive K^+ -channels.

In conclusion, (–)-AZ-DF-265 leads to the inhibition of an ATP-sensitive potassium current in the whole-cell configuration and it also prevents the binding of [3 H]-glibenclamide to the sulphonylurea receptor. We therefore suggest that (–)-AZ-DF-265 binds to the sulphonylurea receptor.

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