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Highly potent and stereoselective effects of the benzoic acid derivative AZ-DF 265 on pancreatic β-cells

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1 Mouse islets were used to define the characteristics and study the mechanisms of the stimulation of insulin release by compound AZ-DF 265, 4-[[N-(α-phenyl-2-piperidino-benzyl) carbamoyl]methyl] benzoic acid, a substituted benzoic acid with an asymmetric carbon atom.

2 At a non-stimulatory concentration of glucose (3 mM), (−)-AZ-DF 265 reversibly inhibited ^{86}Rb efflux from islet cells, depolarized the β-cell membrane, induced electrical activity, stimulated ^{45}Ca efflux, and triggered insulin release. Maximum inhibition of ^{86}Rb efflux occurred at 0.03 μM (−)-AZ-DF 265, whereas the threshold concentration for stimulation of release was 0.1 μM. Omission of extracellular Ca^{2+} abolished all effects of the drug but the inhibition of ^{86}Rb efflux.

3 At a stimulatory concentration of glucose (10 mM), (−)-AZ-DF 265 reversibly increased ^{86}Rb efflux, potentiated electrical activity, augmented ^{45}Ca efflux, and increased insulin release. Maximum stimulation of ^{86}Rb efflux and insulin release was obtained with 0.03 μM (−)-AZ-DF 265. Omission of extracellular Ca^{2+} abolished all effects of the drug.

4 The potency of (−)-AZ-DF 265 was similar to that of glibenclamide, whereas the (+)-enantiomer was about 10 times less potent on ^{86}Rb efflux and insulin release.

5 It is concluded that, like sulphonylureas, compound AZ-DF 265 decreases K^+ permeability of the β-cell membrane and thereby causes depolarization. This activates voltage-dependent Ca channels, permits Ca^{2+} influx and eventually stimulates insulin release. Its stereoselectivity may help to elucidate the mechanisms of K channel blockade and, hence, lead to the design of more potent and specific insulinotropic drugs.

Introduction

Measurements of ionic fluxes in islet cells with radioactive tracers (Henquin, 1980; Hellman, 1981; Henquin & Meissner, 1982; Matthews & Shotton, 1984a), recordings of the β-cell membrane potential with microelectrodes (Matthews *et al.*, 1973; Henquin & Meissner, 1982), characterization of single channel properties with the patch-clamp technique (Sturgess *et al.*, 1985; Trube *et al.*, 1986), and estimations of cytoplasmic free Ca^{2+} concentration with a fluorescent dye (Abrahamsson *et al.*, 1985) have concurred to elucidate the mechanisms by which tolbutamide, the prototype of sulphonylureas of the first generation, increases insulin release. There is now wide agreement that the stimulation of β-cells by tolbutamide involves the following steps: by blocking ATP-dependent K^+ channels, tolbutamide decreases K^+ permeability of the β-cell membrane; this causes a depolarization that activates voltage-dependent Ca^{2+} channels; the ensuing Ca^{2+} influx brings about a rise in cytoplasmic free Ca^{2+} that ultimately triggers exocytosis of insulin granules. It is also believed that the same sequence of events underlies the action of the more potent sulphonylureas of the second generation such as glibenclamide or gliquidone (Matthews & Shotton, 1984a; Ferrer *et al.*, 1984; Garrino *et al.*, 1985; 1986). However, the molecular mechanisms of the interaction of sulphonylureas with their target (K^+ channels) are still unknown.

Two recent studies demonstrated that benzoic acid derivatives corresponding to the non-sulphonylurea moieties of glibenclamide and gliquidone stimulate β-cells by the same mechanism as the parent molecules (Garrino *et al.*, 1985; 1986). It is evident from these observations that the interaction with K^+ channels does not require a sulphonylurea group. However, the relatively low efficacy of these derivatives (100 to 200 times less potent than the parent molecules) does not

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preclude the possibility that such a sulphonylurea group is the optimal chemical structure for blocking K^+ channels in β-cells.

In the present study we show that compound AZ-DF 265 (Figure 1), 4-[[N-(α-phenyl-2-piperidino-benzyl) carbamoyl]methyl] benzoic acid, a substituted benzoic acid with an asymmetric carbon atom, stimulates insulin release with a potency comparable to that of glibenclamide. We also show that this effect is primarily due to a decrease in K^+ permeability of the β-cell membrane, and displays some stereoselectivity.

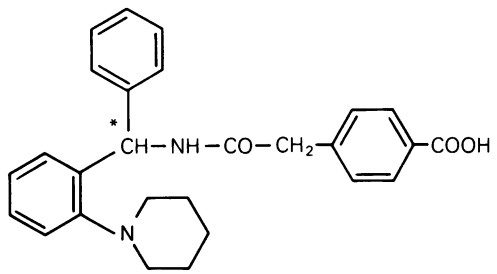

Figure 1 Structural formula of AZ-DF 265.

Methods

All experiments were performed with islets of fed female NMRI mice (25–30 g), killed by decapitation. For electrophysiological experiments, a piece of pancreas was fixed in a small perifusion chamber, and the membrane potential of single β-cells was continuously recorded with microelectrodes (Meissner & Schmelz, 1974). β-Cells were identified by the typical electrical activity that they display in the presence of 10–15 mM glucose (Matthews *et al.*, 1973; Meissner, 1976). For all other experiments, islets were isolated after collagenase digestion of the pancreas. The techniques and the dynamic system of perifusion used to monitor the efflux of ^{45}Ca or ^{86}Rb (used as tracer for K) from preloaded islets have been described (Henquin, 1979). Loading with the tracers was achieved by incubating the islets for 90 min in 0.25 ml medium containing 10 mM glucose. The medium was supplemented with either $^{45}CaCl_2$ (15 MBq ml^{-1}; sp.act. 6 TBq mol^{-1}), or $^{86}RbCl$ (1.5 to 3 MBq ml^{-1}; sp.act. 7.4 to 18.5 TBq mol^{-1}). The concentration of Rb^+ never exceeded 0.4 mM. The fractional rate of efflux was calculated on measured c.p.m. as no experimental condition affected counting efficiency and required quench correction. During the experiments of ^{86}Rb efflux, a portion of each effluent fraction was drawn for measurement of immunoreactive insulin; rat insulin was used as a standard (Henquin & Lambert, 1975).

The medium used had the following ionic composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25. It was gassed with 94% O_2/6% CO_2 to maintain a pH of 7.4, and was supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany), except for electrophysiological recordings. Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ by $MgCl_2$.

The two enantiomers of AZ-DF 265 were synthesized by Dr R. Hurnaus (Dr Karl Thomae GmbH, Biberach an der Riss, Germany). The enantiomeric purity was determined as follows: (−)-enantiomer, ee = 98.6%; (+)-enantiomer, ee = 94.4%. Stock solutions (2 mM) were prepared in NaOH (50 mM) and aliquots were added to the appropriate media. $^{86}RbCl$ and $^{45}CaCl_2$ were purchased from the Radiochemical Centre (Amersham, Bucks, U.K.). All other reagents were from Merck A.G. (Darmstadt, Germany).

Electrophysiological experiments are illustrated by recordings, which are representative of the indicated number of experiments, performed with different mice. Other results are presented as means ± s.e.means for the indicated number of experiments, performed with different islet preparations.

Results

Effects of (−)-AZ-DF 265 in the presence of a non-stimulatory concentration of glucose

Addition of 0.1 μM (−)-AZ-DF 265 to a medium containing 3 mM glucose and 2.5 mM Ca^{2+} inhibited ^{86}Rb efflux, accelerated ^{45}Ca efflux and stimulated insulin release from perifused islets (Figure 2). The inhibition of ^{86}Rb efflux occurred rapidly, but was progressive and stabilized 8–10 min after addition of the drug, when ^{45}Ca efflux and insulin release started to increase. Removal of (−)-AZ-DF 265 from the medium was followed by a delayed reversal of the stimulation of ^{45}Ca efflux and insulin release, that was accompanied by a transient further decrease in ^{86}Rb efflux.

The inhibition of ^{86}Rb efflux was faster and more pronounced when the medium did not contain Ca^{2+}. Its reversal was also delayed but was more complete than in the presence of extracellular Ca^{2+}. Neither ^{45}Ca efflux, nor insulin release was affected by (−)-AZ-DF 265 in the absence of Ca^{2+} (Figure 2).

When the islets were perifused with a medium containing 3 mM glucose and 2.5 mM Ca^{2+}, 0.03 μM (−)-AZ-DF 265 slowly depolarized the β-cell membrane and induced electrical activity (Figure 3a). This activity consisted of small amplitude spikes when the cell membrane was still depolarizing and of much larger spikes when the membrane potential had stabilized at a plateau. After removal of the drug, the

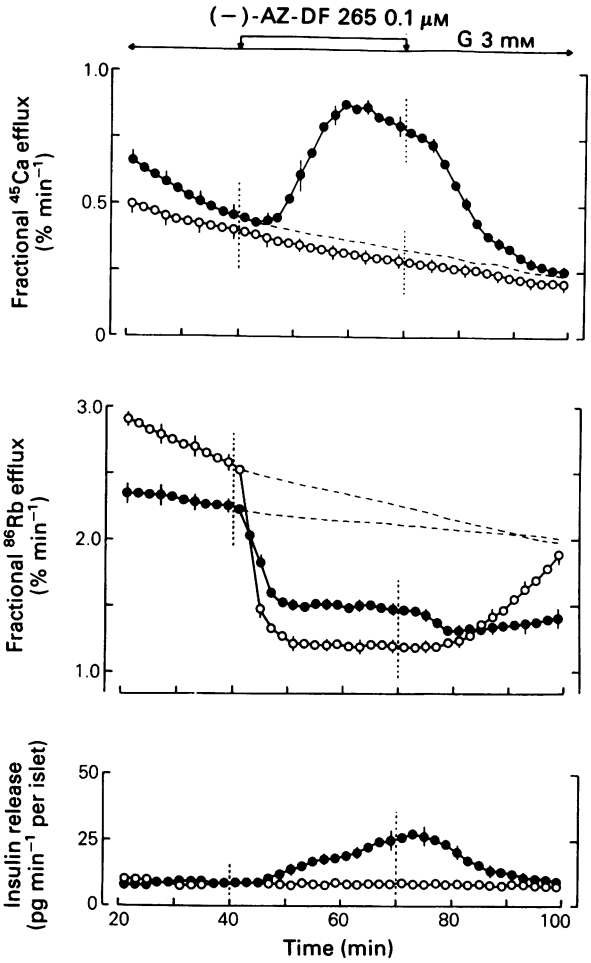

Figure 2 Effects of (−)-AZ-DF 265 on ^{45}Ca or ^{86}Rb efflux and on insulin release from mouse islets perifused with a medium containing a non-stimulatory concentration of glucose (G 3 mM). The experiments were performed in the presence of 2.5 mM Ca^{2+} (●) or in the absence of Ca^{2+} (○). (−)-AZ-DF 265 was added between 40 and 70 min, at a concentration of 0.1 μM. Control experiments without addition of test substance are shown by the broken lines. Values are means of 4–5 experiments; vertical lines show s.e.mean.

spike frequency decreased and the membrane potential started to oscillate in slow waves with bursts of spikes superimposed on the plateau (Figure 3b). Finally, all activity stopped and the membrane progressively repolarized.

Effects of (−)-AZ-DF 265 in the presence of a stimulatory concentration of glucose

When the perifusion medium contained 10 mM glucose and 2.5 mM Ca^{2+}, the rates of ^{45}Ca efflux and of insulin release were higher, whereas the rate of ^{86}Rb efflux was lower than in the presence of 3 mM glucose. Addition of 0.03 μM (−)-AZ-DF 265 increased ^{45}Ca efflux, ^{86}Rb efflux and insulin release (Figure 4). All these effects were slowly reversible after withdrawal of the drug.

When the medium contained 10 mM glucose but no Ca^{2+}, the rate of ^{86}Rb efflux was marginally higher than in the presence of Ca^{2+}, while the rate of ^{45}Ca efflux was markedly decreased and insulin release no longer stimulated. Under these conditions, (−)-AZ-DF 265 was without effect (Figure 4).

The electrical activity induced by 10 mM glucose in β-cells was markedly increased by 0.01 μM (−)-AZ-DF 265 (Figure 3c). This increase was characterized first by a lengthening of the slow waves with spike activity and a shortening of the polarized silent intervals. In all cells tested the membrane eventually remained persistently depolarized at the plateau potential and the spike activity became continuous. Removal of the drug was followed by resumption of slow waves, but after a longer delay than in the presence of 3 mM glucose (not shown).

Stereoselectivity of the effects of AZ-DF 265

Figures 5 and 6 compare the effects of various concentrations of (−)-AZ-DF 265 and (+)-AZ-DF 265 on ^{86}Rb efflux and insulin release from islets perifused with 3 and 10 mM glucose, respectively.

In the presence of 3 mM glucose and 2.5 mM Ca^{2+}, the inhibition of ^{86}Rb efflux by (−)-AZ-DF 265 was already marked at 0.01 μM, was maximal at 0.03 μM, and was less pronounced at higher concentrations, which, on the other hand, stimulated insulin release. At concentrations between 0.01 and 0.3 μM, (+)-AZ-DF 265 brought about a dose-dependent decrease in ^{86}Rb efflux without affecting basal insulin release. This latter was increased by 1 μM (+)-AZ-DF 265, which, however, inhibited ^{86}Rb efflux less potently than did 0.3 μM (Figure 5). The paradoxically smaller effectiveness of the high concentrations of (−)-AZ-DF 265 on ^{86}Rb efflux was not observed in the absence of extracellular Ca^{2+}. Thus, a decrease in ^{86}Rb efflux rate of 1.23 ± 0.05, 1.32 ± 0.06, 1.36 ± 0.04 and $1.31 \pm 0.04\%$ min^{-1} ($n = 4-5$) was induced by 0.03, 0.1, 0.3 and 1.0 μM (−)-AZ-DF 265, respectively.

In the presence of 10 mM glucose and 2.5 mM Ca^{2+}, (−)-AZ-DF 265 increased ^{86}Rb efflux and insulin release at all concentrations tested, the effect plateauing above 0.03 μM (Figure 6). On the other hand, (+)-AZ-DF 265 was ineffective at 0.01 μM and produced

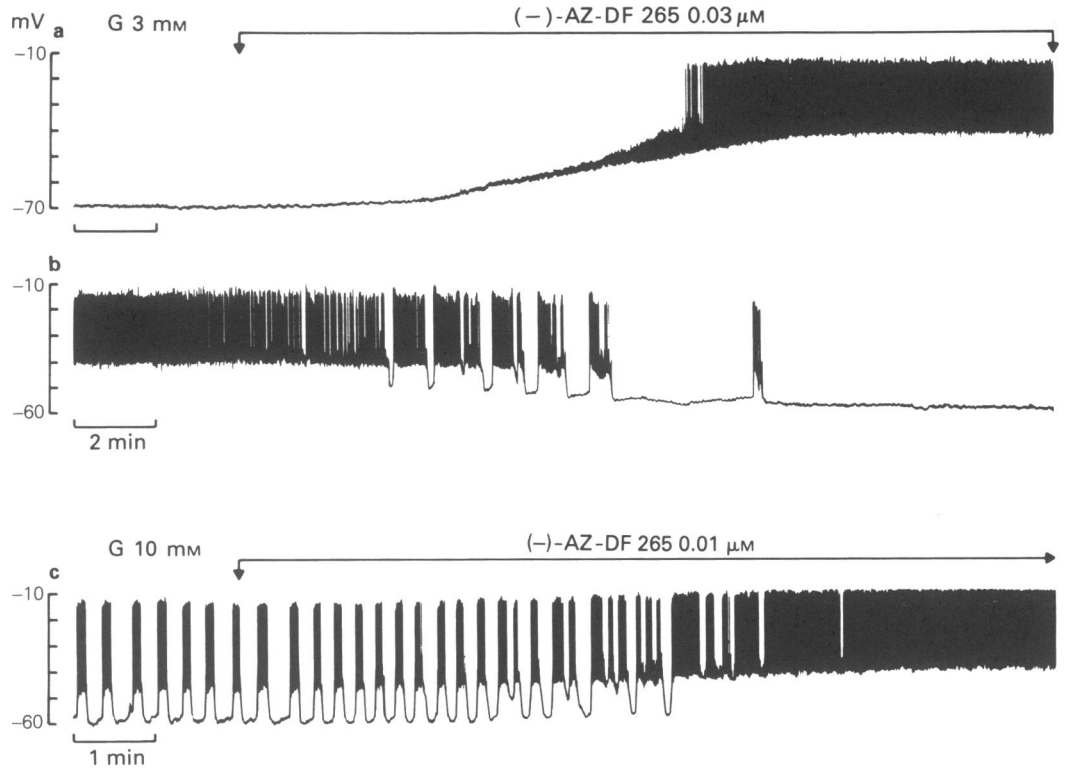

Figure 3 Effects of (−)-AZ-DF 265 on the membrane potential of mouse β-cells perifused with a medium containing 3 mM glucose (G 3 mM) (a and b), or 10 mM glucose (G10 mM) (c). The drug was added at the indicated concentrations, and for the periods shown by the arrows. Record (b) is the direct continuation of (a) and thus starts immediately after withdrawal of (−)-AZ-DF 265. Record (c) was obtained in another cell. These records are representative of 4 experiments at each concentration of glucose.

its maximum effect at 0.3 μM.

The changes in ^{86}Rb efflux and insulin release induced by (+)-AZ-DF 265 followed the same time-course as those induced by an equipotent concentration of (−)-AZ-DF 265. The concentration of each enantiomer also had little influence on that time-course, but the onset of the effect became faster as the concentration was raised. For instance, in the presence of 3 mM glucose, the first significant increase above basal insulin release occurred after 12 min and 6 min with 0.1 μM and 1 μM (−)-AZ-DF 265, respectively. However, the progression of the response, without an initial peak of secretion, persisted. The other significant difference was that the reversal of the effects became slower and less complete as higher concentrations of the drug were used.

Discussion

In a recent communication (Grell *et al.*, 1986) it was reported that compound AZ-DF 265 causes hypoglycaemia in the rat, with an efficacy comparable with that of glibenclamide, the most powerful hypoglycaemic sulphonylurea currently used in clinical practice. The present study demonstrates that AZ-DF 265 is indeed a very potent stimulator of insulin release *in vitro*. A direct action on pancreatic β-cells may thus account for its hypoglycaemic properties.

Comparison with one of our previous studies (Garrino *et al.*, 1985) indicates that, in the presence of 3 mM glucose, the effects of 0.1 μM glibenclamide on ^{86}Rb efflux and insulin release were similar to those of 0.1 μM (−)-AZ-DF 265. It also shows that, in the

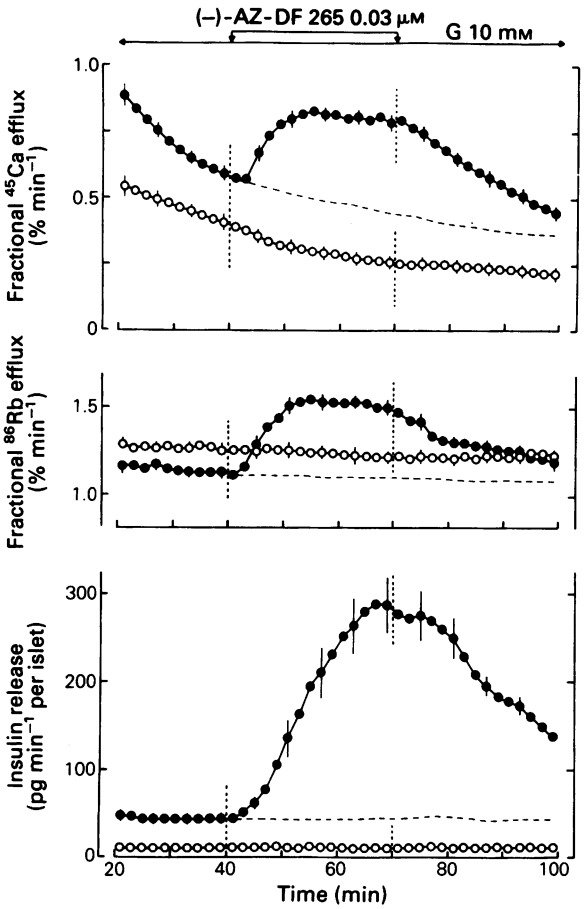

Figure 4 Effects of (−)-AZ-DF 265 on ^{45}Ca or ^{86}Rb efflux and on insulin release from mouse islets perifused with a medium containing a stimulatory concentration of glucose (G 10 mM). The experiments were performed in the presence of 2.5 mM Ca^{2+} (●) or in the absence of Ca^{2+} (○). (−)-AZ-DF 265 was added between 40 and 70 min, at a concentration of 0.03 μM. Control experiments without addition of test substance are shown by the broken lines. Values are means of 4–5 experiments; vertical lines show s.e.mean.

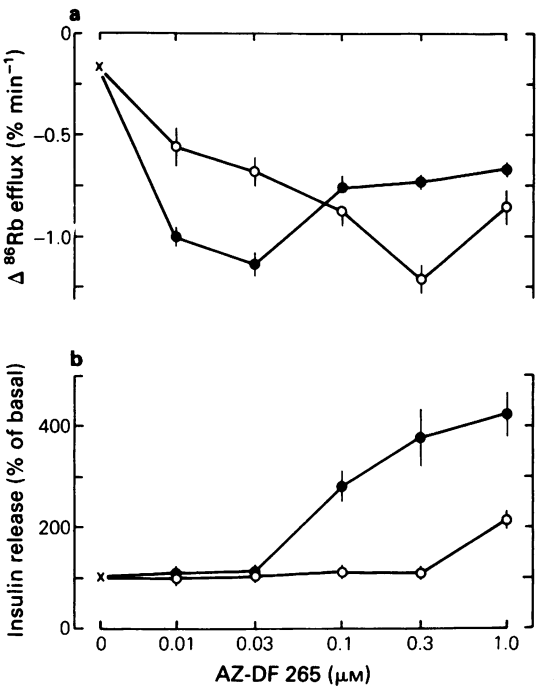

Figure 5 Effects of different concentrations of the two enantiomers (●, (−)-enantiomer; ○, (+)-enantiomer) of AZ-DF 265 on ^{86}Rb efflux (a) and insulin release (b) from mouse islets perifused with a medium containing 3 mM glucose and 2.5 mM Ca^{2+}. The results were obtained in experiments similar to those shown in Figure 2. The effect on ^{86}Rb efflux is given as the difference between the actual efflux rate immediately before and 30 min after addition of the drug. The effect on insulin release was computed by expressing the actual rate of release 30 min after addition of the drug as a percentage of the basal rate of release, measured immediately before addition of the drug. Values are means of 4–5 experiments; vertical lines show s.e.mean.

presence of 10 mM glucose, the effects of 0.02 μM glibenclamide were similar to those of 0.01–0.03 μM (−)-AZ-DF 265. Glibenclamide and (−)-AZ-DF 265 thus appear to have roughly the same potency in our *in vitro* system. This conclusion is unlikely to be biased by important differences in the proportion of free drug in the medium, since AZ-DF 265 is as extensively bound to plasma proteins as glibenclamide (Crooks & Brown, 1974; A. Greischel, personal communication).

Glibenclamide and (−)-AZ-DF 265 not only appear to be equipotent, but their respective effects on ionic fluxes, β-cell membrane potential and insulin release display a fairly similar, progressive time-course (Ostenson *et al.*, 1983; Ferrer *et al.*, 1984; Pace, 1984; Garrino *et al.*, 1985). It should be pointed out that whenever glibenclamide was shown to exert rapid effects on β-cells (Meissner & Atwater, 1976; Ostenson *et al.*, 1983; Matthews & Shotton, 1984a; Ferrer *et al.*, 1984; Pace, 1984), the drug was used at high concentrations (1–100 μM), that considerably exceed the levels ever reached in the plasma of treated patients (Rupp *et al.*, 1972). An important difference exists, however, between AZ-DF 265 and glibenclamide: all

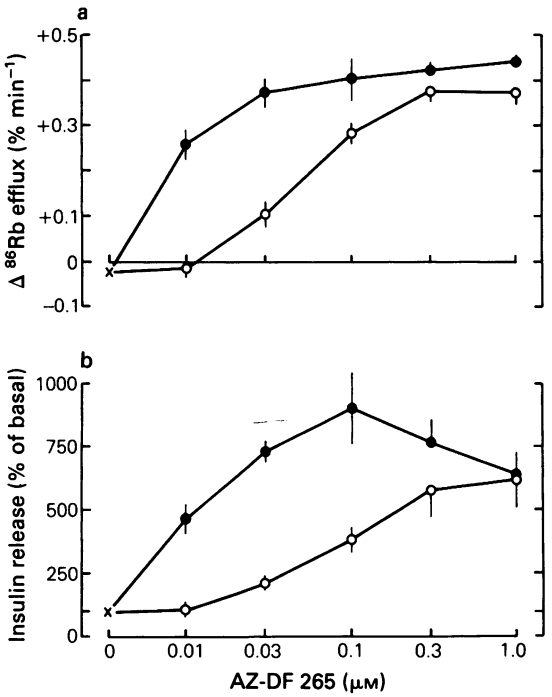

Figure 6 Effects of different concentrations of the two enantiomers (●, (−)-enantiomer; ○, (+)-enantiomer) of AZ-DF 265 on ^{86}Rb efflux (a) and insulin release (b) from mouse islets perifused with a medium containing 10 mM glucose and 2.5 mM Ca^{2+}. The results were obtained in experiments similar to those shown in Figure 4. Same mode of expression as in Figure 5. Values are means of 4–5 experiments; vertical lines show s.e.mean.

effects of AZ-DF 265 were at least partially reversible, whereas the effects of glibenclamide are practically irreversible within the time studied (Meissner & Atwater, 1976; Ostenson *et al.*, 1983; Matthews & Shotton, 1984a, Ferrer *et al.*, 1984), even when the sulphonylurea is used at low concentrations, equipotent to those of (−)-AZ-DF 265 (Garrino *et al.*, 1985).

The progressiveness of the changes recorded upon addition and withdrawal of glibenclamide or AZ-DF 265 contrasts with the rapidity of the changes brought about, under similar conditions, by a less potent sulphonylurea such as tolbutamide (Henquin, 1980; Henquin & Meissner, 1982; Matthews & Shotton, 1984a), or by a benzoic acid derivative such as compound HB 699, which contains the non-sulphonylurea moiety of glibenclamide (Garrino *et al.*, 1985). It is possible that the high potency of drugs active at nanomolar concentrations is due to their progressive accumulation in the β-cell membrane (Deleers & Malaisse, 1984; Gylfe *et al.*, 1984), thereby submitting their target (K^+ channels) to the influence of a concentration substantially higher than that of the medium. Further structure-activity studies will be necessary to establish whether an increase in drug potency is inevitably associated with a decrease in the rapidity of its effects and of their reversal.

The changes in ionic fluxes and β-cell membrane potential brought about by AZ-DF 265 were qualitatively similar to those produced by hypoglycaemic sulphonylureas or by other benzoic acid derivatives (Henquin, 1980; Hellman, 1981; Henquin & Meissner, 1982; Matthews & Shotton, 1984a; Ferrer *et al.*, 1984; Pace, 1984; Garrino *et al.*, 1985; 1986). These analogies make it unnecessary to discuss again these changes in detail. They permit one to conclude that AZ-DF 265 depolarizes the β-cell membrane by decreasing its permeability to K^+ and that this depolarization leads to activation of voltage-dependent Ca^{2+} channels with subsequent influx of Ca^{2+}.

However, two apparently paradoxical observations deserve some comment. The inhibition of ^{86}Rb efflux produced by (−)-AZ-DF 265 at low glucose was larger in the absence than in the presence of Ca^{2+}, and was not directly dose-dependent in the presence of Ca^{2+}. It was maximum at the concentration just below that starting to stimulate insulin release. A similar phenomenon has been observed with sulphonylureas and other benzoic acid derivatives (Henquin & Meissner, 1982; Matthews & Shotton, 1984a; Garrino *et al.*, 1985; 1986). It can be ascribed to activation, by incoming Ca^{2+} and the greater depolarization of the membrane (Henquin, 1979; Matthews & Shotton, 1984b; Trube *et al.*, 1986), of K^+ channels not blocked by AZ-DF 265. These are probably the voltage- and Ca^{2+}-activated K^+ channels identified in islet cells (Cook *et al.*, 1984; Findlay *et al.*, 1985), but the intervention of Ca^{2+}-activated, non-selective cation channels (Sturgess *et al.*, 1986) is also possible. The same explanation holds true for the small acceleration of ^{86}Rb efflux caused by (−)-AZ-DF 265 in the presence of 10 mM glucose. Conversely, the further decrease in ^{86}Rb efflux occurring when (−)-AZ-DF 265 was withdrawn from a medium containing 3 mM glucose and 2.5 mM Ca^{2+} may reflect suppression of the activation of Ca^{2+}-sensitive K^+ channels, while the action of the drug on other K^+ channels, presumably the ATP-sensitive K^+ channels (Sturgess *et al.*, 1985; Trube *et al.*, 1986), still persists. It should be emphasized, however, that the observed changes in ^{86}Rb efflux are only tentatively ascribed to specific types of K^+ channels. Since no pharmacological blocker is sufficiently specific of one type of K^+ channel, single channel studies by the patch clamp technique will be necessary to verify the validity of our interpretation.

Very little is known about the stereoselectivity of hypoglycaemic substances. More than 20 years ago, studies on the metabolites of the sulphonylurea acetohexamide revealed that the (−)-enantiomer of hydroxyhexamide was 2.4 times more potent than the racemic mixture in inducing hypoglycaemia in the rat (McMahon *et al.*, 1965). Stereoselectivity has also been described for sulphonamides of the second generation possessing an asymmetric C atom directly attached to the N atom of the amide group present in their non-sulphonylurea moiety (Rufer *et al.* 1974), as well as for the benzoic acid derivative corresponding to that non-sulphonylurea moiety (Rufer & Losert, 1979). For all tested compounds, the hypoglycaemic activity of the (S)-enantiomer was 20–100 times greater than that of the (R)-enantiomer. A ratio of about 15 characterizes the hypoglycaemic potency of the two enantiomers of AZ-DF 265 (E. Rupprecht, personal communication). Such *in vivo* studies did not identify the site(s) of the stereoselectivity. The present work is, to our knowledge, the first to demonstrate stereoselective stimulation of insulin release *in vitro* by a pharmacological agent of potential clinical usefulness. The potency of (−)-AZ-DF 265 was 10 times higher than that of (+)-AZ-DF 265. This ratio, similar to that found *in vivo*, is likely to be underestimated because of the small contamination of the (+)-enantiomer by the more potent (−)-enantiomer of AZ-DF 265. Most interestingly, this stereoselectivity also characterized the effect of the drug on ^{86}Rb efflux. This lends further support to the above conclusion that the change in K^+ permeability of the β-cell membrane is critically involved in the stimulation of insulin release. This also suggests that blockade of K^+ channels does not merely result from accumulation of the drug in the membrane but involves a much more specific interaction with a reactive site (NH-CO?) in the molecule.

In conclusion, stimulation of insulin release with a very high potency can be achieved by a pharmacological agent that is not a sulphonamide. A sulphonylurea group should no longer be regarded as the optimal chemical function for an interaction with pancreatic β-cells. Pharmacokinetic studies will be necessary to ascertain whether benzoic acid derivatives such as AZ-DF 265 have clinical advantages over currently available drugs. Their stereoselectivity, however, makes them interesting tools to gain insight into the mechanisms of K^+ channel blockade, in particular to determine whether this blockade results from the interaction of various chemical groups with a single binding site of relatively low specificity or with distinct binding sites. It may also help disclose differences between K^+ channels in β-cells and other tissues (in particular heart cells). One may hope that this new information will lead to the synthesis of the optimal drug aimed at stimulating insulin release.

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