British Journal of Pharmacology (1999) 128, 1021–1026
http://www.stockton-press.co.uk/bjp

In vitro mechanism of action on insulin release of S-22068, a new putative antidiabetic compound

[1]Laurence Le Brigand, [1]Anne Virsolvy, [2]Dominique Manechez, [3]Jean-Jacques Godfroid, [2]Beatrice Guardiola-Lemaître, [4]Fiona M. Gribble, [4]Frances M. Ashcroft & *,[1]Dominique Bataille

[1]INSERM U 376, CHU Arnaud-de-Villeneuve, 34295 Montpellier Cedex 05, France; [2]Institut de Recherches Internationales Servier, 92415 Courbevoie Cedex, France, [3]Laboratoire de Pharmacochimie Moléculaire, Université Paris VII-Denis Diderot, 75251 Paris Cedex 05, France, [4]University Laboratory of Physiology, Parks Road, Oxford OX1 3PT

1 The MIN6 cell line derived from *in vivo* immortalized insulin-secreting pancreatic β cells was used to study the insulin-releasing capacity and the cellular mode of action of S-22068, a newly synthesized imidazoline compound known for its antidiabetic effect *in vivo*.

2 S-22068, was able to release insulin from MIN6 cells in a dose-dependent manner with a half-maximal stimulation at 100 μM. Its efficacy (8 fold over the basal value), which did not differ whatever the glucose concentration (stimulatory or not), was intermediate between that of sulphonylurea and that of efaroxan.

3 Similarly to sulphonylureas and classical imidazolines, S-22068 blocked K_{ATP} channels and, in turn, opened nifedipine-sensitive voltage-dependent Ca^{2+} channels, triggering Ca^{2+} entry.

4 Similarly to other imidazolines, S-22068 induced a closure of cloned K_{ATP} channels injected to *Xenopus* oocytes by interacting with the pore-forming Kir6.2 moiety.

5 S-22068 did not interact with the sulphonylurea binding site nor with the non-I_1 and non-I_2 imidazoline site evidenced in the β cells that is recognized by the imidazoline compounds efaroxan, phentolamine and RX821002.

6 We conclude that S-22068 is a novel imidazoline compound which stimulates insulin release *via* interaction with an original site present on the Kir6.2 moiety of the β cell K_{ATP} channels.

Keywords: Imidazoline; S-22068; pancreatic β cell; MIN6 cell line; insulin release; K_{ATP} channel; calcium channel; binding site

Abbreviations: ATP, adenosine 5′ triphosphate; BSA, bovine serum albumin; cRNA, complementary ribonucleic acid; EDTA ethylenediaminetetraacetic acid; IRIS, Institut International de Recherche Servier; K_{ATP}, ATP-dependent potassium channels; KRB, Krebs-Ringer bicarbonate, mRNA, messager ribonucleic acid

Introduction

It is now well established that certain imidazoline compounds such as efaroxan or phentolamine, which belong to a newly defined class of pharmacological agents, are able to stimulate insulin release (Chan *et al*., 1991; 1993). This stimulatory effect implicates the ATP-dependent potassium channels (K_{ATP} channels) present in the β cell plasma membrane (Plant & Henquin, 1990; Dunne 1991). The K_{ATP} channel is made up of two subunits: a sulphonylurea receptor, SUR1, and a pore-forming subunit, Kir 6.2 (Inagaki *et al*., 1995; Sakura *et al*., 1995). Inhibition of these channels leads to cell depolarization, calcium entry and finally insulin secretion. These effects observed both *in vivo* and *in vitro*, result from the interaction with an atypical binding site which differs from the imidazoline I_1 and I_2 sites defined in other tissues. This novel site, associated with the K_{ATP} channel (Olmos *et al*., 1994; Chan *et al*., 1991; 1993; 1994), appears to be localized on the channel itself (Dunne, 1991) and particularly on the Kir6.2 pore-forming subunit (Proks & Ashcroft, 1997).

The aim of the present study was to analyse the property of a new imidazoline derivative, S-22068, on insulin secretion from a β cell line. This molecule was shown to improve glucose tolerance and to increase insulin secretion, *in vivo* in mildly diabetic rats, without causing hypoglycemia (Pelé-Tounian *et al*., 1998). We compared its mechanism of action with that of other secretagogues, such as other imidazoline compounds or sulphonylureas. We finally studied its interaction with the binding sites recognized by other insulin-releasing imidazoline drugs. Owing to its characteristics, this imidazoline derivative may represent a new pharmacological basis for the treatment of type II diabetes.

*Author for correspondence; E-mail: bataille@montp.inserum.fr

Methods

Chemicals

S-22068 (Figure 1) was synthesized by the Laboratory of Molecular Pharmacochemistry, Université Paris VII, and the division of external chemistry of the Institut International de Recherches Servier (IRIS). The other drugs used in this study were as follows: glibenclamide (Guidotti, Italy), [^{3}H]-glibenclamide with a specific activity of 48–51 Ci mmol^{-1} (Amersham, U.K.), efaroxan (Research Biochemicals Incorporated, U.S.A.), clonidine (Sigma, U.S.A.), phentolamine (Sigma), idazoxan (Sigma), [^{3}H]-RX821002 with a specific activity of 56 Ci mmol^{-1} was from Amersham, 2-methoxy-idazoxan, yohimbine, diazoxide, nifedipine, verapamil and oligomycin were all from Sigma. Bovine serum albumin (BSA), fraction V, was from Boehringer (Germany).

Cell culture

MIN6 cells, kindly provided by Dr Ishihara (Third Department of Internal Medicine, University of Tokyo, Japan), were grown as previously described (Ishihara *et al*., 1993) at 37°C in a 5% CO_2 atmosphere in sterile plastic flasks (T75, TPP) in Dulbecco's modified Eagle medium (DMEM, Gibco, U.S.A.) containing 25 mM glucose supplemented with 15% foetal calf serum (FCS, Gibco), 100 u ml^{-1} penicillin (Gibco), 100 $\mu g\ ml^{-1}$ streptomycin (Gibco) and 5 $\mu l\ l^{-1}$ β-mercaptoethanol (Sigma).

Cells were seeded 5 days before each series of studies, in 24-well plates (TPP) at a density of 500,000 cells per well. For the studies described, cells were used between passages 15 and 25.

Studies on insulin release

Eighteen hours before the experiments, the culture medium was renewed. On the day of the experiment, the cells were washed twice with Krebs-Ringer bicarbonate (KRB) buffer, pH 7.5, containing 0.1% BSA (KRB-BSA). They were then preincubated for 1 h in KRB-BSA containing 1 mM glucose at 37°C, 5% CO_2, and incubated for 2 h in KRB-BSA containing various concentrations of glucose and/or other effectors. After incubation, the medium was collected, centrifuged at 600 × *g* for 5 min and stored at −20°C. Insulin release was measured by radioimmunoassay using ^{125}I-porcine insulin, rat insulin (Novo, Denmark) as standard and the guinea-pig anti-porcine insulin antibody 41 previously described (Kervran *et al*., 1976).

Measurement of $^{45}Ca^{2+}$ influx

Twenty-four hours before the experiment, the culture medium was changed. On the day of the experiment, the cells were preincubated for 10 min at 37°C in KRB (Pian-Smith *et al*., 1988). The preincubation solution was then replaced with 250 μl KRB containing 8 $\mu Ci\ ml^{-1}$ $^{45}CaCl_2$ (Amersham, 5–50 mCi mg^{-1} Ca) and the test agents and was incubated for 20 min at 37°C. The reaction was stopped by aspiration of the medium. The cells were rapidly washed four times with ice-cold buffer (in mM: NaCl 35, KCl 5, $CaCl_2$ 2.5, $LaCl_2$ 1, HEPES 10) and then solubilized in 1 ml KRB containing 0.1% Triton for 1 h at room temperature. An aliquot of the solution was then assayed for $^{45}Ca^{2+}$ content after addition of a liquid scintillation medium (PCS, Amersham).

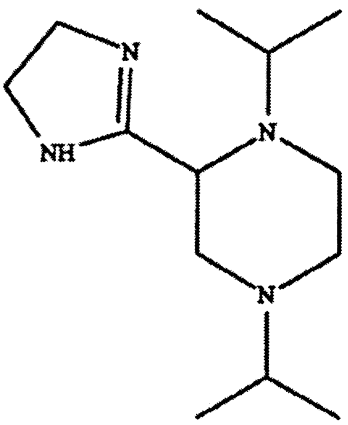

Figure 1 Chemical structure of S-22068.

Measurement of $^{86}Rb^{+}$ efflux

Twenty-four hours before the experiment, the culture medium was replaced and ^{86}RbCl (Amersham, 0.5–10 mCi mg^{-1} Rb) was added to the wells (0.1 μCi per well). Cells were loaded overnight with the isotope (Niki *et al*., 1989). On the day of the experiment, the culture medium was removed and the cells were washed twice with KRB and preincubated for 20 min at 37°C in KRB containing 0.2 $\mu Ci\ ml^{-1}$ ^{86}RbCl. 1.2 $\mu g\ ml^{-1}$ oligomycin and 1 mM 2-deoxy-D-glucose were added in order to decrease the ATP pool and thus open the K_{ATP} channels (Niki *et al*., 1989). The preincubation medium was then replaced with KRB containing various concentrations of the test agents. The incubation was performed at 37°C for 10 min and the medium was then set apart and cells were extracted with 1 ml 0.1 M NaOH for 1 h. The extract and medium were then counted using Cerenkov counting.

Electrophysiological experiments

Xenopus laevis oocytes were injected with mRNA encoding a truncated form of Kir6.2 (Tuker *et al*., 1997) or were coinjected with mouse Kir6.2 and rat SUR1 cRNA (GenBank D50581), (Aguilar-Bryan *et al*., 1995, Sakura *et al*., 1995). Macroscopic currents were recorded 1–4 days later from giant inside-out patches, using an EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) at 20–24°C (Gribble *et al*., 1997a,b). The pipette solution contained (mM): KCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 10 (pH 7.4 with KOH). The internal (bath) solution contained (mM): KCl 110, $MgCl_2$ 1.44, KOH 30, EGTA 10, HEPES 10 (pH 7.2 with KOH). Solutions were exchanged by positioning the patch electrode in the mouth of one of a series of adjacent inflow pipes (Gribble *et al*., 1997a).

Membrane preparation

Cells were grown in plastic flasks (150 TPP). Membranes were prepared as previously described (Göke *et al*., 1989). After 7 days of culture, the culture medium was removed and cells were detached from the surface of the plastic flasks by 30 ml of phosphate buffer/EDTA (in mM: NaCl 136, KCl 27, Na_2HPO_4

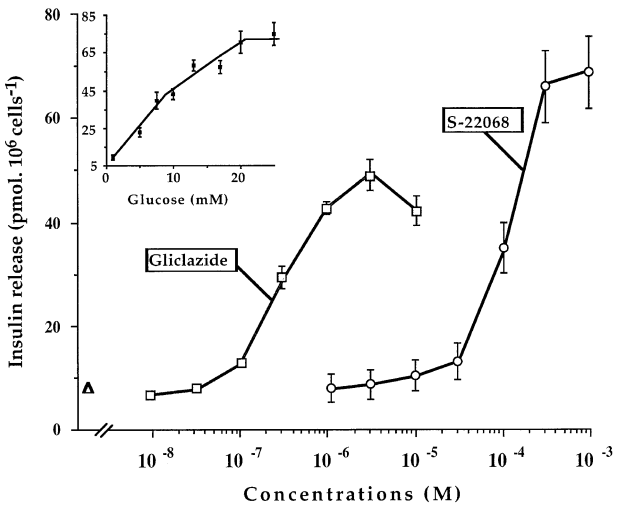

Figure 2 Stimulatory effect of S-22068, in comparison with that of the sulphonylurea gliclazide, on insulin release from MIN6 cells in culture. Experiments were conducted in KRB containing 1 mM glucose (a non-stimulatory concentration). Inset: dose-dependence of glucose on insulin release from MIN6 cells. Data are presented as mean values ± s.e.mean of six experiments.

8.1, EDTA 0.7) pH 7.5 at 37°C, and centrifuged at 4°C for 5 min at 500 × *g*. Cells were disrupted in 15 ml of Tris-HCl homogenization buffer (Tris 10 mM, NaCl 30 mM, Dithiotreitol 1 mM, PMSF 5 mM) pH 7.5 at 4°C, using a glass/glass homogenizer. The homogenate was layered over 15 ml of a 41% (wt v^{-1}) sucrose solution at 4°C and centrifuged at 95,000 × *g* for 60 min at 4°C (Beckman, rotor SW 28). The band at the interface of the layers was collected, diluted with 30 ml of Tris-HCl buffer and centrifuged at 46,000 × *g* for 10 min at 4°C. The pellet was frozen in liquid N_2 and stored at −80°C. Membranes were resuspended in 50 mM Tris-HCl at 50 μg ml^{-1} protein concentrations. This membrane preparation was enchiched in plasma membranes, as shown by specific enzymatic markers for plasma membranes (5′ nucleotidase) and endoplasmic reticulum (cytochrome c reductase) : as compared to the crude preparation, the membrane fraction is enriched 7.5 ± 1.5 fold (mean ± s.e.mean of three determinations) in 5′-nucleotidase, while it is enriched 2.3 fold in cytochrome c reductase.

Binding studies

The radioligand [^{3}H]-RX821002 (Amersham) was used at a final concentration of 2 nM in 50 mM Tris-HCl, pH 7.5. Non-specific binding was defined in the presence of 10 mM unlabelled compound. In this type of experiment, all incubations were performed in the presence of 10 μM yohimbine in order to block α_2-adrenoceptors. Incubation was performed for 60 min at room temperature in the presence of 200 μl of the membrane suspension, 100 μl of the labelled ligand and 200 μl of standard or the test compound in 50 mM Tris-HCl buffer, pH 7.5. The bound radioligand was separated from free by the addition of 3 ml of 50 mM Tris-HCl at 4°C and immediate filtration on Whatman glass fibre filters (GF/B) under vacuum. The filters were quickly washed three times with 3 ml of 50 mM Tris-HCl buffer and counted in a scintillation medium (ACS II, Amersham). Typically, total binding was 300 c.p.m. while non-specific binding was 45 c.p.m.

Results

We first analysed the capacity of S-22068 to induce insulin secretion directly from the MIN6 β-cells in culture. As previously observed, the response of MIN6 cells to glucose lies within the physiological range (Ishihara *et al*., 1993;

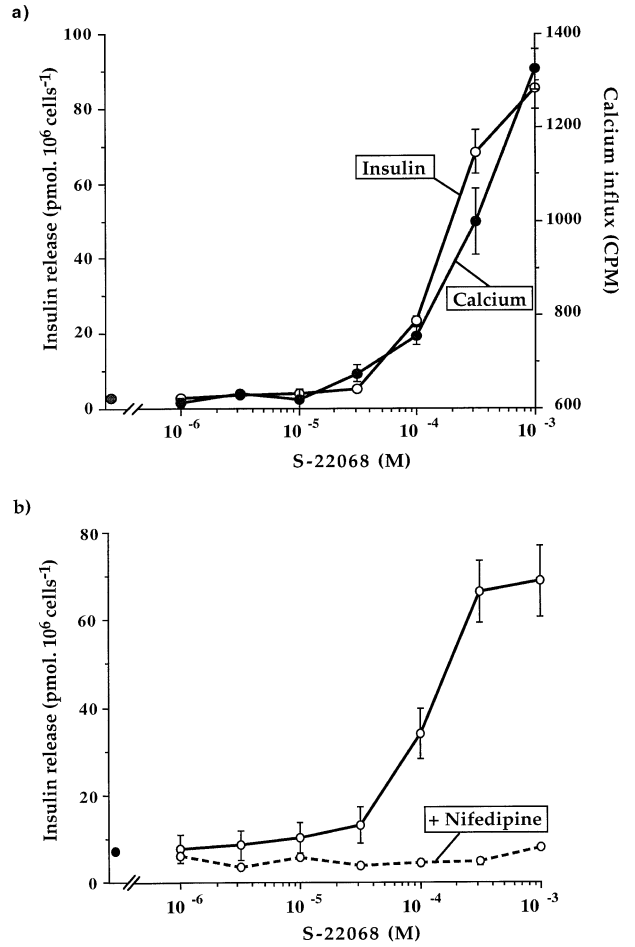

Figure 3 (a) Compared effects of S-22068 on insulin release and on $^{45}Ca^{2+}$ uptake from MIN6 cells on the same passage. Insulin release and $^{45}Ca^{2+}$ uptake were measured in the same medium containing 1 mM glucose after a 2 h incubation. Data are presented as mean values ± s.e.mean of six experiments. (b) Stimulatory effect of S-22068 on insulin release from MIN6 cells in the presence or in the absence of 0.1 mM nifedipine. Data are mean values ± s.e.mean of six experiments.

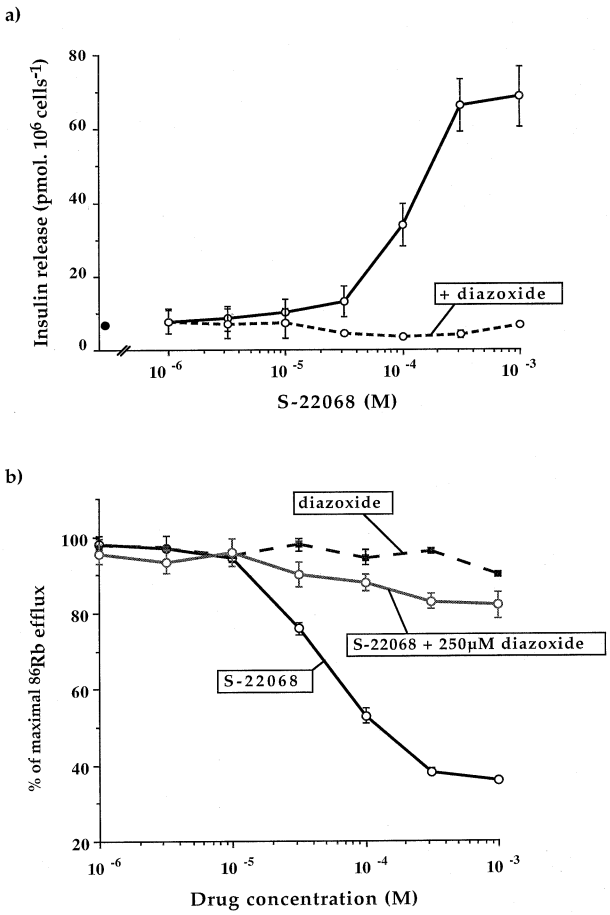

Figure 4 (a) Insulin release from MIN6 cells either in the absence or in the presence of 250 μM diazoxide. Experiments were conducted at 1 mM glucose (a non-stimulatory concentration). Data are presented as mean values ± s.e.mean of six determinations. (b) $^{86}Rb^+$ efflux from MIN6 cells. Experiments were conducted at 1 mM glucose, in the absence or in the presence of various concentrations (broken line labelled 'diazoxide') or in the presence of 250 μM diazoxide together with various concentrations of S-22068. Data are expressed as percentage of the maximal efflux observed in control experiments.

Poitout *et al*., 1996), with a half-maximal stimulation at 7.5 mM and a maximal stimulation of 7.5 fold above basal secretion (Figure 2, inset). As shown in Figure 2, the novel imidazoline derivative S-22068 induced a dose-related increase in insulin release, with a half maximal activation at a concentration of 100 μM. The maximal increase in secretion was 8 fold greater than basal release and higher than that observed for the sulphonylurea gliclazide. When the glucose concentration was raised to 5 mM (stimulatory concentration), both molecules display similar efficacy (not shown).

Since insulin release is a calcium-dependent process (Nelson *et al*., 1987; Hugues *et al*., 1988), we have determined whether S-22068 modulates calcium influx through the β cell membrane. As shown in Figure 3a, S-22068 induced a rise in $[Ca]_i$ with an ED_{50} of 200 μM corresponding to that of insulin secretion. The dose-response curves for insulin release and calcium entry are superimposable (Figure 3a). Since calcium entry associated with insulin release generally results from the activation of L-type calcium channels, we studied the effect of nifedipine, an L-type calcium channel blocker, on S-22068-induced insulin secretion. We observed that the effect of S-22068 was completely suppressed in the presence of 100 μM nifedipine (Figure 3b).

Since activation of calcium channel may be the consequence of an inhibition of K_{ATP} channels, we examined the effect of S-22068 on these channels. We analysed the action of this derivative both indirectly *via* ^{86}Rb efflux (rubidium being used as a substitute for potassium) and directly by electrophysiological measurements (patch-clamp experiments) : we first observed that the molecule was no longer able to stimulate insulin secretion when the K_{ATP} channels were opened in the presence of diazoxide (Figure 4a). Similarly to what is observed for sulphonylurea (Schmid-Automarchi *et al*., 1987; Panten *et al*., 1996) or classical imidazoline compounds, S-22068 reduced ^{86}Rb efflux (Figure 4b). Thus, S-22068 seemed to stimulate insulin secretion through an action on K_{ATP} channels. Binding studies using ^{3}H-glibenclamide indicated that S-22068 does not interfere with the sulphonylurea receptor (data not shown), suggesting an interaction with another component of the channel. To confirm such a mechanism, we analysed the effect of the compound on the activity of K_{ATP} channels reconstituted in *Xenopus* oocytes. We used both the wild-type K_{ATP} (Kir6.2/SUR1) and the Kir6.2ΔC36, a truncated form of Kir6.2 lacking the last 36 residues of the C-terminus which generates a current in the absence of SUR1, in contrast to the full-length kir6.2 (Tuker *et al*., 1997). S-22068 (100 μM) inhibited both types of channels to a similar extent (Figure 5): Kir6.2/SUR1 currents were reduced by 79±4% (n=5) and Kir6.2ΔC36 currents by 82±3% (n=5).

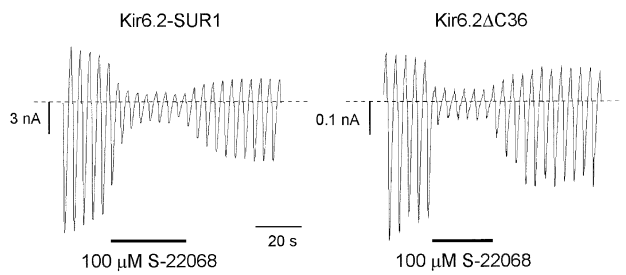

Figure 5 Effects of S-22068 on KIR6.2/SUR1 currents and KIR6.2ΔC26 currents. Macroscopic currents were recorded from giant inside-out patches in response to a series of voltage-ramps from −110 to +100 mV from a holding potential of 0 mV. Oocytes were coinjected with cRNAs encoding KIR6.2 and SUR1, or KIR6.2ΔC26.

This indicated that the site of interaction of S-22068 with the channel is not located on SUR1, but on Kir6.2.

Thus, the mechanism of action of S-22068 implicates a direct interaction with the K_{ATP} channel and, more precisely with the Kir6.2 subunit, as shown earlier for phentolamine (Proks & Ashcroft, 1997). These results led us to compare the properties of S-22068 with those of efaroxan, a classical imidazoline which has been shown to trigger insulin release by interaction with a novel type of imidazoline binding site (Chan *et al*., 1993; 1994). As shown in Figure 6, S-22068 and efaroxan display several differences in their behaviour: in addition to the greater efficacy of efaroxan, the two molecules differ in the glucose-dependence of their action. Indeed, an insulin-stimulatory glucose concentration is not required for the effect of S-22068, in sharp contrast to the strong glucose-dependence of the stimulation by efaroxan, observed in MIN6 cells (Figure 6, inset) and in isolated islets (Chan & Morgan, 1990; Berdeu *et al*., 1994).

It is now well established that several imidazoline compounds act through an atypical binding site present in β cells (Chan *et al*., 1993; 1994) which differs from that of the well characterized I_1 and I_2 sites (Chan *et al*., 1993; Olmos *et al*, 1994). The presence of this site in β cells has been directly demonstrated using the ligand [^{3}H]-RX821002 (Chan *et al*., 1994), a radioactive tracer based on an α_2-antagonist with an imidazoline structure, in the presence of yohimbine for saturating the α_2-receptors. RX821002 is known to stimulate insulin release from isolated rat islets (Lacombe *et al*., 1993), the perfused rat pancreas (Berdeu *et al*., 1994) and MIN6 cells (the present authors, data not shown).

Experiments conducted on MIN6 cell membranes in the presence of 100 μM yohimbine indicated that S-22068 is unable to interfere with [^{3}H]-RX821002 binding (Figure 7). In contrast, phentolamine and efaroxan inhibited this binding in a dose-dependent manner, with similar ED_{50} values (100 μM).

Discussion

Many compounds which contain an imidazoline ring, including several classical α-adrenoceptor-antagonists are able

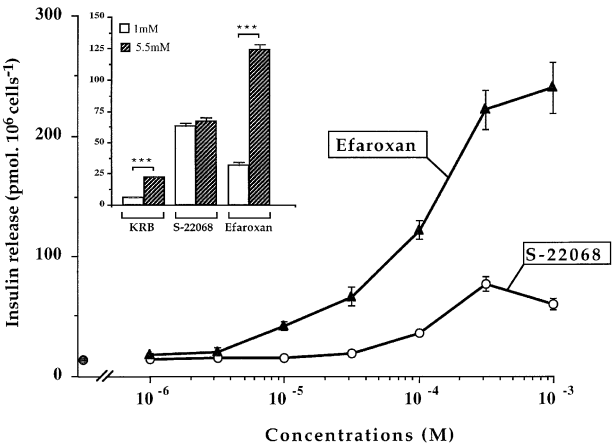

Figure 6 Comparison of the stimulatory effects of S-22068 and efaroxan on insulin release from MIN6 cells. Experiments were conducted at 5.5 mM glucose. Data are presented as mean values±s.e.mean of six experiments. Inset: effect of low (1 mM, open symbols) or high (5.5 mM, hatched symbols) glucose on the ability of 100 μM S-22068 or 100 μM efaroxan to stimulate insulin release from MIN6 cells. Data are presented as mean values±s.e.mean of six experiments. ***P<0.001 versus open symbol.

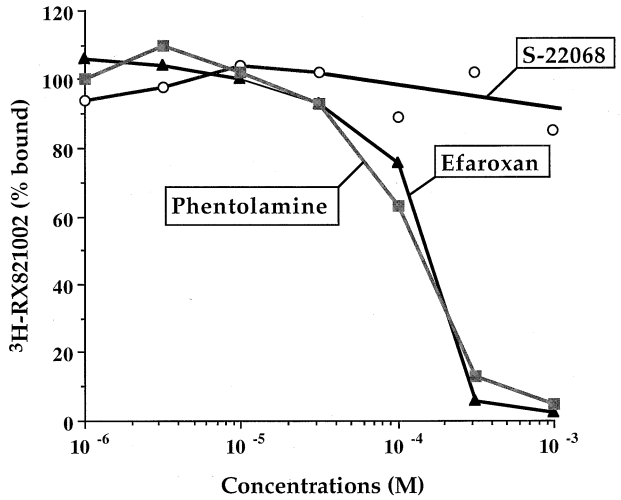

Figure 7 Inhibition of ^{3}H-RX821002 binding to MIN6 membranes by imidazoline compounds. Membranes were incubated with 2 nM ^{3}H-RX821002 in the presence of various concentrations of competitor. In this experiment all incubations were performed in the presence of 10 μM yohimbine for blocking α_2-adrenoceptors. Results are expressed as per cent of specific binding. The figure is representative of six experiments, each point being determined in triplicate.

to enhance the rate of insulin secretion, both *in vivo* and *in vitro* (Schulz & Hasselblatt, 1988; 1989; Chan *et al*., 1988; 1991). The insulin-releasing property of these compounds has been attributed to a blockade of K_{ATP} channels present in the β cells plasma membrane (Plant & Henquin, 1990; Jonas *et al*., 1992; Dunne, 1991; Dunne *et al*., 1995; Proks & Ashcroft, 1997). This inhibition is consecutive to an interaction with binding sites distinct from the major subtypes of imidazoline (I_1 and I_2) sites (Chan *et al*., 1993; Olmos *et al*., 1994). Therefore, imidazoline derivatives active on insulin release were developed as potential candidates for the management of type II diabetes. S-22068, synthesized for this purpose by IRIS, displays several interesting features: it improves glucose tolerance and increases insulin secretion, *in vivo*, in diabetic rats without causing hypoglycemia (Pelé-Tounian *et al*., 1998). Previous studies established that this compound does not display any interaction with either adrenoreceptors or the two established imidazoline receptors types I_1 or I_2 (Pelé-Tounian *et al*., 1998). In our study, we analysed the mechanism of action of S-22068 on the MIN6 cell line which resembles authentic β cells in their secretory response to classical secretagogues including glucose, peptides (glucagon, tGLP-1, somatostatin), cholinergic and adrenergic agents and a wide variety of pharmacological compounds known to affect insulin secretion, such as sulphonylureas (Weng *et al*., 1993; Inagaki *et al*., 1996; Le Brigand *et al*., 1997).

Our data established that the effect of S-22068 on insulin secretion observed *in vivo* results from a direct effect on β-cells and that this release is a consequence of a calcium influx into the β cell. This calcium entry is due to opening of nifedipine-sensitive voltage-dependent L-type Ca^{2+} channels present in the β cell plasma membrane as a result of the membrane depolarization induced by closure of K_{ATP} channels. Therefore, S-22068 affects insulin release *via* a secretory pathway implicating K_{ATP} channels. This mode of action is shared by classical imidazoline compounds (Plant *et al*., 1991; Jonas *et al*., 1992; Chan *et al*., 1991; Dunne *et al*., 1995). Furthermore, electrophysiological data led us to conclude that a site located on Kir6.2 is the target for this compound. Because the imidazoline phentolamine inhibits K_{ATP} channel activity *via* an interaction with the Kir6.2 subunit (Proks & Ashcroft, 1997) and because phentolamine and efaroxan are known to stimulate insulin release through the non-I-1 non I-2 sites, we investigated the possibility that S-22068 shares the same mode of action as those imidazoline compounds. These sites may be directly studied using binding of labelled RX821002 when the α_2-sites are blocked by yohimbine (Chan *et al*., 1995). Our data confirm that phentolamine and efaroxan interact with the non-I1 non-I2 sites present in β cell and indicate that S-22068 does not recognize it. This clear difference between the mode of action of S-22068 and that of the other imidazolines fits well with the fact that, unlike phentolamine and efaroxan, the S-22068 action on insulin release is not dependent upon glucose concentration. Since the site, present on Kir6.2, through which S-22068 acts on the K_{ATP} channel and, eventually on insulin release, is not the non-I1 non I-2 imidazoline site, we can conclude that another type of site for this family of molecules exists on the Kir6.2 moiety of the β cell K_{ATP} channel.

These observations shed a new light on the mode of action of the imidazoline compounds on the β cell, as well as in other fields of pharmacology, and may help in designing new drugs for the management of non-insulin dependent (type II) diabetes mellitus.

References

AGUILAR-BRYAN, L., NICHOLS, C.G., WESCHLER, S.W., CLEMENT IV J.P., BODY III, A.E., GONZALEZ, G., HERRERA-SOSA, H., NGUY, K., BRYAN, J. & NELSON, D.A. (1995). Cloning of the β cell high-sulfonylurea receptor: a regulator of insulin secretion. *Science,* **268,** 423–426.

BERDEU, D., GROSS, R., RIBES, G., LOUBATIERES-MARIANI, M.M. & BERTRAND, G. (1994). Effects of imidazolines and derivatives on insulin secretion and vascular resistance in perfused rat pancreas. *Eur. J. Pharmol.,* **254,** 119–125.

CHAN, S.L., BROWN, C.A. & MORGAN, N.G. (1993). Stimulation of insulin secretion by the imidazoline α2-adrenoceptor antagonist efaroxan is mediated by a novel, stereoselective, binding sites. *Eur. J. Pharmol.,* **230,** 375–378.

CHAN, S.L., BROWN, C.A., SCARPELLO, K.E. & MORGAN, N.G. (1994). The imidazoline site involved in control of insulin secretion: characteristics that distinguish it from I_1- and I_2-sites. *Br. J. Pharmacol.,* **112,** 1065–1070.

CHAN, S.L., BROWN, C.A., SCARPELLO, K.E. & MORGAN, N.G. (1995). Pancreatic β-cells express an imidazoline binding site that is distinct from I1-I2 sites. *Ann. NY. Acad. Sci.,* **763,** 361–373.

CHAN, S.L., DUNNE, J.M., STILLINGS, M.R. & MORGAN, N.G. (1991). The α2-adrenoceptor antagonist efaroxan modulates K^+_{ATP} channels in insulin-secreting cells. *Eur. J. Pharmol.,* **204,** 41–48.

CHAN, S.L.F., HURST, R.D. & MORGAN, N.G. (1988). Effects of α2-adrenergics antagonists on insulin secretion from rat pancreatic islets. *Biochem. Soc. Trans.,* **16,** 1005–1006.

CHAN, S.L. & MORGAN, N.G. (1990). Stimulation of insulin secretion by efaroxan may involve interaction with potassium channels. *Eur. J. Pharmol.,* **176,** 97–101.

DUNNE, M.J. (1991). Block of ATP-regulated potassium channels by phentolamine and other alpha-adrenoceptor antagonists. *Br. J. Pharmacol.,* **103,** 1847–1850.

DUNNE, M.J., HARDING, E.A., JAGGAR, J.H., SQUIRES, P.E., LIANG, R., KANE, C., JAMES, R.F.L. & LONDON, N.J.M. (1995). Potassium channels, imidazolines, and insulin-secreting cells. *Ann. NY. Acad. Sci.,* **763,** 243–261.

GÖKE, R., COLE, T. & CONLON, J.M. (1989). Characterization of the receptor for glucagon-like peptide-1 (7-36) amide on plasma membranes from rat insulinoma derived cells by covalent cross-linking. *J. Mol. Endocrinol.,* **2,** 93 – 98.

GRIBBLE, F.M., ASHFIELD, R., AMMALA, C. & ASHCROFT, F.M. (1997a). Properties of cloned ATP-sensitive K^+ currents expressed in Xenopus oocytes. *J. Physiol.,* **498,** 87 – 98.

GRIBBLE, F.M., TUCKER, S.J. & ASHCROFT, F.M. (1997b). The interaction of nucleotides with the tolbutamide block of cloned ATP-sensitive K^+ channel currents expressed in *Xenopus* oocytes: a reinterpretation. *J. Physiol.,* **504,** 35 – 45.

HUGUES, S.H. & ASHCROFT, S.J.H. (1988). Effect of secretagogues on cytosolic free Ca^{2+} and insulin release in the hamster clonal β-cell line HIT-T15. *J. Mol. Endocr.,* **1,** 13 – 17.

INAGAKI, N., GONOI, T., CLEMENT IV J.P., NAMBA, N., INAZAWA, J., GONZALEZ, G., AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of I-KATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science,* **270,** 1166 – 1169.

INAGAKI, N., KUROMI, H. & SEINO, S. (1996). PACAP/VIP receptors in pancreatic beta-cells: their roles in insulin secretion. *Ann. N. Y. Acad. Sci.,* **26,** 44 – 51.

ISHIHARA, H., ASANO, T., TSUDUKA, K., KATAGARI, H., INUKAI, K., ANAI, M., KIKUCHI, M., YAZAKI, Y., MIYAZAKI, J.I. & OKA, Y. (1993). Pancreatic beta cell line Min6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia,* **36,** 1139 – 1145.

JONAS, J.C., PLANT, T.D. & HENQUIN, J.C. (1992). Imidazolines antagonists of α2-adrenoceptors increase insulin release *in vitro* by inhibiting ATP-sensitive K^+ channels in pancreatic β cells. *Br. J. Pharmacol.,* **107,** 8 – 14.

KERVRAN, A., RIEUTORT, M. & GUILLAUME, M. (1976). A simultaneous radioimmunoassay for growth hormone and insulin in the plasma of rats and rabbits. *Diab. Métabol.,* **2,** 67 – 72.

LACOMBE, C.R., VIALLARD, V.P. & PARIS, H.J. (1993). The properties of imidazoline derivatives to stimulate insulin release by hamster pancreatic islets are probably due to alpha 2-adrenoceptor blockade but not to interaction with non-adrenergic idazoxan binding sites. *Diab. Métabol.,* **19,** 381 – 386.

LE BRIGAND, L., VIRSOLVY, A., PEYROLLIER, K., MANECHEZ, D., GODFROID, J.-J., GUARDIOLA-LEMAÎTRE, B. & BATAILLE, D. (1997). Stimulation of insulin release from the MIN6 cell line by a new imidazoline compound, S-21663. Evidence for the existence of a novel imidazoline site in β cell. *Br. J. Pharmacol.,* **122,** 786 – 791.

NELSON, T.Y., GAINES, K.L., RAJAN, A.S., BERG, M. & BODY III A.E. (1987). Increased cytosolic calcium, a signal for sulphonylurea-stimulated insulin release from beta cells. *J. Biol. Chem.,* **262,** 2608 – 2612.

NIKI, I., KELLY, R.P., ASHCROFT, F.M. & ASHCROFT, S.J.H. (1989). ATP-sensitive K-channels in HIT-T15 β-cells studied by patch-clamp methods, ^{86}Rb efflux and glibenclamide binding. *Pflügers Arch.,* **415,** 47 – 55.

OLMOS, G., KULKARNI, R-N., HAQUE, M. & MACDERMOT, J. (1994). Imidazolines stimulate release of insulin from RIN-5AH cells independently from imidazoline I_1 and I_2 receptors. *Eur. J. Pharmol.,* **262,** 41 – 48.

PANTEN, U., SCHWANSTECHER, M. & SCHWANSTECHER, C. (1996). Sulphonylurea receptors and mechanism of sulphonylurea action. *Exp. Clin. Endocrinol. Diabetes,* **104,** 1 – 9.

PELÉ-TOUNIAN, A., WANG, X., RONDU, F., LAMOURI, A., TOUBOUL, E., MARC, S., DOKHAN, R., PFEIFFER, B., MANECHEZ, D., RENARD, P., GUARDIOLA-LEMAÎTRE, B., GODFROID, J.-J., PENICAUD, L. & KTORZA, A. (1998). Potent antihyperglycaemic property of a new imidazoline derivative S-22068 (PMS 847) in a rat model of NIDDM. *Br. J. Pharmacol.,* **124,** 1591 – 1596.

PIAN-SMITH, M.C., YADA, T., YANEY, G.C., ABDEL-EL-MOTAL, S.M., WIEDENKELLER, D.E. & SHARP, G.W. (1988). Mobilization of Ca^{2+} from intracellular stores of pancreatic β-cells by the calcium store blocker TMB-8. *Endocrinology,* **123,** 1984 – 1991.

PLANT, T.D. & HENQUIN, J.-C. (1990). Phentolamine and Yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β cells. *Br. J. Pharmacol.,* **101,** 115 – 120.

PLANT, T.D., JONAS, J.C. & HENQUIN, J.C. (1991). Clonidine inhibits ATP-sensitive K^+ channels in mouse pancreatic islets. *Br. J. Pharmacol.,* **104,** 385 – 390.

POITOUT, V., OLSON, L.K. & ROBERTSON, R.P. (1996). Insulin-secreting cell lines: classification, characteristics and potential applications. *Diabetes Metab.,* **22,** 7 – 14.

PROKS, P. & ASHCROFT, F.M., (1997). Phentolamine block of KATP channels is mediated by Kir6.2. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 11716 – 11720.

SAKURA, H., ÄMMÄLÄ, C., SMITH, P.A., GRIBBLE, F.M. & ASHCROFT, F.M. (1995). Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic β-cells, brain, heart and skeletal muscle. *FEBS Lett.,* **377,** 338 – 344.

SCHMID-ANTOMARCHI, H., WEILLE, J.-D., FOSSET, M. & LADZUNSKI, M. (1987). The receptor for antidiabetic sulphonylureas controls the activity of ATP-modulated K^+ channel in insulin secreting cells. *J. Biol. Chem.,* **262,** 15840 – 15843.

SCHULZ, A. & HASSELBLATT, A. (1988). Phentolamine a deceptive tool to investigate sympathetic nervous control of insulin release. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **337,** 637 – 643.

SCHULZ, A. & HASSELBLATT, A. (1989). An insulin-releasing property of imidazoline derivatives is not limited to compounds that block alpha-adrenoceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **340,** 305 – 309.

TUKER, S.J., GRIBBLE, F.M., ZHAO, C., TRAPP, S. & ASCHCROFT., F.M. (1997). Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature,* **387,** 179 – 183.

WENG, L., DAVIES, M. & ASHCROFT, S.J. (1993). Effects of cholinergic agonists on diacylglycerol and intracellular calcium levels in pancreatic beta-cells. *Cell Signal.,* **5,** 777 – 786.

(Received June 10, 1999
Revised August 4, 1999
Accepted August 16, 1999)