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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 cells

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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-21663, a newly synthesized imadizoline compound known for its antidiabetic effect *in vivo* and its ability to release insulin from perfused pancreas.

2 S-21663, at concentrations ranging from 10^{-5} M to 10^{-3} M was able to release insulin from MIN6 cells; its activity peaked at 10^{-4} M, a drop in the stimulant factor being noted between 10^{-4} and 10^{-3} M. Its efficacy, which did not differ whatever the glucose concentration (stimulant or not), was higher than that of the other secretagogues tested, glucose, sulphonylureas or the peptide tGLP-1.

3 In contrast to tGLP-1, S-21663 did not change the cyclic AMP content, whereas it increased Ca^{2+} influx via verapamil- and nifedipine-sensitive voltage-dependent calcium channels, the insulin release being a direct consequence of this Ca^{2+} entry. The S-21663-induced Ca^{2+} influx appears to be essentially the consequence of closure of K^+ channels which differ from the ATP-dependent K^+ (K-ATP) channels as determined by measurement of ^{86}Rb efflux and use of a K-ATP channel opener.

4 Comparison of the effects of S-21663 to that of efaroxan, another imidazoline compound shown to act on insulin release in a glucose-dependent way via binding sites distinct from the imidazoline I_1 and I_2 sites, suggested that S-21663 acts through a novel site which displays a remarkably stable expression along the cell culture.

5 It is concluded that S-21663 is a very efficient, glucose-independent insulin secretagogue acting through a novel imidazoline site, linked to K^+ channels, distinct from the I_1, I_2 and 'efaroxan' binding sites. *In vitro* and *in vivo* features of S-21663 indicate that this compound, or new drugs drived from it, might be the basis for a new pharmacological approach to the mangement of type II (non insulin-dependent) diabetes.

Keywords: Imidazoline; S-21663; pancreatic β cell; MIN6 cell line; insulin release; potassium channel; calcium channel

Introduction

Molecules which belong to a new family of imadizoline derivatives, such as compound S-21663, synthesized by Institut de Recherches Internationales Servier (IRIS), displayed interesting features in a rat model of type II diabetes (Thibault *et al*., 1992): S-21663 improved glucose tolerance *in vivo* and increased insulin secretion in mildly diabetic rats, with a lack of hypoglycaemic effect (Wang *et al*., 1996). Owing to its original characteristics, this imidazoline derivative may represent a novel pharmacological basis for the treatment of type II diabetes.

The aim of the present study was to analyse the capacity of S-21663 to release insulin directly from β cells in culture, as well as to understand the cellular mechanism of action of this molecule, in comparison with that of other classical secretagogues, such as glucose or sulphonylureas, the mechanisms of action of which have been extensively studied (Schmid-Antomarchi *et al*., 1987; Ashcroft *et al*., 1988; Panten *et al*., 1996). We also compared the mechanism of action of S-21663 with that of other compounds known to stimulate insulin release through an increase in cellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP), such as the peptide tGLP-1, considerd as a main physiological incretin (Drucker *et al*., 1987). Finally, we compared the biological features of this molecule to that of efaroxan, another imidazoline compound which was shown to stimulate insulin release via a new type of imidazoline site (Chan *et al*., 1991; 1993; 1994) linked to adenosine 5′-triphosphate (ATP)-sensitive K^+ channels (K-ATP channels) present in β cells.

Since calcium is known to play a crucial role in the control of insulin release by many secretagogues (Nelson *et al*., 1987; Hughes & Ashcroft, 1988), we analysed the effect of S-21663 on ^{45}Ca uptake by β cells in culture. To compare further the mechanism of action of S-21663 to that of glucose, sulphonylureas and efaroxan, known to inhibit K-ATP channels and, in turn, to open voltage-sensitive Ca^{2+} channels, we studied the ^{86}Rb efflux from β cells in response to S-21663.

In this study we used a β cell line (MIN6), obtained by use of a transgenic approach (Miyazaki *et al*., 1990; Ishihara *et al*., 1993), which displays most of the characteristics of the normal β cells, in particular a response to glucose within the physiological range.

Methods

Cell culture

MIN6 cells, kindly provided by Dr Ishihara (Third Department of Internal Medicine, University of Tokyo, Japan), were routinely grown as previously described (Ishihara *et al*., 1993)

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at 37°C in a 5% CO_2 atmosphere in sterile plastic flasks (T75, TPP) in Dulbecco's modified Eagle's 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 μg ml^{-1} streptomycin (Gibco) and 5 μl l^{-1} β-mercaptoethanol (Sigma).

Before each series of studies, cells were seeded five days before the experiment 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). The cells were then preincubated for one hour in KRB-BSA containing 1 mM glucose at 37°C, 5% CO_2 and incubated for two hours 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 with [^{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 μCi ml^{-1} $^{45}CaCl_2$ (Amersham, U.K., 5–50 mCi mg^{-1} mg^{-1} Ca) and the test agents. The reaction, developed at 37°C, was stopped by aspiration of the medium. The cells were rapidly washed four times with ice-cold buffer (mM: NaCl 135, KCl 5, $CaCl_2$ 2.5, $LaCl_2$ 1 and HEPES 10). The cells were then solubilized in 1 ml KRB containing 0.1% Triton for one hour 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).

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) and the cells were loaded overnight with the isotope (Nicki *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

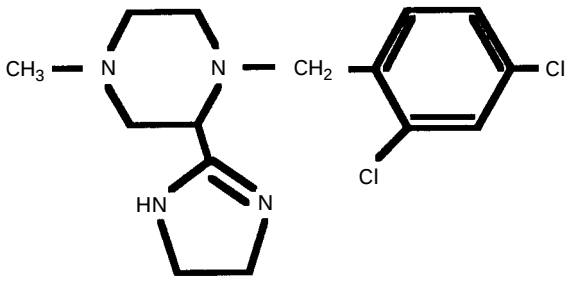

Figure 1 Chemical structure of S-21663.

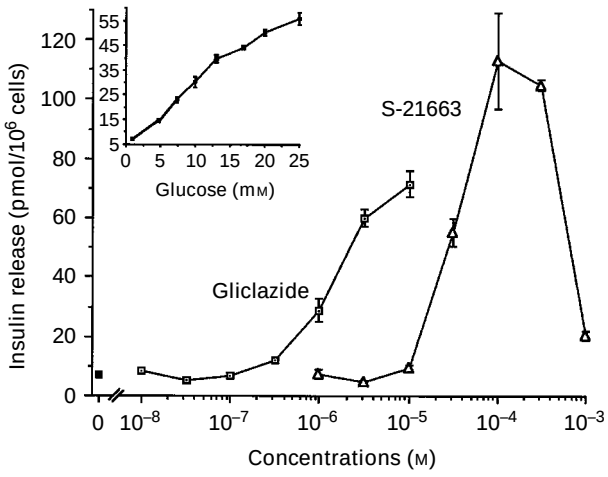

Figure 2 Stimulant effect of S-21663 in comparison with that of the sulphonylurea gliclazide on insulin release from MIN6 cells in culture. Experiments were conducted in KRB containing a non-stimulating (1 mM) concentration of glucose. Inset: dose-dependence of effect of glucose on insulin release from MIN6 cells. Data are presented as mean values of 9 experiments; vertical lines show s.e.mean.

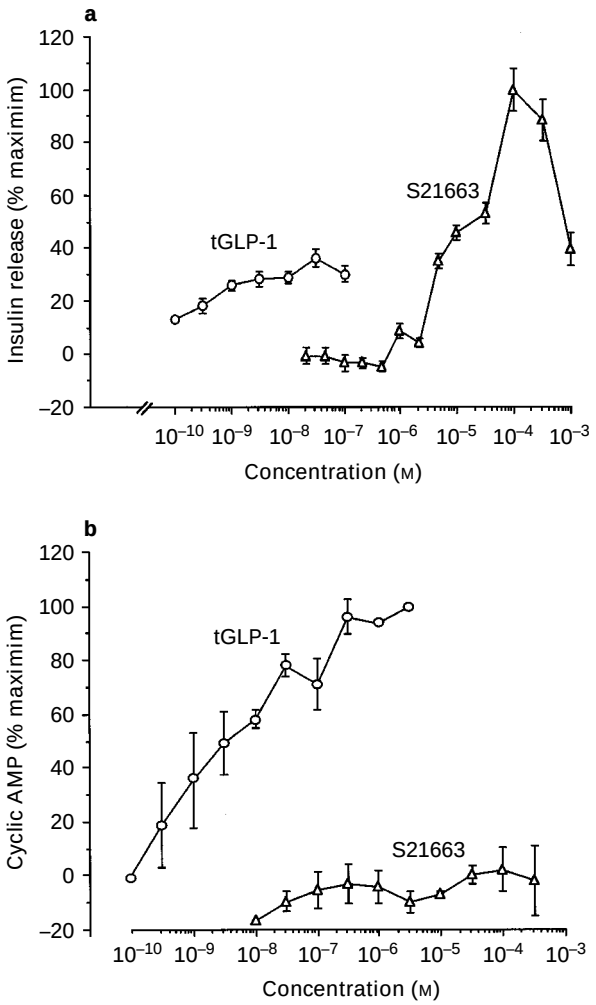

Figure 3 Comparative stimulant effects of S-21663 and glucagon-like-peptide-1 (7–36)-amide (tGLP-1) on: (a) insulin release from MIN6 cells. Experiments were conducted in KRB containing a stimulating concentration (5.5 mM) of glucose. Data are expressed as percentage of the maximal stimulation observed in the experiment (induced by 10^{-4} M S-21663) and presented as mean values of 9 experiments. (b) Cyclic AMP accumulation in MIN6 cells in the presence of an inhibitor of cyclic AMP phosphodieasterase (1 mM IBMX). Data are expressed as ((stimulated-basal)/basal), as % of the maximum (induced by 3×10^{-6} M tGLP1) and presented as mean values of 9 experiments. In (a) and (b) vertical lines show s.e.mean.

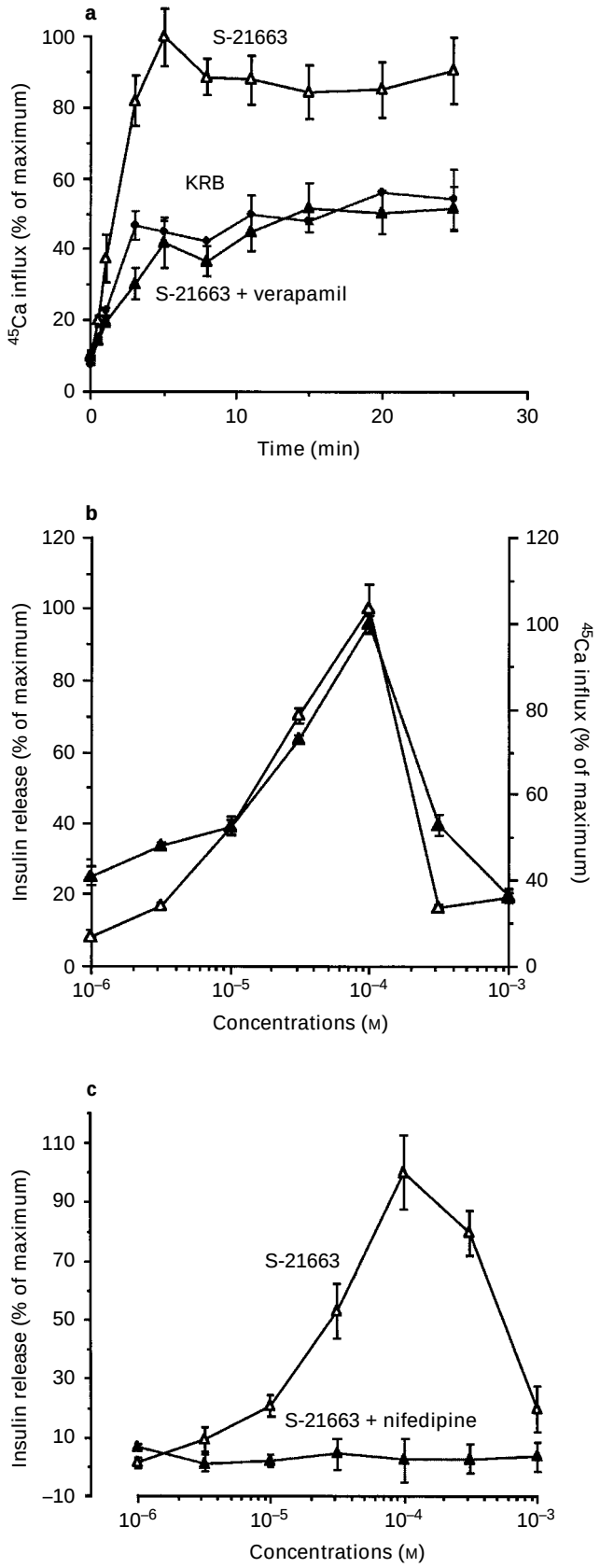

Figure 4 (a) Kinetics of ^{45}Ca influx into MIN6 cells under control conditions (KRB) or in the presence of 100 μM S-21663 with or without 0.1 mM verapamil, a blocker of L-type Ca^{2+} channels. Data are expressed as % of the maximal stimulation (observed at 5 min) with 100 μM S-21663, and presented as mean values of 6 experiments. (b) A comparison of the effects of S-21663 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 two hour incubation. Data are expressed as % of maximal stimulation (observed with 100 μM S-21663) and presented as mean values of 6 experiments. (c) The stimulant effect of S-21663 on insulin release from MIN6 cells in the presence and absence of 0.1 mM nifedipine. Data are expresed as % of maximal stimulation (observed with 100 μM S-21663) and presented as mean values of 3 determinations, representative of 3 repeated experiments. In (a), (b) and (c), vertical lines show s.e.mean.

20 min at 37°C in KRB containing 0.2 μCi ml^{-1} ^{86}RbCl. Oligomycin 1.2 μg ml^{-1} and 1 mM 2-deoxy-D-glucose were added in order to decrease the ATP pool and thus to open the K-ATP channels slightly (Nicki *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 and the medium was then taken and cells were extracted with 1 ml 0.1 M NaOH for one hour. The extract and medium were then counted by use of Cerenkov counting. Efflux was calculated as percentage of total radioactivity.

Determination of cyclic AMP production

The day before the experiment, the culture medium was renewed. On the day of the experiment, cells were washed twice with KRP buffer (mM: NaCl 120, KCl 3, $CaCl_2$ 2.6, $MgCl_2$ 0.67, KH_2PO_4 1.2, glucose 5.2 and HEPES 25, pH 7.4). The incubation was performed in 0.5 ml KRP buffer containing 2% BSA, 0.5 mM IBMX and the test agents for 15 min at 25°C. Cyclic AMP was then extracted with 60% perchloric acid, the sample was neutralized with 9 N KOH and succinylated to increase the sensitivity of the assay (Delaage *et al*., 1978). Cyclic AMP was quantified by radioimmunoassay.

Chemicals

S-21663 (Figure 1) was synthesized by the IRIS chemistry Department. The other drugs used in this study were as follows: glibenclamide (Guidotti, Italy), efaroxan (Research Biochemicals Incorporated, U.S.A.), diazoxide (Sigma, U.S.A.), nifedipine (Sigma), verapamil (Sigma) and oligomycin (Sigma). Bovine serum albumin (BSA), fraction V was from Boehringer, Germany and isobutyl-methyl xanthine (IBMX) from Sigma.

Statistics

Statistical analysis was performed by use of Student's *t* test for unpaired values and results were considered significant when $P<0.05$.

Results

We first analysed the capacity of S-21663 to release insulin directly from the MIN6 cells in culture. As previously observed (Miyazaki *et al*., 1990; Ishihara *et al*., 1993), the response of these cells to glucose was within the physiological range, with a half-maximal stimulation at 7.5 mM and a stimulation factor close to 9 (Figure 2, inset). The capacity of MIN6 cells to behave like normal β cells was further illustrated by the clear-cut response to the sulphonylurea gliclazide (Figure 2). In the same set of experiments, carried out at a non-stimulating glucose concentration (1 mM), compound S-21663 induced a very pronounced insulin release, with a 14 fold increase and a half-maximal effect observed at 20–30 μM. At concentrations higher than 100 μM, a sharp decrease in the stimulation factor was noted up to 1 mM where stimulation fell to negligible values (Figure 2).

In view of a possible cyclic AMP pathway involved in the effect of S-21663, we have compared the effects of S-21663 to that of glucagon-like peptide (7–36)-amide (tGLP-1), known

to increase cyclic AMP in β cells (Drucker *et al*., 1987). As shown in Figure 3a, the stimulation factor for insulin release induced by S-21663 was much higher than that induced by tGLP-1. Furthermore, as shown in Figure 3b, S-21663 failed to induce a significant increase in cyclic AMP, in contrast to that observed for tGLP-1 (Figure 3b). These observations clearly indicated that S-21663 and tGLP-1 trigger insulin release via different intracellular pathways.

Many insulin secretagogues are known to induce an increase in the cytosolic calcium concentration of the β cells, which leads to exocytosis of the insulin secretory granules (Nelson *et al*., 1987; Hughes & Ashcroft, 1988). As shown in Figure 4a, addition of S-21663 to the cells rapidly induced a marked Ca^{2+} influx followed by a steady state. The drug augmented by 65% the Ca^{2+} influx integrated over the first 20 min. This influx was mediated by opening of voltage-dependent Ca^{2+} channels, as demonstrated by the complete suppression of the S-21663-induced Ca^{2+} influx by verapamil (see Figure 4a). Nifedipine, another L type Ca^{2+} channel blocker, displayed very similar effects (not shown). When the S-21663 dose-response curves (Figure 4b) for insulin release and Ca^{2+} influx were superimposed, it was apparent that the two phenomena are closely related. It is of particular interest to note that the decreased stimulating factor at concentrations of S-21663 over 100 μM paralleled the decreased calcium entry. A more direct demonstration of the causal relationship between Ca^{2+} entry and insulin release is shown in Figure 4c: when S-21663-induced Ca^{2+} entry was suppressed by addition of nifedipine, the drug was no longer able to induce insulin release. We conclude that S-21663 triggers insulin release by opening nifedipine (and verapamil)-sensitive L type voltage-dependent Ca^{2+} channels present in the β cell plasma membrane.

Since S-21663 is a new imidazoline derivative, it was of special interest to compare its actions with that of efaroxan, known to act on insulin-releasing β cell via a new type of binding site (Chan *et al*., 1993; 1994). As shown in Figure 5, both S-21663 and efaroxan dose-dependently stimulated insulin release with a marked stimulation factor in the presence of a stimulating (7 mM) glucose concentration. However, while high concentrations of efaroxan still induced a maximal insulin release, an important drop in this parameter was observed for S-21663, as previously noted. The most significant difference observed between the effects of these two molecules was the absence of glucose-dependence of the effect of S-21663. The presence of a stimulating glucose concentration was a prerequisite for observing the effect of efaroxan in this (see inset) and other biological models (Chan & Morgan, 1990; Berdeu *et al*., 1994).

The next step in our study was to determine whether S-21663 acts directly on the Ca^{2+} channel or indirectly by inhibiting K-ATP channels and, in turn, inducing membrane depolarization, as observed, for instance, for sulphonylurease (Schmid-Antomarchi *et al*., 1987; Ashcroft *et al*., 1988; Panten *et al*., 1996) or imidazolines (Plant & Henquin, 1990; Chan *et al*., 1991). Therefore, we compared the extent of S-21663 with that of efaroxan at a stimulating glucose concentration, in the presence and absence of diazoxide, an opener of K-ATP channels.

As shown in Figure 6a, the effect of maximally active concentrations of S-21663 on insulin release was not significantly modified by diazoxide whereas, as expected, the effect of efaroxan was dramatically reduced under the same conditions. When looking at the potassium efflux under the same conditions, it may be seen in Figure 6b that the ^{86}Rb efflux (index of K^+ efflux) was reduced in the presence of efaroxan. S-21663

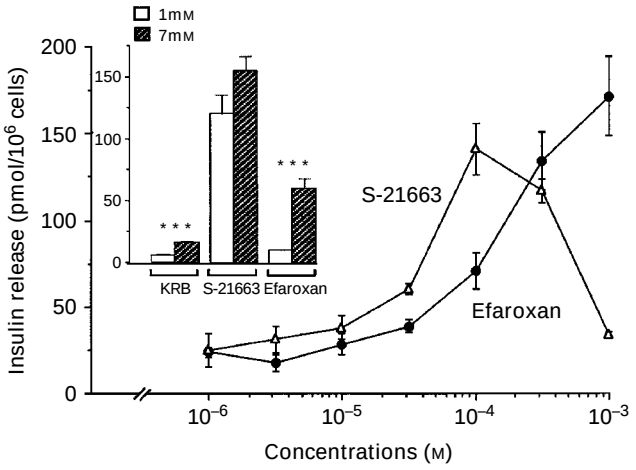

Figure 5 A comparison of the stimulant effects of S-21663 and efaroxan on insulin release from MIN6 cells. Experiments were conducted at 7 mM glucose. Data are presented as mean values of 9 experiments; vertical lines show s.e.mean. Inset: effect of low (1 mM) or high (7 mM) glucose concentration on the ability of 100 μM S-21663 or 100 μM efaroxan to stimulate insulin release from MIN6 cells. Data are presented as mean values ± s.e.mean of 6 experiments. ***$P<0.001$ versus 1 mM glucose.

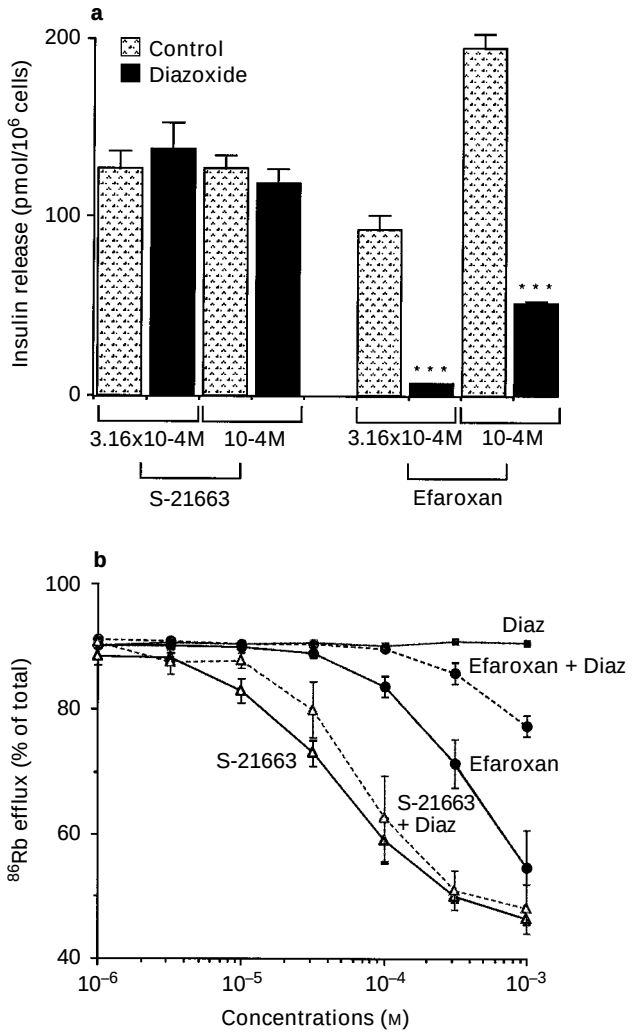

Figure 6 Comparative effects of S-21663 and efaroxan on: (a) insulin release from MIN6 cells either in the absence (control) or presence of 250 μM diazoxide. Experiments were conducted at 7 mM glucose. Data are presented as mean values ± s.e.mean of 9 determinations. ***$P<0.001$ versus the control data. (b) $^{86}Rb^+$ efflux from MIN6 cells. Experiments were conducted at 7 mM glucose, in the absence or presence (broken line) of 250 μM diazoxide (Diaz). Data are expressed as % of total radioactivity present in the cells at the beginning of the test.

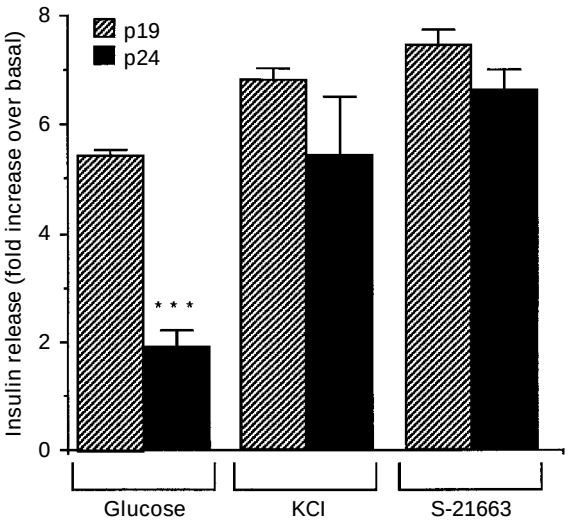

Figure 7 Comparative effects of 15 mM glucose, 15 mM KCl and 10^{-4} M S-21663 on insulin release at late (24) and earlier (19) passages. Data are expressed as fold increase over basal. ***$P<0.001$ versus the data from earlier (19) passages.

also induced an important dose-dependent reduction in ^{86}Rb efflux. Nevertheless, the inhibitory effect of efaroxan on ^{86}Rb efflux was strongly decreased in the presence of diazoxide, in contrast to what was observed with S-21663 (Figure 6b). All these data strongly suggest that S-21663 acts on insulin release by closing potassium channels that differ from the K-ATP channels.

It is also worth noting that, like most insulin-secreting cell lines, MIN6 cells showed a decreased response to most of the stimuli with increasing number of passages. This was reproducibly observed in our laboratory for most of the stimuli including glucose (Figure 7); the same batches of cells still responded to the membrane depolarizing effect of high K^+ or to S-21663 (Figure 7) with a conserved efficiency, confirming the original mode of action of S-21663.

Finally, the complete absence of interaction of S-21663 with the sulphonylurea binding site present in MIN6 cell membranes (not shown) indicated that the S-21663 site of action is distinct from the sulphonylurea receptor. The lack of a significant effect of S-21663 in binding studies conducted on imidazoline sites by use of labelled idazoxan, *P*-aminoclonidine or RX 821002 (Wang *et al*., 1996), excluded the possibility that the observed effects of S-21663 may be mediated by one of the known types of imidazoline sites.

Discussion

While analysing the mode of action of S-21663, a new imidazoline derivative which displays an antidiabetic effect *in vivo*, we observed that this compound releases insulin from the MIN6 β cell line in a dose-dependent manner with a high stimulation factor. At a high concentration (1 mM), we observed a drop in the stimulation factor which was not related to a possible toxic effect of the drug since no sign of cell toxicity was noted during the time of incubation. This type of 'bell-shaped' curve, observed in several *in vitro* and *in vivo* biological models, might be the result of a desensitization or 'down-regulation' process. In contrast to efaroxan, S-21663 stimulated insulin release whatever the glucose concentration; such non glucose-dependent secretagogues are often considered as potential inducers of hypoglycaemia. However, the studies carried out *in vivo* (Wang *et al*., 1996) indicated that this is not the case for S-21663. Such a discrepancy might be explained by the existence in *in vivo* of a compensatory mode of action of S-21663 which may not be seen *in vitro*. Alternatively, the behaviour of our cellular model towards S-21663 might differ from that of normal β cells. Two observations tend to rule out this hypothesis: (1) such a non-classical behaviour of MIN6 cells was never observed for other secretagogues such as glucose, sulphonylureas, efaroxan or peptides tested in our laboratory; (2) this absence of glucose-dependence was also observed in the perfused pancreas (Wang *et al*., 1996).

S-21663 clearly uses an intracellular pathway which is entirely dependent upon Ca^{2+} entry via verapamil- and nifedipine-sensitive L-type channels. This Ca^{2+} entry is not dependent upon the closure of K-ATP channels, in contrast to what is well established for many secretagogues, including sulphonylureas and efaroxan. Since S-21663 decreases very efficiently the K^+ output from the cells, we conclude that S-21663 closes K^+ channels which appear to be distinct from K-ATP channels and consequently induces a membrane depolarization which, in turn, opens voltage-sensitive Ca^{2+} channels. Determining the exact type of K^+ channels involved in this action will be the subject of further investigations.

The binding site through which S-21663 acts also seems to be original. We know that it is not one of the 'peripheral' imidazoline sites I_1 or I_2, confirming previous observations made with other imidazoline compounds (Olmos *et al*., 1994); neither is it the 'efaroxan site' in the pancreatic β cells (Chan *et al*., 1993; 1994) because of the profound discrepancies observed in the mode of action of these two imidazoline molecules. Finally, this novel site as well as its coupling to the effector systems displays an exceptional stability through the passages of cells in culture. It appears, therefore, that it is possible to trigger insulin release from a β cell which has lost totally or partially its sensitivity to physiological and classical pharmacological secretagogues. Further studies will be necessary to confirm the hypothesis raised by our data, that drugs designed to recognize specifically this site might be a novel pharmacological approach for the management of type II diabetes.

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