Antagonism of the stimulatory effects of efaroan and glibenclamide in rat pancreatic islets by the imidazoline, RX801080

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1 The imidazoline α_2 -adrenoceptor antagonist, efaroan, stimulates insulin secretion from rat isolated islets and antagonizes the ability of diazoxide to inhibit glucose-induced insulin secretion. These effects result from closure of ATP-sensitive potassium channels although the mechanisms involved have not been elucidated.

2 In the present work, we have examined the effects of a close structural analogue of efaroan, RX801080, in rat isolated islets of Langerhans. RX801080 was found to be ineffective as a stimulator of insulin secretion and did not prevent the inhibition of insulin secretion mediated by diazoxide.

3 RX801080 acted as an antagonist of the actions of several imidazolines (efaroan, phentolamine and midaglizole) in rat islets. It dose-dependently inhibited the ability of efaroan to antagonize the effects of diazoxide in islets and also completely inhibited the direct stimulation of insulin secretion mediated by efaroan.

4 RX801080 also antagonized the effects of the non-imidazoline, ATP-sensitive potassium channel blocker, glibenclamide, in rat islets. It inhibited both the capacity of glibenclamide to stimulate insulin secretion and the ability of glibenclamide to overcome the inhibitory effects of diazoxide in rat islets.

5 Antagonism of glibenclamide responses by RX801080 was not due to inhibition of binding of the sulphonylurea to its receptor on the pancreatic β -cell.

6 The results suggest that imidazoline compounds and sulphonylureas interact with distinct binding sites on islet cells, but that these sites can interact functionally to control islet cell ATP-sensitive potassium channel activity and insulin secretion.

Keywords: Islets of Langerhans; insulin secretion; imidazoline receptors; potassium channel; efaroan; glibenclamide; α_2 -antagonist; sulphonylurea; RX801080; adrenoceptor

Introduction

The resting potential maintained across the plasma membrane of the pancreatic β -cell is determined primarily by a large efflux of potassium ions (Ozawa & Sand, 1986; Henquin, 1987; Dunne & Petersen, 1991; Ashcroft *et al.*, 1992; Boyd, 1992). This efflux is facilitated by a range of potassium channels in the β -cell plasma membrane, which remain open in the resting state. The most important of these is a channel the open state of which varies according to the ATP/ADP ratio within the cell (the K-ATP channel; Cook *et al.*, 1988; Dunne & Petersen, 1991; Ashcroft *et al.*, 1992; Boyd, 1992). Thus, the open state of this channel is determined by the metabolic status of the β -cell and, under conditions of increased glucose oxidation, the potassium permeability of the plasma membrane is reduced by closure of the channel (Cook & Hales, 1984; Ashcroft *et al.*, 1984; 1992; Dunne & Petersen, 1991; Ashcroft & Ashcroft, 1990; Boyd, 1992). Under these conditions the membrane depolarizes, allowing the gating of voltage-sensitive calcium channels and a rise in the rate of calcium influx. This, in turn, acts as the trigger for insulin secretion.

In addition to their sensitivity to the ATP/ADP ratio, β -cell K-ATP channels are also subject to modulation by a variety of pharmacological agents. These can either close (e.g. hypoglycaemic sulphonylureas; Sturgess *et al.*, 1985; Panten *et al.*, 1989; Ashcroft & Ashcroft, 1990; Nelson *et al.*, 1992) or open (e.g. diazoxide; Trube *et al.*, 1986; Dunne, 1989; Dunne *et al.*, 1990; Kozłowski & Ashford, 1992) the channels. It has been established recently that the activity of

β -cell K-ATP channels can also be controlled by certain imidazoline compounds (Plant & Henquin, 1990; Chan *et al.*, 1991a; Jonas *et al.*, 1992). These were initially identified as a group of α_2 -adrenoceptor antagonists (including efaroan, phentolamine and midaglizole (DG-5128)) which share the unusual property of eliciting a direct increase in insulin secretion in the absence of any adrenoceptor agonist (Schultz & Hasselblatt, 1988; Smith & Furman, 1988; Chan & Morgan, 1990; Chan *et al.*, 1991a). It has now been established, however, that the primary determinant of this secretagogue activity is not α_2 -antagonism *per se*, but rather the possession of an imidazoline ring within the molecule. Thus, certain other imidazolines which are not α_2 -antagonists also have insulin secretagogue activity (Schulz & Hasselblatt 1989a); as does clonidine, which is an α_2 -adrenoceptor agonist (Schulz & Hasselblatt, 1989b; Plant *et al.*, 1991).

Taken together, these data suggest that K-ATP channels in the pancreatic β -cell may be subject to regulation by a component which contains a binding site for imidazoline compounds. This site does not appear to belong to either of the recently designated I_1 or I_2 subclasses of 'imidazoline receptor' (although such sites may be present on islet cells (Remaury & Paris, 1992; Brown *et al.*, 1993)) since it appears to have very low affinity for idazoxan (Brown *et al.*, 1993). However, the putative 'imidazoline binding site' responsible for control of K-ATP channel activity in the β -cell shows stereoselectivity and the response is subject to down-regulation by agonists, suggesting that the binding site may represent a new type of imidazoline receptor (Chan *et al.*, 1993).

In order to characterize this islet imidazoline receptor fur-

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ther, and to study its role in the regulation of insulin secretion, we have attempted to identify compounds that may act as antagonists at this site. In the present paper, we describe the results of studies with RX801080, an imidazoline compound that is related in structure to efaroxan.

Methods

Isolation of islets of Langerhans

Islets of Langerhans were isolated by collagenase digestion (Montague & Taylor, 1968) from the pancreata of male Wistar rats (180–250 g body weight) allowed free access to food and water. The isolation medium was a bicarbonate buffered physiological saline solution equilibrated to pH 7.4 by gassing with O₂:CO₂ (95:5) (Gey & Gey, 1936). The buffer was supplemented with 4 mM glucose and 1 mM CaCl₂. Islets were selected individually under a binocular dissecting microscope and were used within 2 h of isolation.

Insulin secretion experiments

The method for islet incubation has been described in detail previously (Morgan & Montague, 1985). Briefly, groups of 3 islets were incubated in bicarbonate buffered medium, supplemented with bovine serum albumin (1 mg ml⁻¹) and appropriate test reagents. Islets were incubated for 1 h at 37°C and samples were then removed for measurement of insulin by radioimmunoassay.

Preparation of HIT-T15 cell membranes

Crude membranes were prepared from HIT-T15 cells cultured in RPMI-1640 containing 10% (v:v) foetal calf serum (Ashcroft *et al.*, 1986). The tissue culture medium was removed from the flasks, and the cells rinsed with phosphate buffered saline. Cells were then scraped from the flask surface and suspended in ice cold Tris (50 mM)/EDTA (1 mM)/MgCl₂ (10 mM) buffer (TEM) pH 7.5. The suspension was homogenized in a Teflon/glass Potter Elvehjem homogenizer (6 passes) and the homogenate centrifuged at 40000 g for 20 min (4°C). The membrane pellet was washed by resuspension in 40 ml TEM buffer and recentrifuged at 40000 g for 20 min (4°C). The final pellet was resuspended in TEM buffer and stored at -80°C until required.

Radioligand binding experiments

[³H]-glibenclamide (11 Ci mmol⁻¹) was used to label glibenclamide binding sites in HIT cell membranes (Niki & Ashcroft, 1991). Membranes (approx 400 µg protein), [³H]-glibenclamide (2 nM) and competing agents were incubated in a final volume of 0.5 ml TEM buffer pH 7.5, for 1 h at 37°C. Non-specific binding was determined in the presence of 10 µM glibenclamide. Incubations were terminated by rapid vacuum filtration through Whatman GF/B filters. Filters were washed with 10 ml of ice cold Tris (50 mM)/EDTA (5 mM) buffer pH 7.4, and their radioactivity measured after addition of scintillant.

Protein determination

The protein content of HIT-T15 cell membrane preparations was measured by the bicinchoninic acid method of Smith *et al.* (1985).

Statistics

Results were analysed by Student's *t* test for unpaired data and differences were considered to be significant if the *t* value corresponded to a probability of 1:20 or less (*P* < 0.05).

Materials

Efaroxan, RX801080 and RX831003 (Figure 1) were synthesized as described by Chapleo *et al.* (1984) and were provided by Reckitt & Colman Products. Diazoxide was obtained from Glaxo Pharmaceuticals, phentolamine from Pfizer and midaglizole from Reckitt & Colman Products. Glibenclamide was provided by SmithKline Beecham and [³H]-glibenclamide was a gift from Hoechst Pharmaceuticals. Radioactive [¹²⁵I]-iodine (for radioimmunoassay) was from ICN Biomedicals, and anti-bovine insulin serum from Sigma. Culture medium RPMI-1640 and foetal calf serum were purchased from Gibco. Bicinchoninic acid reagents, for assay of protein, were purchased from Pierce. All other reagents were of analytical reagent quality.

Results

Screening of imidazoline compounds for activity in the pancreatic β-cell

In initial experiments two imidazoline compounds that are related in structure to efaroxan (Figure 1) were tested for activity in the pancreatic β-cell. In order to assess the effects of these compounds on islet function, their ability to reverse the inhibitory effect of 250 µM diazoxide on insulin secretion was studied. It has been shown previously that imidazoline compounds which can stimulate insulin secretion directly, are also able to overcome the inhibitory effects of diazoxide (Chan & Morgan, 1990; Chan *et al.*, 1991a,b; Plant & Henquin, 1990). Therefore the use of diazoxide provides a convenient assay system for determining the effectiveness of imidazolines to interact functionally with islet cells.

The results presented in Table 1 show that while efaroxan was able to reverse the inhibitory effects of diazoxide on insulin secretion, two close structural analogues, RX801080 and RX831003, were devoid of activity. We selected one of these (RX801080) for more detailed analysis and proceeded to investigate the effects of RX801080 on responses mediated by other imidazoline compounds in islets.

Figure 1 Structures of the imidazoline compounds tested for activity in pancreatic islets. The functional interactions of three imidazoline compounds with pancreatic islet cells were studied. The compounds employed were efaroxan, RX801080 and RX831003. Routes for synthesis of these compounds have been described by Chapleo *et al.* (1984).

Table 1 Effects of imidazolines on the inhibition of glucose-induced insulin secretion mediated by diazoxide

Imidazoline (100 μ M)	Diazoxide (250 μ M)	Insulin secretion (ng/islet h ⁻¹)
-	-	2.97 $\pm$ 0.22
-	+	0.52 $\pm$ 0.06
Efaroxan	+	2.80 $\pm$ 0.30*
RX801080	+	0.54 $\pm$ 0.09
RX831003	+	0.52 $\pm$ 0.06

Groups of 3 rat isolated islets were incubated for 1 h in the presence of 20 mM glucose. Diazoxide (250 μ M) was included as shown, in the presence of each of three different imidazoline compounds (100 μ M). Samples of medium were removed and insulin secretion measured by radioimmunoassay. Data are presented as mean rates of insulin secretion $\pm$ s.e.mean from 12 observations.

* $P < 0.001$ relative to 20 mM glucose + diazoxide.

Effects of RX801080 on responses mediated by imidazoline compounds in rat islets of Langerhans

Initially, it was established with several different islet preparations that RX801080 (up to 100 μ M) did not modify insulin secretion induced by 20 mM glucose confirming that the compound lacks significant α_2 -adrenoceptor agonist activity (Chapleo *et al.*, 1984) and does not exert other deleterious effects in the β -cell. When RX801080 was included in the incubation medium with efaroxan, it exerted a dose-dependent antagonistic effect, such that when present at an equimolar concentration with efaroxan, RX801080 completely prevented the reversal of diazoxide inhibition of secretion, mediated by efaroxan (Figure 2). The IC₅₀ for this response was approximately 40 μ M (Figure 2).

To examine this relationship further, we studied the effect of RX801080 on direct stimulation of insulin secretion induced by efaroxan (Table 2). Addition of 100 μ M efaroxan to islets incubated in the presence of 4 mM glucose resulted in a 3 fold increase in insulin secretion, and introduction of 100 μ M RX801080 abolished this response (Table 2). RX801080 did not alter basal insulin secretion in the absence of efaroxan (not shown).

Figure 2 Antagonism of the effects of efaroxan in rat islets by RX801080. Rat isolated islets were incubated in groups of 3 for 1 h in the presence of 20 mM glucose, 250 μ M diazoxide and 100 μ M efaroxan (●). Increasing concentrations of RX801080 were included as shown. The rate of insulin secretion in response to 20 mM glucose alone (■) and 20 mM glucose plus diazoxide (▲) is also shown. Each value represents the mean rate of insulin secretion $\pm$ s.e.mean from a minimum of 10 observations.

Table 2 Effects of RX801080 on direct stimulation of insulin secretion by efaroxan

Glucose (mM)	Efaroxan (100 μ M)	RX801080 (100 μ M)	Insulin secretion (ng/islet h ⁻¹)
4	-	-	1.20 $\pm$ 0.15
20	-	-	4.85 $\pm$ 0.35*
4	+	-	3.65 $\pm$ 0.40*
4	+	+	1.45 $\pm$ 0.20**

Groups of 3 rat isolated islets were incubated for 1 h under the conditions shown. The incubation medium was then sampled and its insulin content measured by radioimmunoassay. Results are mean values $\pm$ s.e.mean from 10 observations.

* $P < 0.001$ relative to 4 mM glucose alone; ** $P < 0.001$ relative to efaroxan alone.

The ability of RX801080 to antagonize stimulatory effects in islets was not restricted to responses mediated by efaroxan. Both phentolamine and midaglizole (100 μ M) effectively reversed the inhibition of insulin secretion mediated by 250 μ M diazoxide (Figure 3) and addition of 100 μ M RX801080 antagonized the effect of each of these compounds. These results therefore suggest that RX801080 is an effective antagonist of responses mediated by a range of imidazoline compounds in the pancreatic β -cell.

Effects of RX801080 on responses mediated by glibenclamide in pancreatic islets

In view of the evidence that efaroxan may exert its effects on insulin secretion by inducing the closure of K-ATP channels, the effects of RX801080 on a non-imidazoline K-ATP channel blocker, glibenclamide, were studied.

As shown previously (Panten *et al.*, 1989) glibenclamide

Figure 3 Antagonism of the effects of imidazoline compounds by RX801080. Groups of 3 rat islets were incubated at 37°C for 1 h in the presence of 20 mM glucose. Diazoxide (250 μ M) was included as indicated and phentolamine (Phentol) or midaglizole (Midag) were each present at a final concentration of 100 μ M. When present, RX801080 was included at a concentration of 100 μ M (▨). Samples of the medium were removed and assayed for insulin content by radioimmunoassay. Each value represents the mean $\pm$ s.e.mean from at least 10 observations. * $P < 0.001$ relative to 20 mM glucose alone (open column). ** $P < 0.001$ relative to test agent in the absence of RX801080 (▨).

was able to overcome significantly the inhibitory effect of diazoxide on glucose-induced insulin secretion from isolated islets (Table 3). Addition of RX801080 (100 μ M) prevented this response (Table 3) suggesting that the compound was able to antagonize the effect of glibenclamide directly. In order to verify this conclusion, the effect of RX801080 on direct stimulation of insulin secretion by glibenclamide was studied (Table 4). Glibenclamide promoted a dose-dependent increase in insulin secretion from islets incubated in the presence of 4 mM glucose; this response was significantly inhibited in the presence of 100 μ M RX801080 (Table 4).

Effects of RX801080 on binding of [3 H]-glibenclamide to HIT-T15 cell membranes

In order to examine the antagonistic effects of RX801080 on glibenclamide responses further, ligand binding studies were performed using [3 H]-glibenclamide to label sulphonylurea receptors on membranes from cultured β -cells. For these studies HIT-T15 cells were employed since they express abundant sulphonylurea receptors that have been well characterized pharmacologically (Boyd, 1988; Gaines *et al.*, 1988; Ashcroft & Ashcroft, 1990; Niki & Ashcroft, 1991; Schwansstecher *et al.*, 1992a,b).

The K_D for interaction of [3 H]-glibenclamide with its receptor in HIT cell membranes is in the sub-nanomolar range (Boyd, 1988; Gaines *et al.*, 1988) and concentrations up to 13 nM have been used successfully in displacement studies (Niki & Ashcroft, 1991). On the basis of preliminary experiments we found that a radioligand concentration of 2 nM was convenient for this purpose. Non-specific binding was determined in the presence of 10 μ M unlabelled glibenclamide, and was found to represent approximately 20% of the total binding (not shown).

Table 3 Effects of RX801080 on the ability of glibenclamide to inhibit the action of diazoxide in glucose-stimulated islets

Glibenclamide (1 μ M)	Diazoxide (250 μ M)	RX801080 (100 μ M)	Insulin secretion (ng/islet h $^{-1}$)
—	—	—	3.4 $\pm$ 0.20
—	+	—	1.2 $\pm$ 0.15*
+	+	—	2.6 $\pm$ 0.35**
+	+	+	0.8 $\pm$ 0.10***

Groups of 3 islets were incubated in the presence of 20 mM glucose for 1 h at 37°C. Glibenclamide, diazoxide and RX801080 were included as shown and the extent of insulin secretion measured. Results represent mean values $\pm$ s.e. mean from 12 observations.

* P < 0.001 relative to 20 mM glucose alone; ** P < 0.01 relative to 20 mM glucose + diazoxide; *** P < 0.001 relative to glibenclamide + diazoxide.

Table 4 Effects of RX801080 on stimulation of insulin secretion by glibenclamide in rat islets

Glucose (mM)	Glibenclamide (μ M)	RX801080 (100 μ M)	Insulin secretion (ng/islet h $^{-1}$)
4	—	—	0.27 $\pm$ 0.06
20	—	—	1.30 $\pm$ 0.19*
4	0.1	—	0.85 $\pm$ 0.12*
4	0.1	+	0.23 $\pm$ 0.09**
4	1	—	0.91 $\pm$ 0.13*
4	1	+	0.35 $\pm$ 0.10**

Groups of 3 islets were incubated with test reagents as shown for 1 h at 37°C. After this time samples of the incubation medium were removed and assayed for insulin content. Results represent mean values $\pm$ s.e. mean from 10 observations.

* P < 0.001 relative to 4 mM glucose alone; ** P < 0.001 relative to glibenclamide in the absence of RX801080.

Figure 4 Effects of imidazoline compounds on the binding of [3 H]-glibenclamide to membranes from HIT-T15 cells. Crude membranes from HIT-T15 cells were incubated with 2 nM [3 H]-glibenclamide for 1 h at 25°C in the absence (open columns) or presence (cross hatched columns) of 1 mM MgATP. Efaroxan (Efx, 300 μ M), RX801080 (RX801, 300 μ M) or diazoxide (Dz, 250 μ M) were included as shown. Bound and free ligand were separated by vacuum filtration. Each value represents the mean binding $\pm$ s.e. mean, from at least 8 determinations. * P < 0.001 relative to total specific binding in the absence of ATP. ** P < 0.001 relative to total specific binding in the presence of ATP.

Competition studies were performed to assess the ability of imidazolines to displace [3 H]-glibenclamide binding (Figure 4). These experiments revealed that neither efaroxan nor RX801080, at concentrations up to 300 μ M, was able to displace [3 H]-glibenclamide from its binding site.

It has been shown previously that certain ligands require the presence of MgATP in order to displace [3 H]-glibenclamide binding effectively from β -cell membranes (Niki & Ashcroft, 1991; Schwansstecher *et al.*, 1991; 1992a,b). Therefore, we also examined the effect of ATP on [3 H]-glibenclamide binding. Addition of MgATP (1 mM) resulted in approximately 40% displacement of specific [3 H]-glibenclamide binding to HIT cell membranes (Figure 4). Under these conditions, diazoxide (250 μ M) caused a further significant displacement of the radioligand (Figure 4). However efaroxan and RX801080 were still entirely ineffective in displacing [3 H]-glibenclamide binding, despite the presence of ATP.

Discussion

It has become evident from recent studies that the gating of K-ATP channels in the pancreatic β -cell can be regulated by a variety of pharmacological agents. The most potent of these are the sulphonylureas which are used widely in the management of Type II diabetes mellitus (reviewed by Ashcroft & Ashcroft, 1990; Nelson *et al.*, 1992). These agents control K-ATP channel activity by interacting with a specific receptor site located close to, or within, the channel (Sturgess *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987; Kramer *et al.*, 1988). However, the mechanisms by which occupation of this receptor results in channel closure have not been defined, and the receptor itself has eluded complete characterization.

By contrast with the sulphonylureas, certain other K-ATP channel blockers are less specific in their actions (e.g. tetraethylammonium, 9-aminoacridine; Henquin *et al.*, 1979; Karlsson & Ahren, 1992) and may not interact with specific receptors associated with the channels. Thus, both specific

and non-specific mechanisms have been implicated in K-ATP channel blockade.

In the present work we have extended our studies of the mechanisms involved in control of insulin secretion by imidazoline reagents. Previous results have shown that these compounds stimulate insulin release by inducing the closure of β -cell K-ATP channels (Chan *et al.*, 1991a; Plant & Henquin, 1990; Jonas *et al.*, 1992), but the nature of the interaction between imidazoline compounds and K-ATP channels has not been clarified. We have recently presented evidence that the ability of imidazolines to regulate insulin secretion is stereospecific and subject to agonist down-regulation (Chan *et al.*, 1993). These characteristics suggest that a functional receptor may be an important component of their site of interaction with the β -cell. The present results further support this hypothesis since they demonstrate that RX801080 can antagonize the stimulatory effects of other imidazoline compounds in islets.

The results described here also reveal that there are quite marked structural requirements determining efficacy at islet imidazoline receptors. For example, extension of the aliphatic chain on the chiral carbon of efaroxan (ethyl; Figure 1) by addition of 3 further carbon atoms (RX831033, Figure 1) resulted in loss of activity (Table 1). Presumably, this is due to the steric effect of an extended hydrocarbon chain reducing access of the molecule to the putative agonist binding site. RX801080 lacks substitution at the equivalent carbon atom of efaroxan (Figure 1) but is also inactive as an agonist. This lack of activity is unlikely to reflect reduced access to a binding site since any steric effects of substituents would be minimized in this molecule. Therefore, we investigated the possibility that RX801080 might interact with the efaroxan binding site in an antagonistic manner. The results obtained suggest that this may be the case, since RX801080 was found to antagonize the direct stimulation of insulin secretion mediated by efaroxan (Table 2) and also to prevent the effects of efaroxan and other imidazolines on responses induced by diazoxide in islets (Figures 2 and 3). Since the effects of efaroxan are attributable to changes in the open state of K-ATP channels, these results are consistent with the possibility that RX801080 acts antagonistically to other imidazolines at a regulatory site associated with K-ATP channels.

Confirmation of this hypothesis will require the development of a high affinity ligand that can be used to label the imidazoline binding sites in islets. In other tissues imidazoline sites can be labelled with [3 H]-idazoxan (Michel & Ernster, 1992; Kilpatrick *et al.*, 1992). However, although

[3 H]-idazoxan will label non-adrenoceptor sites in rat islets (Brown *et al.*, 1993) and cultured β -cell lines (Remaury & Paris, 1992), it neither stimulates insulin secretion nor antagonizes the effects of other imidazolines in rat islets (Chan & Morgan, 1990; Brown *et al.*, 1993). Thus, it appears that the functionally important binding site for imidazolines in islets differs from those described previously in other tissues, and that [3 H]-idazoxan is not a suitable ligand with which to label this site.

Surprisingly, RX801080 not only antagonized the responses to imidazolines in islets but also inhibited the ability of glibenclamide to stimulate insulin secretion (Tables 3 and 4). This was an unexpected observation in that there is no obvious structural similarity between the sulphonylurea and any of the imidazolines tested. Indeed, neither RX801080 nor efaroxan was able to compete with [3 H]-glibenclamide for its binding site on β -cell membranes (Figure 4) even when present at concentrations more than 5 orders of magnitude greater than that of the radioligand. Thus, the inhibition of the effects of glibenclamide by RX801080 does not result from direct competition for the same binding site. Furthermore, RX801080 did not inhibit glucose-induced insulin secretion (Figure 3) emphasizing that RX801080 does not alter the regulation of islet K-ATP channels mediated by endogenous ATP.

Taken together, the results of the present study imply that sulphonylureas and imidazoline compounds may both interact with regulatory sites associated with K-ATP channels. These sites are clearly independent with respect to ligand binding but they appear to be related in a functional manner. The molecular basis of this interaction remains to be established but it may prove to be important in the context of diabetes therapy, since recent studies have demonstrated that administration of low doses of glibenclamide and efaroxan to rats *in vivo* leads to a synergistic increase in insulin secretion (Berridge *et al.*, 1992). Characterization of the islet imidazoline binding site may, therefore, yield important information about the regulation of K-ATP channels and also provide a new target for development of novel anti-hyperglycaemic agents.

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