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Characterisation of new efaroxan derivatives for use in purification of imidazoline-binding sites

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Abstract

The insulin secretagogue activity of certain imidazoline compounds is mediated by a binding site associated with ATP-sensitive K^+ (K_{ATP}) channels in the pancreatic β-cell. We describe the effects of a series of structural modifications to efaroxan on its activity at this site. Substitution of amino-, nitro- or azide- groups onto the 5-position of the benzene ring of efaroxan did not significantly affect the functional interaction of the ligand with the islet imidazoline binding site. Modification of the imidazoline ring to an imidazole to generate 2-(2-ethyl-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazole (KU14R) resulted in loss of secretagogue activity. Indeed, this reagent appeared to act as an imidazoline antagonist since it blocked the secretory responses to imidazoline compounds and also inhibited the blockade of β-cell K_{ATP} channels by efaroxan in patch clamp experiments. Application of KU14R alone resulted in a modest reduction in K_{ATP} channel opening, suggesting that it may display weak partial agonism, at least in patch-clamp experiments.

Keywords: Insulin secretion; Endocrine pancreas; Efaroxan; Imidazoline; I-site; K_{ATP} channel

1. Introduction

It has become increasingly well established during recent years that the rate of insulin secretion from pancreatic β-cells can be increased by certain compounds bearing an imidazoline ring within their structure (e.g., efaroxan, phentolamine) (Chan, 1993; Morgan et al., 1995). This response occurs both in vivo (in animals and man) (Ahrén and Lundquist, 1985; Broadstone et al., 1987) and in vitro (Schulz and Hasselblatt, 1988, 1989; Smith and Furman, 1988; Plant and Henquin, 1990; Chan and Morgan, 1990; Jonas et al., 1992; Berdeu et al., 1994). Although the majority of imidazoline compounds which exhibit this activity also bind to α_2-adrenoceptors, there is clear evidence that their effects are not mediated by α_2-adrenoceptors (Chan et al., 1988; Schulz and Hasselblatt, 1989). Rather, they stimulate insulin secretion by virtue of an interaction with a binding site which displays many characteristics common to the class of 'imidazoline receptors' described in other tissues. These have now been subclassified into two major groups (imidazoline I_1 and I_2 receptors) on the basis of pharmacological criteria (Michel and Ernsberger, 1992; Regunathan and Reis, 1996), although atypical imidazoline receptors which do not readily fit this classification have also been described (Chan et al., 1994; Molderings and Göthert, 1995; Coupry et al., 1996; Molderings, 1997). The imidazoline receptor involved in control of insulin secretion represents an atypical site (Chan et al., 1994; Olmos et al., 1994) since its characteristics are distinctly different from those of imidazoline I_2 receptors and, although it has some similarity with imidazoline I_1 receptors, it also displays significant differences. One of the most important of these is the finding that efaroxan acts a potent antagonist at I_1 sites in the brain and the periphery (Ernsberger et al., 1992), whereas it is an agonist in the endocrine pancreas (Chan and Morgan, 1990; Chan et al., 1991; Berdeu et al., 1994). Furthermore,

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idazoxan binds to I_1 sites in most tissues but is not active in the pancreatic β-cell (Smith and Furman, 1988; Östenson et al., 1988; Chan et al., 1988; Chan and Morgan, 1990). Recently the putative islet imidazoline receptor has been termed 'I_3' to denote its atypical pharmacology (Eglen et al., 1998).

The imidazoline binding site present in pancreatic β-cells is associated with ATP-sensitive K^+ (K_{ATP}) channels and binding of agonists to the site results in K_{ATP} channel closure, membrane depolarisation, gating of voltage-sensitive Ca^{2+} channels and an increase in insulin secretion (Chan and Morgan, 1990; Plant and Henquin, 1990; Chan et al., 1991; Dunne, 1991; Proks and Ashcroft, 1997). Thus, agonists at the islet imidazoline binding site potentiate nutrient-induced insulin secretion suggesting that the site may represent a useful target for development of new insulin secretagogues for patients with type II (non-insulin-dependent) diabetes mellitus. Indeed, several imidazoline insulin secretagogues have already been shown to have efficacy in such patients (reviewed by Morgan, 1994; Molderings, 1997).

In order to develop new oral hypoglycaemic agents directed to the islet imidazoline receptor it is necessary to gain a firm understanding of the structure–activity relationships required for activity at this site. It is also important that the receptor is cloned and expressed in order to allow the full characterisation of its properties and to determine its relationship with other components of the K_{ATP} channel system. To this end, we have undertaken the development and functional characterisation of novel imidazolines with the aim of generating new ligands for use in analysis of the structure and function of the islet imidazoline binding site. In the present work we describe the effects of a series of structural modifications to efaroxan (an effective imidazoline agonist in pancreatic β-cells) one of which generates a compound (2-(2-ethyl-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazole; KU14R) having functional antagonist activity at the islet imidazoline site (Chan et al., 1997a). As such, KU14R is only the second example of a drug exhibiting antagonist activity at this site and represents a new ligand which will be useful for further characterisation of the site in pancreatic β-cells. Parts of this work have been reported in abstract form (Chan et al., 1997b).

2. Methods

2.1. Synthesis of efaroxan derivatives

The methods for synthesis and full characterisation of the derivatives of efaroxan will be described elsewhere (manuscript in preparation). Briefly, nitration of 2-ethyl-2,3-dihydrobenzo[*b*]furan-2-carboxylic acid (Edwards et al., 1987) gave the 5-nitro derivative which was transformed into the imidazolines **2** (2-(2-ethyl-5-nitro-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazoline; KU06M) **3** (2-(5-amino-2-ethyl-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazoline; KU08C) and **4** (2-(5-azido-2-ethyl-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazoline; KU10A) using standard methods (Chapleo et al., 1984). KU14R (2-(2-ethyl-2,3-dihydrobenzo[*b*]furan-2-yl)-1*H*-imidazole; **5**) was prepared from 2-cyano-2-ethyl-2,3-dihydrobenzo[*b*]furan by transformation to the imidate ester and reaction with aminoacetaldehyde diethylacetal.

2.2. Preparation of efaroxan conjugates

5-Amino-efaroxan (KU08C) was coupled to either bovine serum albumin (Type V), thyroglobulin, carbonic anhydrase or trypsinogen with glutaraldehyde. Solutions of amino–efaroxan (0.5 ml at 5 mg/ml) and protein (0.5 ml at 10 mg/ml) in 0.01 M phosphate buffered saline (PBS, pH 7.4) were carefully mixed with 0.25 ml of glutaraldehyde (0.5%, v/v). After incubating at room temperature for 60 min, the reaction was stopped by the addition of 70 μl 1 M lysine (pH 7.0). The complexes were then dialysed extensively with PBS at 4°C for 24 h.

Control protein conjugates (without efaroxan) were crosslinked with glutaraldehyde as described above.

2.3. Immunisations

5-Amino–efaroxan coupled to bovine serum albumin (with glutaraldehyde) was used as immunogen (Harlow and Lane, 1988). Primary sub-cutaneous injections (three separate sites; 0.2 ml per site) in rabbits of the immunogen (protein concentration 5 mg/ml) emulsified 1:1 (v/v) in Freund's complete adjuvant was followed at 3–4 week intervals by booster injections of immunogen in Freund's incomplete adjuvant (1:1, v/v). Marginal ear vein bleeds were taken 1 week after each booster injection.

The presence of anti-efaroxan antibodies in the sera was determined by ELISA. This ELISA was also used to assess the specificity of the antibodies in competition experiments.

2.4. ELISA

5-Amino–efaroxan coupled to thyroglobulin was used as the coating molecule. Use of a different efaroxan–protein conjugate eliminates the necessity to remove antibodies generated against the BSA carrier during immunisation. Efaroxan–thyroglobulin conjugate (20 μg/ml) was adsorbed overnight at 4°C to wells of 96-well ELISA plates in 0.1 M carbonate/bicarbonate buffer (pH 9.6). After washing with 50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.1% (v/v) Tween-20, wells were blocked with 2% (v/v) goat serum in 50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.1% (v/v) Tween-20 (blocking solution), for 60 min at 37°C. Following two washes with 50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM KCl,

0.1% (v/v) Tween-20, 100 μl antiserum (1:100 dilution in blocking solution) and test reagents were added. Following incubation for 2 h at 37°C, the wells were washed three times for 5 min each wash with 50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.1% (v/v) Tween-20. Goat anti-rabbit Ig G antibody coupled to alkaline phosphatase (Sigma) was added (1: 10 000 in blocking solution) and incubated for 60 min, 37°C. Following washes with 50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.1% (v/v) Tween-20, colorimetric detection using *p*-nitrophenyl phosphate as substrate was performed, and absorbance measured at 405 nm.

In competition experiments, a decrease in optical density reading correlates with recognition of antibodies for the competitor.

2.5. Membrane preparations

Rat brain membranes were prepared from whole rat brain removed immediately following sacrifice. The brains were chopped finely and homogenised in ice-cold TEM buffer (50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$, pH 7.4), supplemented with 50 μM phenylmethyl sulphonylfluoride, 2 μg/ml aprotonin, 2 μg/ml leupeptin) and then centrifuged at $3000 \times g$ for 10 min, 4°C. The resultant supernatant was centrifuged at $40\,000 \times g$ for 20 min, 4°C. The pellet was resuspended in 50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$, pH 7.4 and re-centrifuged. The final crude membrane fraction was resuspended in 50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$, pH 7.4 and aliquots were snap-frozen and stored at −80°C. Protein content was measured by the bicinchoninic acid method (Smith et al., 1985).

2.6. Radioligand binding studies

Aliquots of rat brain membranes (100 μg protein) were incubated with 5 nM [^{3}H]RX821002 in the absence or presence of efaroxan or other competing drugs (total incubation volume, 100 μl) for 90 min at 25°C in 50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$, pH 7.5. After this time, bound and free ligand were separated by rapid vacuum filtration through Whatman GF/C filters. The filters were washed (2×3 ml) with ice-cold TEM buffer and their radioactivity determined by liquid scintillation counting. Non-specific binding was defined in the presence of 100 μM yohimbine.

2.7. Preparation of islets of Langerhans

Islets of Langerhans were isolated from Wistar rats of either sex (170–250 g body weight) by collagenase digestion (Montague and Taylor, 1968). Islets were selected by hand under a binocular dissecting microscope and were incubated in groups of three in 0.5 ml of bicarbonate buffered saline solution (Gey and Gey, 1936) containing 1 mM $CaCl_2$ and bovine serum albumin (1 mg/ml). The medium was gassed with O_2:CO_2 (95:5) to pH 7.4 and contained 4 mM glucose during islet isolation. During insulin secretion studies the islets were incubated with appropriate test reagents for 1 h at 37°C and the medium was then sampled for assay of insulin by radioimmunoassay.

2.8. Electrophysiology

Primary cultures of rat pancreatic β-cells were prepared for patch-clamp studies using methods described previously (Hamill et al., 1981; Findlay et al., 1985). Single channel current events from K_{ATP} channels were recorded using the inside-out configuration with the micropipette filled with a 140 mM sodium-rich solution and the bath contained a 140 mM K^+-rich solution (Lebrun et al., 1996). The osmolarity of all solutions was 290 ± 5 mosm/kg. All studies were carried out at 0 mV voltage clamp. Under these experimental conditions, with the intracellular Ca^{2+} concentration buffered to < 10 nM, openings from calcium- and voltage-dependent K^+ channels are not observed. The recording apparatus, data storage and analytical procedures have been described previously (Lebrun et al., 1996).

2.9. Materials

Efaroxan and derivatives were synthesised in house, as outlined above. Anti-bovine insulin serum for radioimmunoassay was from ICN Biomedicals and [^{125}I]iodine for radioiodination was from Dupont-NEN. Crystalline biosynthetic human insulin was used for radioiodination and was a gift from Eli Lilly (Indianapolis, USA). Reagents for protein conjugation were purchased from Pierce Chemicals and ELISA/immunoblot reagents were from Sigma [^{3}H] RX821002 was purchased from Amersham International (UK) and unlabelled RX821002 was a gift from Reckitt and Colman Products (Hull, UK). All general laboratory reagents were of analytical grade.

2.10. Statistical analysis

Differences between groups of samples were examined by either analysis of variance or by Student's *t*-test and data were considered significant when $P < 0.05$ or lower.

3. Results

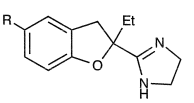
3.1. Effects of substitution on the benzene ring of efaroxan to the functional activity of the molecule

Initially a series of substituted efaroxan derivatives was synthesised in which an amino-, nitro- or azide- group was

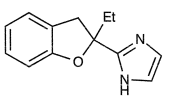
successively substituted onto the 5-position of the parent molecule in order to generate derivatives which could, in principle, be coupled to other molecules (e.g., via the amino or azide groups). Nuclear magnetic resonance analysis of each purified product confirmed the location of the substituents on the benzene ring (Fig. 1). The functional activity of each of these derivatives was then tested in insulin secretion experiments. These were performed using an assay in which glucose-induced insulin secretion was first inhibited with diazoxide and then the ability of the substituted derivatives to reverse this inhibition examined. This assay has been used extensively to study imidazoline responses in islet cells (Chan and Morgan, 1990; Plant and Henquin, 1990; Chan et al., 1991).

All three derivatives (KU06M, KU08C and KU10A) significantly reversed the inhibitory effect of diazoxide on glucose-induced insulin secretion (Fig. 2a–c). These effects were dose-dependent although small differences in potency between the compounds were evident (EC_{50} values: KU10A—15 μM; KU06M—30 μM; KU08C—60 μM). The potential photoaffinity reagent, KU10A, also retained the α_2-adrenoceptor antagonist properties of efaroxan in that it dose-dependently antagonised the inhibition of insulin secretion mediated by 1 μM noradrenaline (Fig. 3) and this response occurred at lower concentrations ($EC_{50} \sim 2$ μM) than those required to reverse the inhibitory effect of diazoxide (Fig. 2c). Thus, it appears that addition of substituents to position 5 of the benzene ring did not prevent the interaction of efaroxan with either the islet imidazoline binding site or with islet α_2-adrenoceptors.

In order to confirm the α_2-adrenoceptor binding properties of KU10A, displacement experiments were performed in brain membranes using the selective α_2-adrenoceptor antagonist [^{3}H]RX821002 as radioligand (Fig. 4). Efaroxan caused a dose-dependent reduction in [^{3}H]RX821002 binding over the concentration range 10–1000 nM (Fig. 4) whereas unusual results were obtained with KU10A (Fig. 4). Addition of between 10 nM and 10 μM KU10A resulted in a paradoxical increase in binding of [^{3}H]RX821002 to the membranes. This was not due to a significant change in non-specific binding since yohimbine was still able to displace [^{3}H]RX821002 even in the presence of 100 nM KU10A. Thus, it appears that the presence



(1) R = H, efaroxan
(2) R = NO_2, KU06M
(3) R = NH_2, KU08C
(4) R = N_3, KU10A



(5) KU14R

Fig. 1. Structure of efaroxan and derivatives.

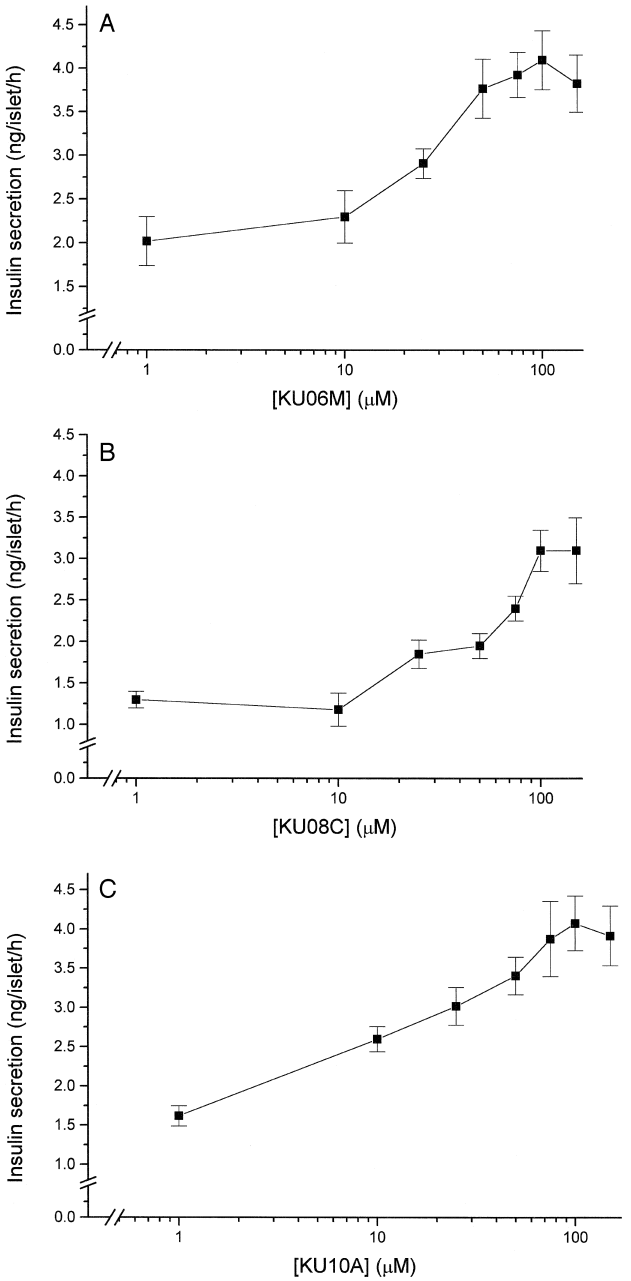


Fig. 2. Effect of efaroxan derivatives on diazoxide-induced inhibition of insulin secretion from rat islets. Groups of three islets were incubated in the presence of 20 mM glucose plus 200 μM diazoxide for 1 h at 37°C. Increasing concentrations of KU06M (panel A) KU08C (B) and KU10A (C) were included, as shown. Samples of medium were collected and assayed for insulin by radioimmunoassay. All data are mean values ± S.E.M. from replicates of 12. The rates of insulin secretion measured in the presence of 20 mM glucose alone averaged 4.0 ± 0.35 ng/islet per h (panel A) 3.95 ± 0.40 ng/islet per h (panel B) and 4.10 ± 0.26 (panel C) ng/islet per h.

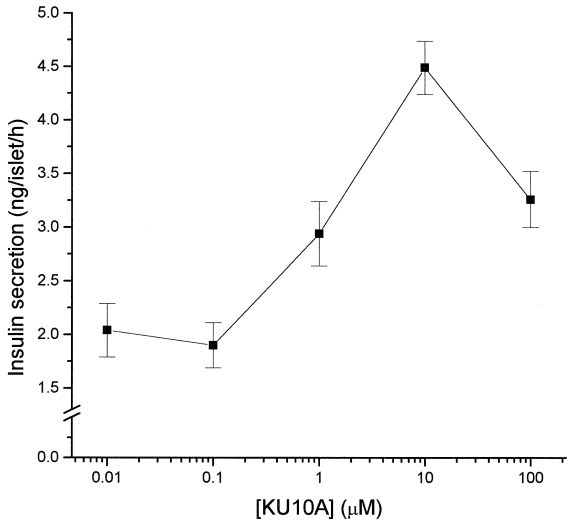


Fig. 3. Effects of KU10A on inhibition of glucose-induced insulin secretion mediated by noradrenaline. Groups of three isolated islets were incubated in medium containing 20 mM glucose and 1 μM noradrenaline in the absence or presence of increasing concentrations of KU10A, as shown. After incubation for 1 h, samples of medium were removed and insulin release measured. Results are mean values ± S.E.M. from 12 observations. The rate of insulin secretion measured in the presence of 20 mM glucose alone averaged 3.3 ± 0.3 ng/islet per h. Noradrenaline (1 μM) reduced this to 1.6 ± 0.15 ng/islet per h.

of KU10A resulted in exposure of binding sites which are normally masked within the membrane preparation. These additional sites retained the characteristics of α_2-adrenoceptors since they were labelled by low concentrations of [^{3}H]RX821002 and were sensitive to yohimbine.

3.2. Development of an antiserum having high affinity for efaroxan derivatives

We exploited the utility of 5-amino-efaroxan (KU08C) to raise a polyclonal antiserum capable of high-affinity binding to the conjugated ligand. Using an ELISA, it was established that binding of the antiserum to conjugated efaroxan was inhibited by several structural analogues including efaroxan itself, KU08C and KU10A (Fig. 5b) but not by structurally unrelated compounds such as

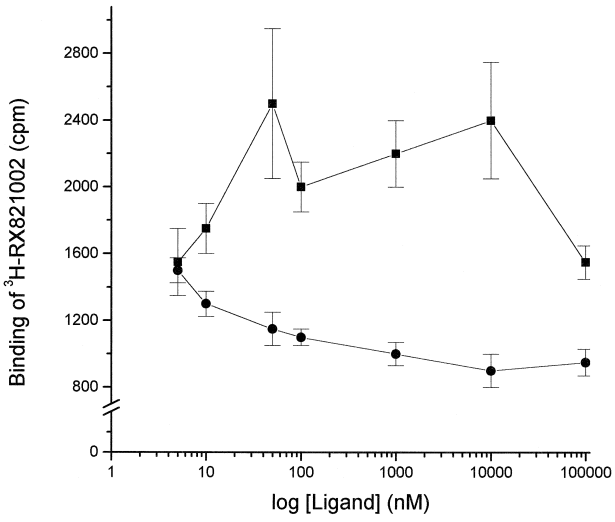


Fig. 4. Effects of efaroxan and KU10A on binding of [^{3}H]RX821002 to rat brain α_2-adrenoceptors. Rat brain membranes were incubated with [^{3}H]RX821002 in the absence or presence of increasing concentrations of either efaroxan (circles) or KU10A (squares) for 90 min at 25°C. After this time, bound from free ligand was separated by rapid vacuum filtration. Each point represents the mean value ± S.E.M. obtained from six separate experiments each performed in replicates of five. The binding of [^{3}H]RX821002 measured in the absence of competing ligands averaged 1550 ± 120 cpm.

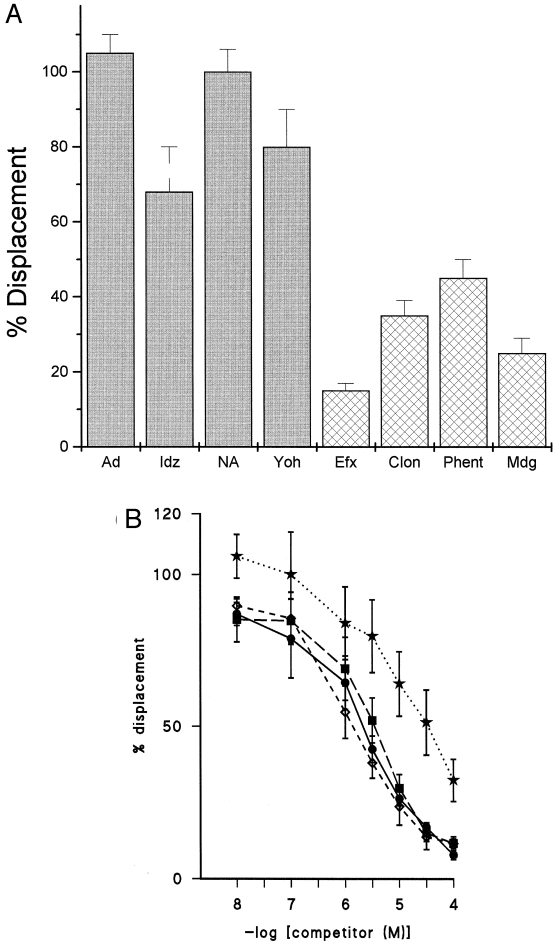


Fig. 5. (a) Specificity of anti-efaroxan antibodies. Competitors were included in the ELISA at concentration of 100 μM (adrenaline, Ad; efaroxan, Ef; idazoxan, ldz; clonidine, Clon; phentolamine, Phent; noradrenaline, NA; yohimbine, Yoh; midaglizole, Mdg). A decrease in the absorbance reading correlates with the displacement of antibody binding to the efaroxan conjugate coating the bottom of the well. Data are expressed as mean percent of displacement for 3–6 separate experiments. (b) Antiserum displacement curves for efaroxan and derivatives. The ELISA was performed as described in Section 2. Efaroxan and its derivatives were included as shown (Efaroxan, ●; KU10A, ■; KU08C,; KU14R, ☆). Data are presented as mean percent of displacement for three or four separate experiments.

adrenaline, noradrenaline and yohimbine (Fig. 5a). It is noteworthy that those drugs that can interact at the islet imidazoline receptor were recognised well by the antiserum suggesting that it may recognise structural features that are important for imidazoline receptor binding (Fig. 5a). Fig. 5b reveals that the antiserum recognised efaroxan, KU08C and KU10A with similar affinities (EC_{50} approx. 5 μM at 1:100 antiserum). KU14R was also recognised strongly by the antibodies but with lower affinity (EC_{50} approx. 30 μM).

3.3. Effects of modification to the imidazoline ring on the functional activity of efaroxan

Having established that addition of substituents to the 5-position of efaroxan did not result in significant loss of biological activity, we next investigated the effect of structural modification of the imidazoline ring. A number of derivatives were synthesised, of which one in particular (KU14R, Fig. 1) displayed unusual activity in functional assays. KU14R contains an imidazole substituent in place of the imidazoline ring and was lacking in agonist activity in insulin secretion assays (Table 1). By contrast, inclusion of KU14R with efaroxan resulted in significant antagonism of the effect of the imidazoline secretagogue (Table 1).

KU14R was also able to antagonise the stimulatory effects of several other imidazoline secretagogues in the endocrine pancreas (Fig. 6) including phentolamine and RX821002 but it did not alter the rate of secretion in response to 20 mM glucose (Table 1) nor did it inhibit insulin secretion mediated by depolarisation of the β-cell membrane with 50 mM KCl (not shown). Thus, the inhibitory effect of KU14R on insulin secretion was selective for imidazoline responses and did not reflect a generalised inhibition of islet secretion. Despite its opposite functional activity at the imidazoline receptor, KU14R retained the α_2-adrenoceptor antagonist properties of efaroxan in islets (Chan and Morgan, 1990) in that it blocked the inhibition of glucose-induced insulin secretion mediated by noradrenaline (Fig. 7).

Table 1
Effect of KU14R on insulin secretory responses to glucose and diazoxide in isolated rat islets

[Glucose] (mM)	Diazoxide 200 μM	Efaroxan 100 μM	KU14R 100 μM	Insulin secretion (ng/islet per h)
6	–	–	–	0.92 ± 0.21
6	–	+	–	1.65 ± 0.21
6	–	–	+	0.95 ± 0.10[a].
6	–	+	+	0.72 ± 0.18[b]
20	–	–	–	2.34 ± 0.55
20	–	–	+	2.38 ± 0.24[a].
20	+	–	–	0.79 ± 0.15
20	+	+	–	2.60 ± 0.14
20	+	–	+	1.28 ± 0.23[a]
20	+	+	+	1.23 ± 0.18[b]

Groups of three isolated rat islets were incubated in the presence of either 6 mM or 20 mM glucose. Diazoxide (200 μM), KU14R (100 μM) and efaroxan (100 μM) were included as shown. After incubation for 1 h, samples of medium were removed for assay of insulin by radioimmunoassay. Each point is the mean ± S.E.M. for 12 replicates in each case.
[a]Not significantly different from the equivalent incubation condition in the absence of KU14R.
[b]$P < 0.01$ Relative to equivalent response mediated by efaroxan in the absence of KU14R.

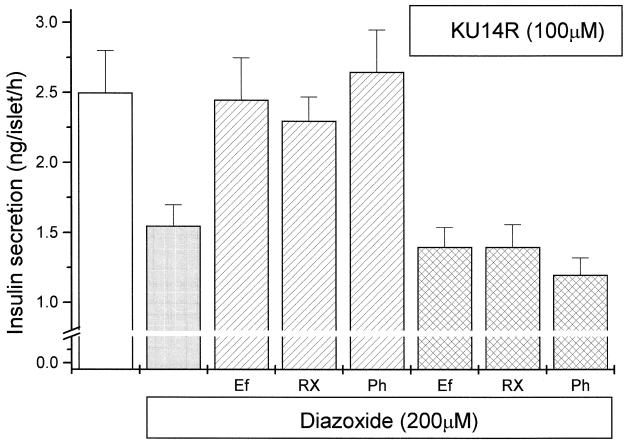


Fig. 6. Antagonism of imidazoline-mediated responses by KU14R in rat islets of Langerhans. Groups of three islets were incubated in the presence of 20 mM glucose, 200 μM diazoxide and imidazoline drugs (efaroxan, Ef; RX821002, RX; phentolamine, Ph; 100 μM) as shown. KU14R was included at 100 μM (double hatches). Insulin secretion was measured after incubation for 1 h at 37°C. In each case the inclusion of KU14R significantly antagonised the secretory response to the imidazoline compound ($P < 0.005$ for all conditions with KU14R, relative to appropriate control in its absence). Each point is the mean ± S.E.M. from 12 replicates.

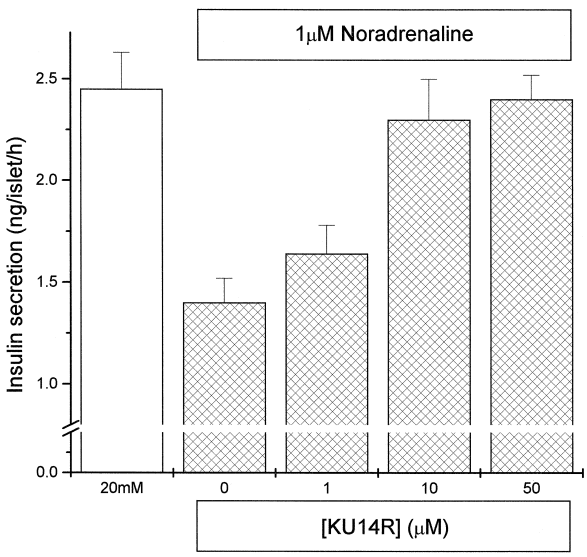


Fig. 7. Effects of KU14R on inhibition of glucose-induced insulin secretion mediated by noradrenaline. Groups of three isolated islets were incubated in medium containing 20 mM glucose and 1 μM noradrenaline in the absence or presence of increasing concentrations of KU14R, as shown. After incubation for 1 h, samples of medium were removed and insulin release measured. Results are mean values ± S.E.M. from 12 observations.

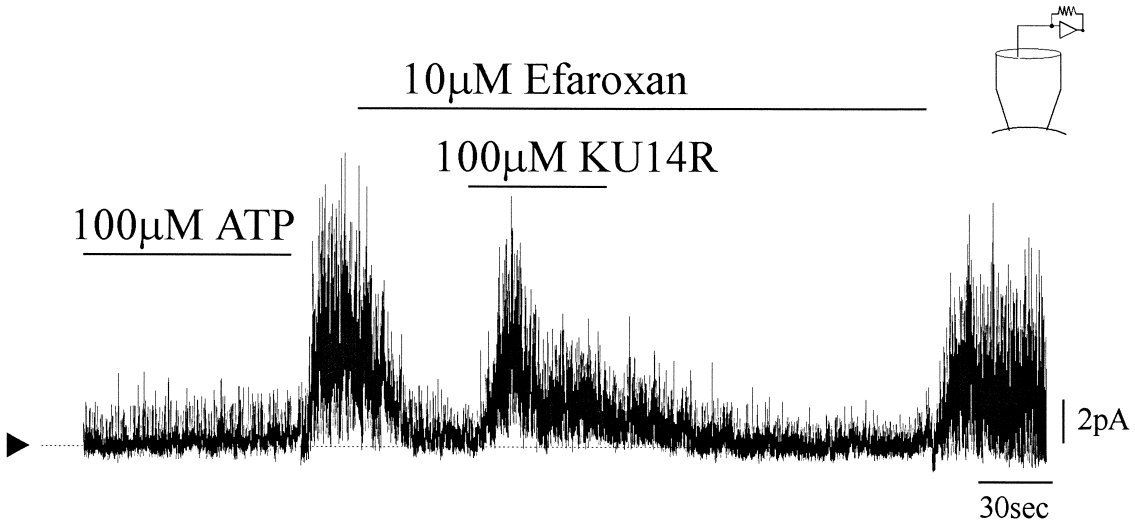


Fig. 8. Modulation of imidazoline-induced inhibition of K_{ATP} channels by KU14R. The continuous current recording illustrated was taken from an inside-out patch on an isolated rat β-cell. The record shows upward deflections as outward K_{ATP} channel currents from the zero current level. The presence of K_{ATP} channels was confirmed by blockade with ATP. Efaroxan also caused channel blockade and this was relieved in a reversible manner by 100 μM KU14R.

In order to confirm the observations made with intact islets, the effects of KU14R on ATP-sensitive K^+ channel activity in pancreatic β-cells was also investigated directly by patch-clamp techniques. A total of 28 inside-out patches were studied and the presence of K_{ATP} channels confirmed by the inhibitory effects of ATP (Fig. 8). As expected, addition of 10 μM efaroxan to rat pancreatic β-cells resulted in substantial inhibition of the gating of K_{ATP}

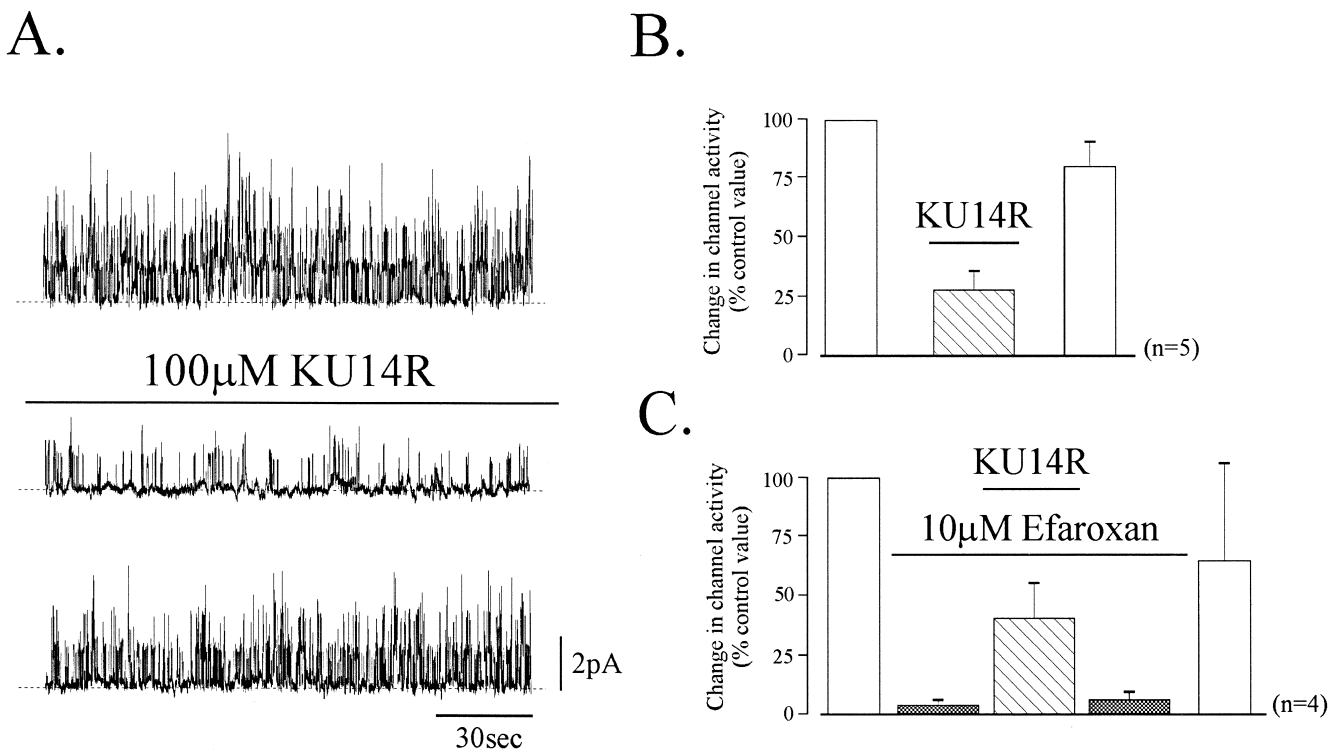


Fig. 9. Effects of KU14R on β-cell K_{ATP} channels. Panel A shows the direct effects of 100 μM KU14R on K_{ATP} channel activity in pancreatic β-cells. Each of the data panels is taken from the same patch of membrane and the interval between panels was 5 s. When added directly to the membrane in the absence of efaroxan, KU14R inhibited K_{ATP} channels. Panel B shows a summary of the direct effects of KU14R on K_{ATP} channels. Data points are mean values ± S.E.M. obtained from separate additions of KU14R to five inside-out patches. Panel C summarises the effects of KU14R on the closure of K_{ATP} channels mediated by efaroxan. Each value is the mean ± S.E.M. obtained from separate additions of KU14R to four inside-out patches.

channels ($n = 17/17$ occasions in eight patches). The mean decrease in channel activity induced by 10 μM efaroxan was to $4 \pm 2\%$ of control. Subsequent introduction of an excess of KU14R was associated with rapid recovery of channel opening, consistent with antagonism of the effects of efaroxan (Fig. 8 and Fig. 9; $n = 16/17$ in eight patches). This was readily reversible upon removal of KU14R and still later removal of efaroxan was accompanied by complete recovery of channel activity. These data suggest that the presence of efaroxan or KU14R did not cause any long term functional change to the K_{ATP} channels. Although KU14R did not, itself, directly alter insulin secretion under any conditions studied, addition of 100 μM of the compound to pancreatic β-cells under patch clamp conditions resulted in a reduction in channel opening (Fig. 9A,B). This was seen consistently in 13 patches on 17/17 occasions and resulted in an average inhibition of channel activity to $27.8 \pm 9\%$ of the immediate control value. These results indicate that KU14R was not entirely devoid of channel blocking activity but suggest that it may display weak partial agonism, at least in patch-clamp experiments.

4. Discussion

While it is well established that imidazolines can cause closure of K_{ATP} channels, raise cytosolic Ca^{2+} and stimulate insulin secretion in pancreatic β-cells (Schulz and Hasselblatt, 1988, 1989; Plant and Henquin, 1990; Chan, 1993; Dunne et al., 1995; Morgan et al., 1995; Shepherd et al., 1996; Zaitsev et al., 1996; Molderings, 1997; Efanova et al., 1998) the binding site(s) responsible for mediating these effects has not been identified unequivocally. Proks and Ashcroft (1997) have suggested that imidazolines may interact directly with the pore-forming subunit of the K_{ATP} channel, Kir6.2, but evidence has also been presented to indicate that intracellular imidazoline binding sites may also be involved (Zaitsev et al., 1996; Efanova et al., 1998). In the present work, we have developed a series of reagents, based on the structure of the imidazoline insulin secretagogue efaroxan, which may be useful for characterisation of imidazoline binding sites expressed in pancreatic β- (and other) cells. We demonstrate that introduction of structural changes to the insulin secretagogue efaroxan can lead to marked alterations in functional activity. Substitution of the benzene ring of the molecule was not associated with significant changes in the ability of efaroxan to stimulate insulin secretion suggesting that this moiety is not crucial for ligand recognition. Conversely, alteration of the imidazoline ring by introduction of an additional double bond (generating an imidazole; KU14R) yielded a compound which lacked insulin secretagogue activity. This reagent appeared to act as an imidazoline antagonist in rat islets since it blocked the secretory response to efaroxan and several other imidazoline secretagogues (Table 1, Fig. 6; Chan et al., 1997a). The effect was selective in that stimulation of insulin secretion by either 20 mM glucose or depolarisation with KCl was not inhibited by the compound, suggesting that it did not cause any generalised functional impairment of the islets. The most straightforward interpretation of these data is that KU14R acts as an antagonist at the islet imidazoline receptor and thereby prevents the stimulatory effects of efaroxan. On this basis, KU14R would occupy the islet imidazoline binding site without eliciting an increase in insulin secretion. To our knowledge, only one other antagonist of the islet imidazoline receptor has been reported (RX801080; Brown et al., 1993) and this reagent is no longer available for general use. Thus, KU14R represents an important new addition to the range of reagents available for characterisation of imidazoline-binding proteins.

It has been suggested that the binding site for imidazolines may lie on the intracellular face of the plasma membrane (Dunne et al., 1995; Rustenbeck et al., 1995), raising the possibility that the lack of functional activity of KU14R could reflect poor accessibility to the binding site. However, the finding that KU14R was able to modify the effects of efaroxan in intact islets confirms that the compound did gain access to the binding site.

The effects of KU14R described in the present work offer new insights into the mechanisms which underlie the functional effects of imidazoline drugs in islets. Thus, one possibility is that imidazolines may elicit the closure of K_{ATP} channels by physically occluding the channel pore. We have challenged this view by demonstrating that the insulin secretory response to efaroxan in islets displays marked stereospecificity and can be downregulated (Chan et al., 1993, 1994); characteristics which suggest the involvement of a functional receptor. Furthermore, we have also provided recent evidence that the site may be a target for physiological ligands (Chan et al., 1997c). The present results add further support to the hypothesis that the islet imidazoline receptor has an independent functional status by demonstrating that KU14R lacks secretagogue activity in its own right but can antagonise the effects of other imidazolines. Thus, it appears to occupy the imidazoline receptor in intact islets in a manner which impedes the action of other imidazolines. This was confirmed in patch-clamp studies of K_{ATP} channel activity which revealed that KU14R was able to significantly antagonise the inhibition of channel activity by efaroxan (Fig. 8). Surprisingly, these experiments also revealed that, when added alone, KU14R caused some inhibition of β-cell K_{ATP} channel activity (Fig. 9). This effect was much less marked than with efaroxan and was not associated with stimulation of insulin secretion. Thus, KU14R appears to exhibit the characteristics of a weak partial agonist in electrophysiological studies but displays only antagonism at the level of insulin secretion. It is of interest, in this context, that idazoxan also acts as a weakly effective blocker of β-cell K_{ATP} channels (Chan et al., 1991) but does not stimulate insulin

secretion (Chan et al., 1988; Östenson et al., 1988; Chan and Morgan, 1990).

We cannot draw definitive conclusions about the affinity of KU14R for the islet imidazoline binding site but the functional data suggest that it may be a more potent ligand than efaroxan since, at equimolar concentrations, KU14R was able to inhibit the effect of efaroxan almost completely in insulin secretion experiments. This was the case whether direct stimulation of insulin secretion (at 6 mM glucose) or reversal of the effects of diazoxide were measured.

The finding that attachment of any one of several functional groups to the benzene ring of efaroxan did not alter its activity as an insulin secretagogue provides a potential means to synthesise ligands which can be covalently coupled to the target receptor upon binding. In particular, we have been able to synthesise a derivative which bears a photoreactive azide moiety (KU10A) and can, in principle, be cross-linked to efaroxan-binding proteins. Moreover, we have also developed a high affinity polyclonal antiserum capable of recognising efaroxan after covalent attachment to protein. This antiserum displays a high level of selectivity for derivatives of efaroxan (Fig. 4b) and it is suitable for detection of efaroxan after conjugation to protein on both Western blots and in ELISA.

Both KU14R and KU10A retained the ability of the parent compound efaroxan, to act as α_2-adrenoceptor antagonists in rat islets (Figs. 3 and 7). This suggests that the structure–function relationships for the islet imidazoline receptor are quite different from those that characterise α_2-adrenoceptors. These observations also serve to emphasise the fact that the ability of imidazoline drugs to stimulate insulin secretion does not result from blockade of islet α_2-adrenoceptors. In radioligand binding studies, KU10A displayed unusual characteristics in that it appeared to increase the specific binding of [^{3}H]RX821002 at certain concentrations. [^{3}H]RX821002 is considered to be a selective α_2-adrenoceptor antagonist, although we have shown that, at high concentrations, it can also label imidazoline binding sites with low affinity (Chan et al., 1994). Thus, the increase in binding induced by KU10A could reflect a change in interaction with either of these sites. However, the measured increase in binding was displaced completely by yohimbine suggesting that KU10A facilitates binding of [^{3}H]RX821002 to a population of α_2-adrenoceptors rather than to a mixed population which includes imidazoline binding sites. The mechanisms by which [^{3}H]RX821002 was able to label α_2-adrenoceptor sites differentially in the presence and absence of KU10A remain unclear but it is noteworthy that the increase in labelling was seen at low concentrations (10–100 nM) of KU10A.

5. Conclusion

The current work demonstrates that the islet imidazoline binding site displays strict structure–activity relationships. Modifications to the benzene ring of the insulin secretagogue efaroxan can be tolerated without loss of functional activity whereas changes to the imidazoline moiety have greater impact. Using this information, we have developed an antagonist of the islet imidazoline receptor (KU14R) which is functional under both whole cell and patch-clamp conditions, and a novel photoreactive agonist, KU10A. These reagents should be valuable probes for use in characterisation and purification of the islet imidazoline binding site.

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