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The effect of the putative endogenous imidazoline receptor ligand, clonidine-displacing substance, on insulin secretion from rat and human islets of Langerhans

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1 The effects of a rat brain extract containing clonidine-displacing substance (CDS), a putative endogenous imidazoline receptor ligand, on insulin release from rat and human isolated islets of Langerhans were investigated.

2 CDS was able to potentiate the insulin secretory response of rat islets incubated at 6 mM glucose, in a dose-dependent manner. The magnitude of this effect was similar to that in response to the well-characterized imidazoline secretagogue, efaroxan.

3 CDS, like other imidazoline secretagogues, was also able to reverse the inhibitory action of diazoxide on glucose-induced insulin release, in both rat and human islets.

4 These effects of CDS on secretion were reversed by the imidazoline secretagogue antagonists, RX801080 and the newly defined KU14R, providing the first evidence that imidazoline-mediated actions of CDS can be blocked by specific imidazoline antagonists.

5 The effects of CDS on insulin secretion were unaffected when the method of preparation involved centri-filtration through a 3,000 Da cut-off membrane or when the extract was treated with protease. These results confirm that the active principle is of low molecular weight and is not a peptide.

6 Overall, the data suggest that CDS behaves as a potent endogenous insulin secretagogue acting at the islet imidazoline receptor.

Keywords: Islets of Langerhans; insulin secretion; imidazoline; I-site; endogenous ligand

Introduction

Imidazoline compounds such as efaroxan and phentolamine represent a newly defined class of pharmacological agents capable of modulating the open state of adenosine 5′-triphosphate (ATP)-sensitive potassium channels in the pancreatic B-cell (Chan & Morgan, 1990; Plant & Henquin, 1990; Dunne, 1991; Chan *et al*., 1991; Chan, 1993). Like sulphonylureas, which are used widely in the management of Type II diabetes mellitus, imidazoline compounds stimulate insulin release by inducing the closure of B-cell K-ATP channels, leading to membrane depolarization, opening of voltage-sensitive calcium channels and thus, initiation of exocytosis. The nature of the interaction between imidazoline compounds and K-ATP channels has not been clarified, but we have provided evidence that a functional receptor may be involved (Morgan *et al*., 1995). Although full characterization of this putative receptor has not yet been achieved, it is clearly distinct from the sulphonylurea binding site (Brown *et al*., 1993a). The imidazoline binding site involved in potentiation of insulin secretion also displays pharmacological characteristics (Chan *et al*., 1994; Olmos *et al*., 1994) that distinguish it from the I_1 and I_2 subclasses of imidazoline receptor (I-receptor) defined in certain other tissues (Michel & Ernsberger, 1992; Regunathan & Reis, 1996). Thus, the pancreatic B-cell imidazoline receptor can be classified as an atypical receptor and, as such, it may represent a selective target for a new generation of oral anti-hyperglycaemic agents.

The description of a new class of physiologically relevant binding sites for imidazoline compounds raises the possibility that an endogenous ligand may exist which can act agonistically at these sites. Although the existence of such a molecule has not been confirmed definitively, a substantial body of evidence has accumulated which suggests that an endogenous ligand is present in certain tissues (Atlas, 1990; 1995; Reis *et al*., 1995). This compound has been termed clonidine-displacing substance (CDS) (Atlas & Burstein, 1984) and has been found to be of low molecular weight (587 Da) and is a non-catecholamine, non-peptide substance (Atlas & Burstein, 1984; Atlas *et al*., 1987). It was originally identified in extracts of rat and bovine brain (Atlas & Burstein, 1984) but may also be present in peripheral tissues and in the circulation (Hensley *et al*., 1989; Meeley *et al*., 1992). CDS can bind to α_2-adrenoceptors and to I_1 and I_2-sites with high affinity, but it does not interact with α_1- or β-adrenoceptors (Atlas, 1995). It may behave as an agonist at α_2-adrenoceptors under certain conditions, since the inhibition of electrically elicited contraction of rat vas deferens by CDS is sensitive to yohimbine (Diamant & Atlas, 1986). CDS also appears to act as an agonist at I-sites since it reproduces certain imidazoline-mediated responses including contraction of rat gastric fundus (Felsen *et al*., 1987) and aorta (Synetos *et al*., 1991), and stimulation of catecholamine release from bovine adrenal chromaffin cells (Regunathan *et al*., 1991).

Attempts to isolate and identify the active constituent(s) of extracts containing CDS have been largely unsuccessful although a recent study has suggested that the presence of agmatine (de-carboxylated arginine) may account for the majority of the activity of CDS (Li *et al*., 1994). Indeed, agmatine binds to both I-sites and α_2-adrenoceptors (Li *et al*., 1994), and is, by definition, a CDS molecule. However, agmatine (and its biosynthetic enzyme arginine decarboxylase) has a different distribution in the brain and periphery from that of CDS, and it binds to I-sites and α_2-adrenoceptors with much lower affinities than CDS (Reis *et al*., 1995). Moreover, agmatine appears to lack efficacy at α-adrenoceptors and its effects on imidazoline-mediated responses do not directly parallel those induced by other preparations of CDS (Reis *et*

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al., 1995). In the endocrine pancreas, agmatine is able to elicit a slight stimulation of insulin secretion but this effect requires very high concentrations ($\geqslant$1 mM) and develops only slowly after agmatine addition (Sener et al., 1989; Chan et al., 1995). Furthermore, agmatine only reversed partially the inhibitory actions of diazoxide, a K-ATP channel opener, and the imidazoline secretagogue antagonist RX801080 failed to block the responses to agmatine in islets (Chan et al., 1995). Thus, despite evidence that agmatine can block ATP-sensitive potassium channels in B-cells (Chan et al., 1995; Dunne et al., 1995), the weight of evidence indicates that agmatine is unlikely to be an endogenous ligand acting at islet imidazoline receptors.

In the present study, we have attempted to determine whether extracts of rat brain containing CDS have functional activity in the endocrine pancreas *in vitro*. The effects of CDS on insulin release from isolated islets of Langerhans from rat and human were investigated to assess whether CDS might be an endogenous ligand having activity at the islet imidazoline receptor.

Methods

Preparation of clonidine-displacing substance

Whole rat brains (in groups of 10) were removed immediately after death and chopped into small pieces for homogenization in 3 volumes (v/w) of distilled water (4°C) in a motor driven teflon-glass homogenizer. The homogenate was centrifuged at $100{,}000 \times g$ for 30 min at 4°C and the supernatant denatured by boiling for 15 min. After being cooled, the boiled extract was re-centrifuged and the resulting supernatant frozen at −20°C and freeze-dried. The lyophilized powder was reconstituted in 4 ml of ice-cold distilled water and centrifiltered, first through Centricon-10 (10,000 molecular weight cut-off) then through Centricon-3 (3,000 molecular weight cut-off) concentrators. Following freeze-drying, the lyophilized preparation was extracted by bath-sonication in 10 volumes (v/w) of analytical grade methanol. Methanol-insoluble particulate was removed by filtering the preparation through Whatman no. 1 filter paper. Aliquots of the filtrate were freeze-dried and stored at −20°C. Samples of the dried methanolic extract containing CDS were reconstituted in distilled water immediately before use.

Isolation of islets of Langerhans

Rat 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 (Gey & Gey, 1936) containing 4 mM D-glucose and 1 mM $CaCl_2$. Islets were selected under a binocular dissecting microscope and were used within 2 h of isolation.

Human islets of Langerhans were isolated from heart-beating cadaver organ donors by collagenase digestion and density gradient centrifugation as described previously (Lake et al., 1989). Islets were cultured in RPMI-1640 plus 10% (v/v) foetal calf serum for up to 4 days before use in secretion experiments.

Insulin secretion experiments

Incubations were performed in 96-well plates to minimize the quantity of test reagents used. Groups of three isolated islets were incubated in 100 μl buffer solution supplemented with 1 mg ml^{-1} bovine serum albumin (Type V), for 60 min in a humidified air: CO_2 (95 : 5) atmosphere at 37°C, in the presence of test reagents. Yohimbine (10 μM) was included in all incubations to block α_2-adrenoceptor activation. After incubation, samples of the medium were removed for measurement of insulin content by radioimmunoassay.

Protein digestion of CDS

Fifty microlitres of reconstituted methanolic extract containing 5 units of CDS activity (see below) was incubated with an equal volume of saline solution, in the presence of 100 μg ml^{-1} of the broad activity protease, proteinase K. Following incubation for one hour at 37°C, the protease was inactivated by boiling for 10 min. After being cooled, the treated CDS was tested for insulin secretagogue activity.

Membrane preparation

Rat brains were chopped and homogenized in ice-cold TEM buffer (50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$), supplemented with 50 μM phenylmethylsulphonylfluoride, 2 μg ml^{-1} aprotonin, 2 μg ml^{-1} leupeptin and centrifuged at $3{,}000 \times g$ for 10 min at 4°C. The resultant supernatant was centrifuged at $40{,}000 \times g$ for 20 min at 4°C. The pellet was resuspended in TEM buffer and re-centrifuged. The final crude membrane fraction was resuspended in TEM buffer and aliquots were snap-frozen and stored at −80°C. Protein content was measured by the bicinchoninic acid method (Smith et al., 1985).

Flame photometry

The potassium and sodium ion contents of the reconstituted methanolic extract of rat brain containing CDS were assessed by flame photometry, with standard solutions for calibration.

Radioligand binding sites

Assessment of the amount of CDS present in each preparation was assayed with the membrane fraction of rat brain prepared as described above. One unit of CDS activity is defined as the amount required to displace 50% of specifically bound [^{3}H]-clonidine (2 nM) in the presence of 10 μM noradrenaline, from rat brain membranes (100 μg) incubated in a volume of 100 μl of 50 mM Tris-HCl buffer, pH 7.5, at 25°C for 40 min (Atlas & Burstein, 1984). Thus, varying dilutions of each CDS preparation were incubated with the radioligand and brain membranes. Incubations were terminated by rapid vaccuum filtration through Whatman GF/C filters. Filters were washed with 5 ml of ice-cold Tris buffer and their radioactivity measured after addition of scintillant.

To study binding at the sulphonylurea receptor, 2 nM [^{3}H]-glibenclamide was incubated with 100 μg rat brain membranes and competing drugs, for 40 min at 25°C.

Materials

Collagenase (type XI), adrenaline, noradrenaline, clonidine, diazoxide and yohimbine were purchased from Sigma Chemical Co. (Dorset, U.K.). Efaroxan and RX801080 (2-(2,3 dihydro-2-benzofuranyl)-2-imidazoline HCl) were gifts from Reckitt & Colman Products (Kingston-upon-Hull, U.K.). KU14R (2-(2,3 dihydro-2-benzofuranyl)-2-imidazole HCl) was synthesized in the Dept. of Chemistry, University of Keele, U.K. (Pallett et al., 1996). [^{3}H]-clonidine was purchased from NEN Products. [^{3}H]-glibenclamide was a gift from Hoechst Pharmaceuticals, [^{125}I]-iodine and anti-bovine insulin antiserum (for radioimmunoassay, RIA) were from ICN Biomedicals. Centricon-10 and -3 were from Amicon. Bicinchoninic acid reagents for assay of protein content were purchased from Pierce Chemicals. All other reagents were of analytical reagent grade.

Statistics

Data are presented as means ± s.e.mean. The statistical significance of difference between means was assessed by analysis of variance followed by Tukey's multiple comparison test or by Student's *t* test for unpaired data when only two groups were compared.

Results

Several batches of CDS were prepared over the course of these studies. On average approximately 10 units of CDS activity were obtained per gram wet weight of rat brain, although the spread ranged from 4.6 to 16 units g^{-1} in different preparations. Upon assessment of the amount of CDS present in aliquots of the methanolic extract, subsequent aliquots within a batch were dissolved in an appropriate volume of distilled water to give a 'stock' concentration of 1 unit 10 μl^{-1} (100 u ml^{-1}). Each experimental protocol was followed with 2 or 3 different batches of CDS.

We have previously shown that imidazoline-mediated augmentation of insulin release is glucose-dependent (Chan & Morgan, 1990), such that imidazoline secretagogues act as potentiators of secretion rather than initiators. Thus, the effects of CDS on insulin release were studied initially at a threshold concentration of glucose, 6 mM. Under these conditions, CDS caused a dose-dependent increase in insulin release from rat isolated islets (Figure 1), with an EC_{50} of approximately 2 u ml^{-1} and a maximal stimulating effect at about 10 u ml^{-1}. The maximum extent of potentiation was similar to that induced by 100 μM efaroxan, a well-characterized imidazoline secretagogue (Chan, 1993).

It has been well established that imidazoline secretagogues are able to reverse the inhibitory effects of the K-ATP channel opener diazoxide on glucose-induced insulin release (Chan & Morgan, 1990; Plant & Henquin, 1990; Chan *et al*., 1991) thus the use of diazoxide provides a convenient assay system for determining the effectiveness of imidazolines to interact functionally with islet cells. In Figure 2, the insulin secretory response of islets incubated at 20 mM glucose, a maximally stimulating concentration, was diminished by inclusion of 200 μM diazoxide. As expected, efaroxan abolished the inhibition of glucose-induced insulin release mediated by diazoxide, and a similar response was seen with CDS. Indeed, CDS induced a dose-dependent reversal of the effects of diazoxide (Figure 2), the dose curve of which was very similar to that for direct stimulation of secretion (Figure 1).

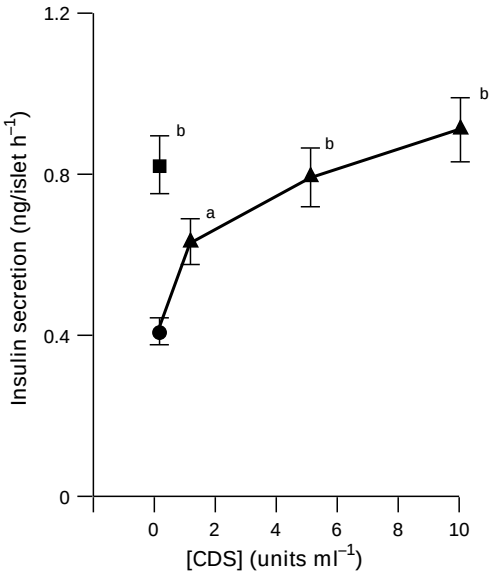

Figure 1 Dose-dependent stimulation of glucose-induced insulin release by clonidine-displacing substance. Rat isolated islets were incubated with 6 mM glucose (●) and with either CDS (▲) or 100 μM efaroxan (■) for 60 min at 37°C. After this time, the medium was sampled and its insulin content determined by radioimmunoassay. Data are expressed as mean rates for 18–22 observations; vertical lines show s.e.mean. $^{a}P<0.005$, $^{b}P<0.001$ relative to 6 mM glucose alone.

The effects of CDS on insulin secretion were sensitive to antagonism by the imidazoline secretagogue antagonist, RX801080 and a newly synthesized molecule, KU14R (Pallett *et al*., 1996) (Table 1). As shown previously, 100 μM RX801080 or KU14R alone was without effect on insulin release (Brown *et al*., 1993a; Pallett *et al*., 1996). However, 100 μM RX801080 and KU14R were each able to reverse the direct stimulant effects of 5 u ml^{-1} CDS from islets incubated in 6 mM glucose and they also blocked CDS-mediated antagonism of the inhibitory actions of diazoxide on glucose-induced secretion (Table 1). These data strongly suggest that the secretory actions of CDS are not mediated by non-specific mechanisms but imply that an imidazoline receptor may be involved.

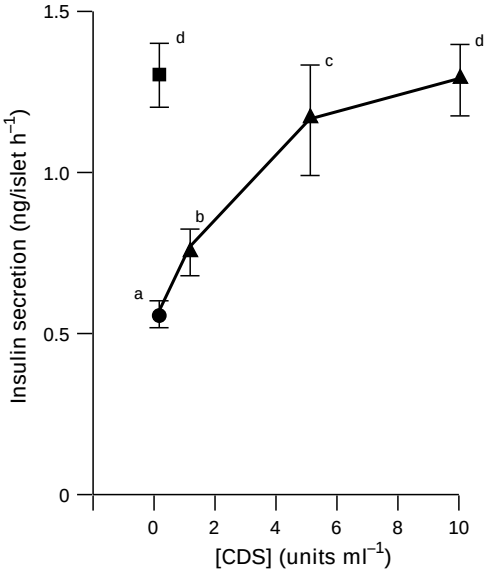

Figure 2 Reversal of diazoxide-induced inhibition of insulin secretion by CDS. Rat isolated islets were incubated in the presence of 20 mM glucose and 200 μM diazoxide (●) with either CDS (▲) or 100 μM efaroxan (■). The secretion rate was measured after 60 min of incubation at 37°C. The secretion rate measured in the presence of 20 mM glucose alone averaged 1.36 ± 0.12 ng islet h^{-1}. Results are mean values for 18–22 observations; vertical lines show s.e.mean. $^{a}P<0.001$ relative to 20 mM glucose alone; $^{b}P<0.05$, $^{c}P<0.005$, $^{d}P<0.001$ relative to 20 mM glucose + diazoxide alone.

Table 1 Effects of the imidazoline secretagogue antagonists RX801080 and KU14R on clonidine-displacing substance-induced responses on insulin secretion

[Glucose] (mM)	*CDS*	*Diazoxide* 200 μM	*Imidazoline antagonist*	*Insulin secretion* (ng/islet h^{-1})
6	–	–	–	0.48 ± 0.05
6	+	–	–	0.72 ± 0.09^{a}
6	+	–	RX801080	0.44 ± 0.05^{d}
6	+	–	KU14R	0.47 ± 0.04^{d}
20	–	–	–	1.64 ± 0.14
20	–	+	–	0.62 ± 0.05^{b}
20	+	+	–	1.02 ± 0.12^{c}
20	+	+	RX801080	0.55 ± 0.05^{d}
20	+	+	KU14R	0.63 ± 0.06^{d}

Groups of three isolated islets from rats were incubated for 60 min at 37°C under the conditions shown. Insulin levels were measured by radioimmunoassay. Data are presented as mean values ± s.e.mean for 12–14 observations. $^{a}P<0.05$ relative to 6 mM glucose alone; $^{b}P<0.001$ relative to 20 mM glucose; $^{c}P<0.01$ relative to 20 mM glucose + diazoxide; $^{d}P<0.01$ relative to absence of antagonist.

Figure 3 shows that CDS also has functional activity in human islets of Langerhans. CDS-R is a crude preparation from rat brain (as described above). As with rat islets, CDS (5 u ml^{-1}) was able to antagonize the actions of diazoxide on glucose-induced insulin release from human islets.

During the routine preparation of CDS from rat brain, the extracts were centrifiltered through membranes with a molecular weight cut-off of 10,000 Da. However, to eliminate any possibility that these preparations might contain small amounts of the recently defined putative endogenous ligand for sulphonylurea receptors (a protein of 13,000 Da, 'endosulphine' (Virsolvy-Vergine *et al*., 1996)), some preparations of CDS were further passed through Centricon-3 membranes (molecular weight cut-off of 3,000 Da). Figure 4 illustrates the insulin secretory responses of rat islets incubated with either CDS passed through Centricon-10 only or with CDS centrifiltered through both Centricon-10 and -3. When islets were incubated with 6 mM glucose, efaroxan and the two CDS preparations were all able to enhance the insulin secretory response further (Figure 4a). Efaroxan and both CDS preparations also attenuated the inhibited secretion rate in the presence of diazoxide (Figure 4b). It is noteworthy that the extent of direct stimulation of secretion and the reversal of the actions of diazoxide induced by the two preparations of CDS were very similar, suggesting that the active principle has a molecular weight <3000 Da and is unlikely to be endosulphine. In support of this, CDS-10 (CDS passed through Centricon-10 membrane only) did not displace the binding of [^{3}H]-glibenclamide from rat brain membranes (in contrast to glibenclamide which caused complete displacement), thus confirming the lack of contamination by endosulphine (data not presented).

Furthermore, in order to eliminate the possibility that the active component of CDS may be a peptide, CDS, pre-digested with proteinase K, was able to reverse the inhibitory action of diazoxide on insulin release just as effectively as untreated CDS (Figure 5), suggesting that the active principle is not a peptide.

By use of flame photometry, the K^+ content of one unit of the reconstituted methanolic extract of rat brain CDS was

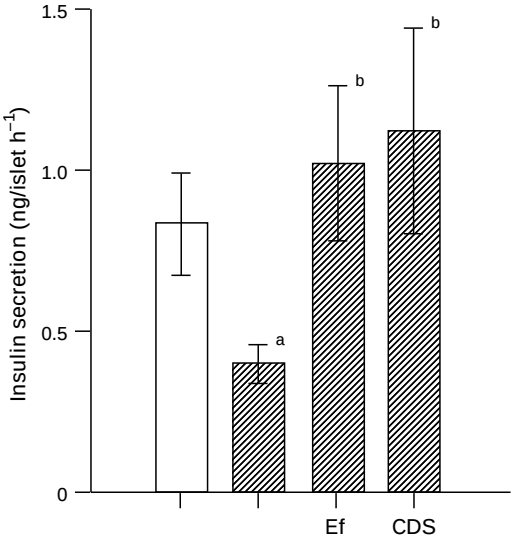

Figure 3 The effects of clonidine-displacing substance on insulin secretion in human islets of Langerhans. Groups of three human islets were incubated with 20 mM glucose (open column) in the presence of 200 μM diazoxide (hatched columns) and test reagents as shown: efaroxan (Ef, 100 μM) and CDS at 5 units ml^{-1} (CDS). After being incubated for 60 min at 37°C, samples of the medium were assayed for insulin. Results are mean rates ± s.e.mean for 7 observations. $^aP<0.05$ relative to 20 mM glucose alone; $^bP<0.05$, $^cP<0.005$ relative to 20 mM glucose + diazoxide alone.

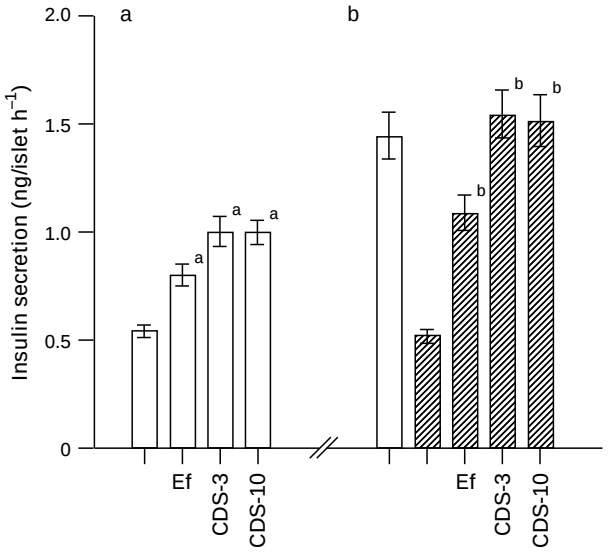

Figure 4 The effects of clonidine-displacing substance on insulin release following differential centrifiltration. Crude methanolic CDS preparations from rat brain were centrifiltered through either Centricon-10 only (CDS-10) or sequentially passed through Centricon-10 and Centricon-3 (CDS-3). Samples of each were then tested on insulin secretion in rat isolated islets. Data are means ± s.e.mean for 13–17 observations. (a). Islets were incubated in 6 mM glucose and with test reagents as indicated: efaroxan (Ef, 100 μM) and CDS (5 units ml^{-1}). (b) Reversal of the effects of diazoxide. Islets were incubated with 20 mM glucose with the inclusion of 200 μM diazoxide (hatched columns) and test reagents as shown: efaroxan (Ef, 100 μM) and CDS (5 units ml^{-1}). $^aP<0.001$ relative to 6 mM glucose alone; $^bP<0.001$ relative to 20 mM glucose + diazoxide alone.

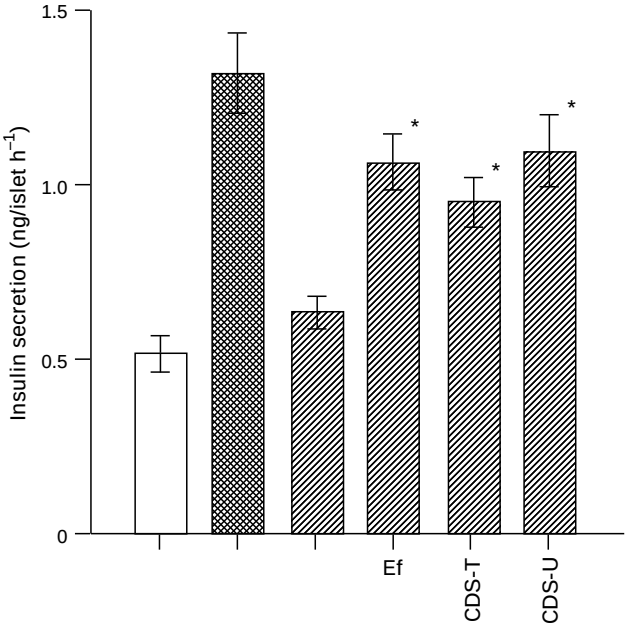

Figure 5 CDS-induced reversal of diazoxide-mediated inhibition of insulin release following protease treatment with proteinase K (see Methods). Rat islets were incubated with either 4 mM glucose (open column), 20 mM glucose (cross-hatched column) or 20 mM glucose plus 200 μM diazoxide (hatched columns) and test reagents as indicated: efaroxan (Ef, 100 μM); proteinase k-treated CDS (CDS-T, 5 units ml^{-1}); untreated, control CDS (CDS-U, 5 units ml^{-1}). After being incubated for 60 min at 37°C, samples of the medium were assayed for insulin. Results are mean rates ± s.e.mean for 15–17 observations. $^*P<0.001$ relative to diazoxide alone.

calculated to equal 1.11 ± 0.25 μmol ($n=4$) and the Na^+ ion content averaged 0.89 ± 0.20 μmol ($n=4$). Thus, when used at a maximal stimulating concentration of 10 units ml^{-1} a K^+ concentration of 11 mM and an average Na^+ concentration of 9 mM would be achieved. As shown in Table 2, islets incubated with KCl were not responsive to K^+ until a depolarizing concentration of 20 mM KCl was employed. More strikingly, unlike the response to CDS, insulin release induced by KCl was not antagonised by RX801080. Thus, the presence of K^+ ions in the preparation was not responsible for the secretagogue activity of CDS, and the addition of small amounts of Na^+ (against a background concentration of 150 mM in the incubation buffer) did not alter insulin secretion (not shown).

In the experiments described above with CDS, the α_2-antagonist, yohimbine (10 μM), was included in all incubation buffers to mask possible binding of components of the CDS preparations to α_2-adrenoceptors. This was done because CDS has, itself, been shown to exhibit α_2-agonist properties in some cells (Diamant & Atlas, 1986) and also because crude preparations of CDS may contain catecholamines (Singh *et al*., 1995). Activation of α_2-adrenoceptors in pancreatic B-cells would result in inhibition of insulin release (Nakaki *et al*., 1980; Ismail *et al*., 1981) and mask any imidazoline-mediated stimulation of secretion. Indeed, this is the case with the imidazoline α_2-agonist clonidine, where secretagogue activity is only revealed under conditions of α_2-blockade (Schulz & Hassleblatt, 1989). Therefore, in order to extend the study, the actions of CDS preparations on islet α_2-receptors were investigated. Rat islets were incubated with CDS in the presence of 20 mM glucose but in the absence of yohimbine. In these experiments, CDS 5 u ml^{-1}, an effective secretagogue concentration, did not alter glucose-induced insulin secretion from rat islets (control 1.54 ± 0.12 ng/islet h^{-1} compared to CDS 1.70 ± 0.12 ($n=20$)). The inclusion of yohimbine failed to modify the secretion rate in the presence of 20 mM glucose and CDS (1.95 ± 0.15 ng/islet h^{-1}).

Discussion

The insulin secretagogue activity of imidazoline compounds is mediated by an imidazoline binding site (I-site) that is associated with ATP-sensitive potassium channels in pancreatic B-cells (Chan *et al*., 1991; Dunne, 1991; Jonas *et al*., 1992). The functional pharmacology of this binding site appears to differentiate it from the I_1 and I_2-sites described in a wide variety of tissues (Chan *et al*., 1994), although both of these may also be present in insulin-secreting cells (Remaury & Paris, 1992; Brown *et al*., 1993b; Lacombe *et al*., 1993; Ernsberger *et al*., 1995). We have provided previous evidence that imidazoline-mediated effects on insulin release reflect the activation of a biologically relevant receptor molecule since, at least for some agonists, the responses are stereoselective and can be down-regulated (Chan *et al*., 1993; 1994). Moreover, imidazoline antagonists that block the secretagogue activity of agonists such as efaroxan and phentolamine have been described (Brown *et al*., 1993a; Pallett *et al*., 1996). The evidence provided in this paper, showing that a putative endogenous ligand for imidazoline receptors (CDS) is a potent insulin secretagogue, further substantiates this hypothesis.

We have shown that crude preparations of CDS from rat brain, can potentiate glucose-induced insulin and reverse the effects of diazoxide in rat and human islets of Langerhans. These effects were subject to blockade by two separate imidazoline antagonists, RX801080 and KU14R. To our knowledge, this is the first description an imidazoline-mediated action of CDS which can be blocked by imidazoline secretagogue antagonists. Taken as a whole, the data presented here suggest that preparations of CDS contain an active compound capable of interacting agonistically with the islet imidazoline receptor.

In most of the experiments, a relatively crude methanolic extract of rat brain containing CDS activity, as defined in radioligand binding assays, was used. The protocols employed to extract CDS were similar to those described in several other studies (methanolic extraction of a boiled brain homogenate) which have generated CDS preparations having activity in a number of bioassays (Felsen *et al*., 1987; Regunathan *et al*., 1991; Synetos *et al*., 1991; Meeley *et al*., 1992). However, since the structure of the active principle remains obscure, it is important to consider whether the presence of likely contaminants could be responsibile for the CDS bioactivity observed.

The presence of catecholamines (noradrenaline and adrenaline) has been shown in some CDS preparations from bovine brain and lung, albeit at very low levels (Singh *et al*., 1995). Nevertheless, it has been argued that catecholamines may account for the α_2-adrenoceptor-mediated actions of CDS, in tissues such as the rat vas deferens (Diamant & Atlas, 1986). To minimise the impact of contaminant catecholamines in insulin secretion experiments, we included yohimbine as a routine precaution to block islet α_2-adrenoceptors. However, when yohimbine was omitted from the islet incubation buffer, glucose-induced insulin secretion was not inhibited upon addition of CDS. This suggests that there were no significant amounts of adrenaline or noradrenaline in our preparations and also demonstrates that, in agreement with findings in certain other tissues (Diamant *et al*., 1987; Pinthong *et al*., 1995a,b), CDS does not have direct α_2-agonist activity in pancreatic islets.

A second contaminant of some preparations of CDS is histamine. This can be present at μM concentrations (Singh *et al*., 1995) but these levels cannot account for the secretory effects of CDS in islets since, although histamine can stimulate insulin secretion, concentrations in the mM range are required. Another amine, agmatine, has also been identified recently in preparations of CDS (Li *et al*., 1994) and a pathway for its biosynthesis and degradation has been defined in mammalian tissues, including brain (Reis *et al*., 1995). Agmatine is a weak insulin secretagogue which induces a slowly developing secretory response (Sener *et al*., 1989; Chan *et al*., 1995) but, unlike CDS, its effects are not inhibited by the imidazoline antagonist RX801080 (Chan *et al*., 1995). Thus, although agmatine has been shown to fulfil some of the requirements of a CDS (in that it displaces the binding of [^{3}H]-clonidine from both α_2-adrenoceptors and imidazoline receptors) it has already been excluded as a candidate for an endogenous imidazoline insulin secretagogue.

A final group of contaminants which are known to be present in CDS extracts are cations, especially potassium and sodium. Indeed, in one study, the functional response observed with crude CDS was attributed to the high potassium content of an extract of CDS (Meeley *et al*., 1992). Since potassium depolarization is a potent stimulus for insulin secretion, we

Table 2 Effects of RX801080 on KCl-induced insulin release

[Glucose] (mM)	*[KCl]* (mM)	*Efaroxan* (100 μM)	*RX801080* (100 μM)	*Insulin secretion* (ng/islet h^{-1})
6	–	–	–	0.78 ± 0.06
6	10	–	–	0.78 ± 0.07
6	20	–	–	1.11 ± 0.11[a]
6	30	–	–	1.12 ± 0.10[b]
6	30	–	+	1.14 ± 0.12
6	–	+	–	1.46 ± 0.18[c]
6	–	+	+	0.91 ± 0.05[d]

Groups of three isolated islets from rats were incubated for 60 min at 37°C under the conditions shown. Insulin levels were measured by RIA. Data are presented as mean values ± s.e.mean for 12 or 13 observations. [a]$P<0.02$, [b]$P<0.01$, [c]$P<0.005$ relative to 6 mM glucose alone; [d]$P<0.01$ relative to efaroxan alone.

considered it important to determine the cation content of our CDS preparations. Both potassium and sodium ions were present, although the concentrations achieved were too low to account for the effects of the extracts on either [^{3}H]-clonidine binding (Sing *et al*., 1995) or insulin secretion. Rat islets are unresponsive to small changes in the concentration of Na^+ ions, and, although an increase in insulin release was induced by potassium depolarisation, this effect was seen only when the concentration of KCl was increased by 20 mM or more. Levels as high as this were never reached in experiments with crude CDS preparations, even when the maximally effective concentration of CDS (10 u ml^{-1}) was used. In addition, the secretion response to KCl was not affected by the presence of the antagonist RX801080 whereas this agent markedly inhibited the action of CDS. Thus, we conclude that the presence of sodium and potassium ions cannot account for the ability of CDS-containing extracts to stimulate insulin secretion.

On the basis of the data presented herein, we suggest that the active component of our CDS preparations is a low molecular weight molecule which stimulates insulin release via the islet imidazoline binding site. Although the structure of CDS still awaits confirmation, plasma desorption mass spectrometry indicate a molecular weight of 587.8 daltons. One unit of CDS activity has been calculated to correspond to approximately 5 pmol (Atlas, 1995) and thus, this implies that the concentration of the active component in the experiments was in the 5–50 nM range, making CDS the most potent imidazoline receptor ligand yet identified in pancreatic islets. Structural analysis of the purified component must now become a major priority since the availability of a high affinity ligand is crucial to the further characterization and cloning of the islet imidazoline receptor.

One further pertinent consideration in the present context, is the relationship between the insulin secretagogue activity of CDS and a recently identified putative ligand for the islet sulphonylurea receptor. Since sulphonylurea receptors and imidazoline receptors are both involved in control of ATP-sensitive potassium channels in pancreatic B-cells, it is conceivable that the receptors may be structurally related. Indeed, there is evidence that imidazoline insulin secretagogues and sulphonylureas can interact functionally in islets (Berridge *et al*., 1992), although they are unlikely to occupy the same binding site. A putative endogenous ligand for sulphonylurea receptors has been described ('endosulphine') which is a polypeptide existing in two forms (Virsolvy-Vergine *et al*., 1992; 1996). The larger of these has an apparent molecular weight of 13 kDa, although this may serve as the precursor of a smaller, more active, form (Virsolvy-Vergine *et al*., 1996).

We have used several means to examine whether the presence of a peptide such as endosulphine could account for the activity of CDS in methanolic extracts of brain. This is important since the procedure used to isolate endosulphine involves several steps which are similar to those used in preparation of CDS, including heat coagulation followed by acid and methanol extraction (Virsolvy-Vergine *et al*., 1988). The most convincing evidence that CDS differs from endosulphine derives from the observation that CDS is resistant to incubation with the broad activity protease Proteinase K (Figure 5), confirming previous suggestions that the active component is not a peptide. This contrasts with the activity of endosulphine, which is destroyed by protease treatment (Virsolvy-Vergine *et al*., 1988). Additionally, we have confirmed that CDS retains full insulin secretagogue activity after passage through a 3000 molecular weight cut-off membrane (Centricon-3) whereas even the smaller form of endosulphine (endosulpine-β) is excluded by low molecular weight cut-off dialysis membrane. Finally, CDS did not displace [^{3}H]-glibenclamide from the sulphonylurea receptor whereas the activity of endosulphine is defined by its ability to displace this radioligand. Therefore, although the extraction procedures have certain similarities, we consider it extremely unlikely that the insulin secretagogue activity of CDS is due to the presence of endosulphine.

Overall, therefore we conclude that CDS displays many characteristics expected of an endogenous regulator of insulin secretion acting through the islet imidazoline receptor. It is a potent insulin secretagogue and its activity can be inhibited by imidazoline antagonists. This evidence strengthens the hypothesis that the imidazoline binding site mediating control of insulin release in the endocrine pancreas is a biologically relevant receptor. Although a potential physiological role for CDS in the endocrine pancreas remains speculative, it is noteworthy that the molecule has a broad distribution within peripheral tissues and is also present in the circulation. Thus, the possibility that CDS may be involved in the homeostatic control of insulin secretion *in vivo* should not be excluded.

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