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Identification of the monomeric G-protein, Rhes, as an efaroxan-regulated protein in the pancreatic β-cell

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1 Efaroxan induces membrane depolarization by interaction with the pore forming subunit of the ATP-sensitive potassium channel, Kir6.2. However, this effect is not responsible for its full secretory activity.

2 In this study we have used an anti-idiotypic approach to generate antibodies that recognize additional proteins that may be regulated by efaroxan in pancreatic β-cells.

3 Using these antisera in an expression cloning strategy we have identified a monomeric GTP-binding protein, Rhes, as a potential target for regulation by imidazoline ligands. Rhes is shown to be expressed in β-cells and its expression is regulated by efaroxan under conditions when a structurally related molecule, KU14R, is ineffective.

4 The results reveal that β-cells express Rhes and suggest that changes in the expression of this molecule may regulate the sensitivity of β-cells to imidazoline secretagogues.
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Abbreviations: FITC, fluorescein dithiocyanate; PBS, phosphate-buffered saline; PVDF, polyvinylidenedifluoride; Rhes, Ras-homologue expressed in striatum

Introduction

Recent studies have established that the imidazoline compound, efaroxan, has the capacity to potentiate nutrient-induced insulin secretion from pancreatic β-cells (reviewed by Morgan *et al*., 1999; Morgan & Chan, 2001). As such, efaroxan is representative of a group of structurally similar molecules that can increase insulin release in a glucose-dependent manner (Efanov *et al*., 2001a; Eglen *et al*., 1998; Jonas *et al*., 1992; Mest *et al*., 2001; Rustenbeck *et al*., 1997; Schulz & Hasselblatt, 1988; Zaitsev *et al*., 1996). Electrophysiological (Proks & Ashcroft, 1997) and biochemical studies (Monks *et al*., 1999) have provided convincing evidence that imidazolines, including efaroxan, can bind to the pore-forming subunit of the ATP-sensitive potassium channel, Kir6.2, in the β-cell. However, it is now apparent that blockade of the K_{ATP} channel cannot account completely for the ability of efaroxan to potentiate insulin secretion and recent data strongly suggest the existence of a second, more important, molecular target (Chan *et al*., 2001). This target has not been defined in molecular terms but functional data imply that it is associated with a distal component(s) of the exocytotic pathway involved in the enhancement of Ca^{2+} dependent secretion (Chan *et al*., 2001; Efanov *et al*., 2001a, b).

Since efaroxan is an 'imidazoline' ligand, the assumption has been made that its molecular target in the β-cell represents one member of the wider family of imidazoline binding proteins (Eglen *et al*., 1998; Morgan & Chan, 2001). However, it is also clear that the β-cell efaroxan-binding protein is pharmacologically different from the I_1 and I_2 subclasses defined elsewhere, suggesting that a third subclass (I_3) may be expressed in this tissue (Chan *et al*., 1994).

Classical ligand binding experiments have not been useful for the identification of efaroxan-binding proteins involved in the control of insulin secretion since the binding affinity of the target protein(s) for the imidazoline ligand is relatively low (Brown *et al*., 1993; Monks *et al*., 1999). Thus, it is accepted that it will be necessary to adopt a different approach in order to identify the targets for efaroxan. To this end, we have developed a panel of anti-idiotypic antisera (Erlanger *et al*., 1986) to identify potential efaroxan-regulated proteins in pancreatic β-cells and have now employed these in expression cloning experiments. As a result, we have identified a monomeric G-protein whose expression is controlled in an efaroxan-sensitive manner in β-cells.

Methods

Materials

Collagenase (type XI), diazoxide, phenylmethylsulphonylfluoride, leupeptin, aprotinin, bovine serum albumin (type V) were purchased from Sigma Chemical Co. (Dorset, U.K.). Efaroxan hydrochloride was generously provided by RBI (Natick, MA, U.S.A). KU14R & KU08C were synthesized in the Department of Chemistry, University of Keele, U.K. (Chan *et al*., 1998). [^{125}I]-insulin was purchased from Linco and anti-bovine insulin antiserum (for radioimmunoassay, RIA) was from ICN Biomedicals. All other reagents were of analytical reagent grade.

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Immunizations

Polyclonal anti-efaroxan antibodies (Chan *et al*., 1998), were affinity-purified using protein A and an efaroxan-affinity column, prepared by covalent linkage of 5-amino-efaroxan (KU08C) to AminoLink Gel (Pierce). The purified antibodies were used to immunize a second set of rabbits to generate anti-idiotypic antibodies. Primary subcutaneous injections of the immunogen emulsified in (1 : 1, v v^{-1}) Freund's complete adjuvant (Sigma) were followed at 3–4 week intervals by booster injections of immunogen in Freund's incomplete adjuvant (Sigma). The presence and specificity of anti-idiotypic antibodies was determined by ELISA.

RINm5F cell culture and membrane preparation

RINm5F cells were cultured in RPMI-1640 supplemented with 5% (v v^{-1}) foetal calf serum, 2 mM glutamine at 37°C in a humidified atmosphere of air:CO_2 (95 : 5). Membranes were prepared by homogenization of cells in ice-cold buffer (50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$) containing 50 μM phenylmethylsuphonylfluoride, 2 μg ml^{-1} aprotonin, 2 μg ml^{-1} leupeptin. Membranes were harvested by centrifugation at 40,000 × *g* for 20 min, 4°C and stored at −80°C. Protein content was measured by the bicinchoninic acid method (Pierce).

ELISA

RINm5F cell membranes (20 μg ml^{-1}) was adsorbed overnight at 4°C to 96-well ELISA plates in 0.1 M carbonate/bicarbonate buffer (pH 9.6). After washing in TBS-T (50 mM Tris, pH 8.0; 140 mM NaCl; 3 mM KCl; 0.1% (v v^{-1}) Tween-20), wells were blocked with 2% (v v^{-1}) goat serum in TBS-T (blocking solution) for 60 min at 37°C. Following two washes with TBS-T, 100 μl antiserum (1 : 100 dilution in blocking solution) was added and incubated for 2 h at 37°C. After three washes with TBS-T, goat anti-rabbit-IgG-coupled alkaline phosphatase was added and binding detected colorimetrically.

Immunofluorescence and Western blotting studies

RINm5F cells were fixed with 3% (w v^{-1}) paraformaldehyde and permeabilized with ice-cold methanol. After blocking with 10% (v v^{-1}) goat serum in phosphate-buffered saline (PBS) antiserum (or pre-immune serum) was applied at 1 : 50 dilution (60 min, 37°C). A secondary antibody–fluoroscein dithiocyanate (FITC) conjugate was then applied and the cells viewed under a fluorescence microscope. For Western blotting RINm5F cell membranes were electrophoresed on 10% SDS–PAGE gels, transferred to polyvinylidenedifluoride (PVDF) membranes and probed with 1 : 1000 pre-immune or test antiserum (as indicated).

Islet isolation and incubation

Rat and human islets of Langerhans were isolated by collagenase digestion (Chan *et al*., 2001; Lacey *et al*., 1993). Human islets were provided from within the Diabetes U.K. Core Islet Transplant Programme and were isolated from heart-beating cadaver organ donors at University of Leicester. Where appropriate, rat and human islets were cultured in RPMI-1640 plus 10% (v v^{-1}) foetal calf serum. Following culture, the islets were washed with 3 × 5 ml of islet incubation medium (Gey & Gey, 1936) containing 4 mM glucose and they were then pre-incubated in a further 20 ml of this medium for 30 min prior to hand-picking for secretion experiments. Insulin secretion studies were performed in 96-well microtitre plates (Chan *et al*., 2001) and insulin was measured by radioimmunoassay.

Expression cloning

A RINm5F cell cDNA library (kindly provided by Prof F.M. Ashcroft, University of Oxford) was excised to yield phagemids which were then transformed into *E. coli*. Amplified phagemids were isolated from aliquots of bacteria and were transcribed and translated *in vitro* using a rabbit reticulocyte lysate system. Peptide products were screened by dot-blotting after transfer to PVDF membranes and incubation with anti-idiotypic antisera. Samples yielding positive signals were subdivided and re-screened sequentially until single clones were obtained. These cDNAs were sequenced and their homology with known sequences determined by interrogation of the GenBank database using BLAST software.

Semi-quantitative RT–PCR

Islets and clonal cell lines were cultured in RPMI-1640 and total RNA extracted with TRIzol reagent (GIBCO–BRL) and quantified spectrophotometrically. RT–PCR was performed in a single tube system (Advanced Biotechnologies, U.K.) using either Ras Homologue Expressed in Striatum (Rhes) (F: gcaagagctccattgtctcc; R: cgtgttcttcttggctgaca) or β-actin specific primers. The PCR reaction was run for 25 cycles (95°C-1 min; 55°C-1 min; 72°C-1 min) and the products fractionated on 1% agarose gels. The relative densities of individual bands were determined with Scion Image software.

Statistics

The statistical significance of differences between mean values was assessed by analysis of variance with Newman-Keuls Multiple Comparison test.

Results and Discussion

Polyclonal anti-idiotypic antisera were generated in rabbits by injection with purified anti-efaroxan serum previously raised in separate animals. This approach was employed since it was anticipated that the anti-idiotypic antisera would contain a high titre of antibodies directed against efaroxan-binding proteins, thereby allowing their use to identify such proteins in pancreatic β-cells. The anti-idiotypic antisera were screened for immunoreactivity against β-cell proteins by an ELISA method in which multi-well plates were coated with membranes prepared from RINm5F cells. It was quickly ascertained that the sera contained antibodies that were immunoreactive with RINm5F cell proteins since strongly positive signals were obtained (not presented). Pre-immune sera from the same animals failed to generate positive signals

in the ELISA protocol. Direct confirmation that the sera were reactive against β-cell proteins was obtained by immunostaining of RINm5F cells growing in culture (Figure 1). Cells were positively stained upon exposure to the anti-idiotypic antisera (Figure 1a) whereas no staining was seen with preimmune serum or if the primary antiserum was omitted (not shown). Significant staining was observed at the periphery of the cells suggesting that at least one of the immunoreactive proteins may be associated with the cell surface.

Functional studies confirmed that an immunoreactive cell surface molecule could be involved in the actions of efaroxan since incubation of isolated islets with the anti-idiotypic sera resulted in an attenuated insulin secretory response to efaroxan in either the absence (Figure 2a) or the presence of diazoxide (Figure 2b). Pre-immune sera were without effect on insulin secretion under either condition and the selectivity of the response to the anti-idiotypic antiserum was confirmed by the demonstration that glucose (20 mM)-induced secretion was unaffected by the presence of the serum (Figure 2a). These results imply that ligation of one or more cell surface protein by the antiserum had prevented the actions of efaroxan, which would be consistent with the possibility that the immunoreactive protein(s) is required for either imidazoline transport or for control of insulin secretion by the drug.

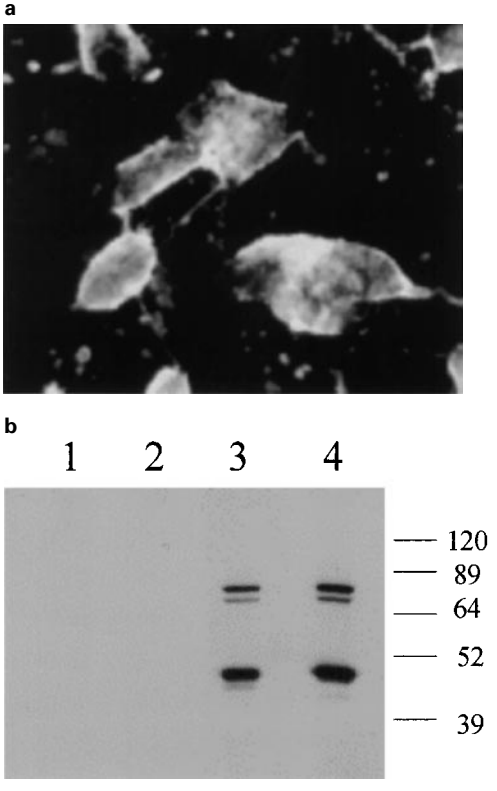

Figure 1 Detection of proteins immunoreactive against an anti-idiotypic antiserum in RINm5F cells. (a) Immunoreactive proteins were visualized by fluorescence microscopy after addition of anti-idiotypic antiserum and a secondary-antibody-FITC conjugate. (b) Immunoreactive proteins were visualized by Western blotting of RINm5F cell proteins with pre-immune (1 and 2) or anti-idiotypic antiserum (3 and 4). The major bands had molecular weights of 40–45 kDa, 75 kDa and 80 kDa as judged by reference to molecular weight markers run in parallel.

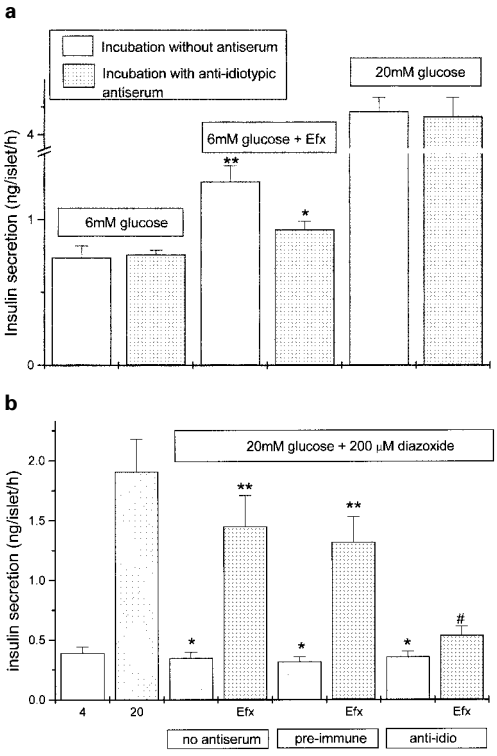

Figure 2 The effect of anti-idiotypic antiserum on efaroxan-induced insulin secretion from isolated human islets. (a) Isolated islets were incubated in the presence of 6 mM glucose, 6 mM glucose plus efaroxan (Efx; 100 μM) or 20 mM glucose, as shown. Control incubations were performed in the absence of added antiserum (open bars) while test incubations contained anti-idiotypic antiserum at a dilution of 1 : 1000 (filled bars). (b) Isolated islets were incubated in the presence of 4 mM glucose alone (4) 20 mM glucose alone (20) or 20 mM glucose plus 200 μM diazoxide. The ability of 100 μM efaroxan (Efx; filled bars) to antagonize the inhibitory effect of diazoxide on 20 mM glucose-induced insulin release was examined in the absence of antiserum or in the presence of either pre-immune or anti-idiotypic antisera (each at 1 : 1000) as shown. Results are mean rates of insulin secretion $\pm$ s.e.means ($n=14$ observations). (a) $^{**}P<0.01$ relative to 6 mM glucose alone; $^{*}P<0.05$ relative to efaroxan in the absence of anti-idiotypic antiserum. (b) $^{*}P<0.001$ relative to 20 mM glucose alone; $^{**}P<0.001$ relative to the equivalent condition in the absence of efaroxan; $\#P<0.01$ relative to efaroxan in the presence of pre-immune serum.

Use of the antisera in Western blotting experiments revealed that several different immunoreactive molecules are expressed in RINm5F cells (Figure 1b). The immunoreactive bands ranged in size from 40 to 80 kDa but none corresponded in molecular weight to that expected of Kir6.2, a molecule that has been previously implicated as a β-cell efaroxan binding protein (Monks *et al.*, 1999). This does not exclude the possibility that the surface immunoreactivity seen in immunofluorescence studies may have derived from the labelling of K_{ATP} channels since it is possible that the antiserum may recognize a native conformation of the protein that is not preserved after denaturing gel electrophoresis. Western blotting of RINm5F cell proteins with preimmune sera did not lead to detection of any immunoreactive proteins (Figure 1b).

In an attempt to identify critical immunoreactive β-cell proteins that were recognized by the anti-idiotypic antisera, an expression cloning approach was adopted. A number of

positive clones were identified by this technique and several of these yielded cDNA sequences that were highly homologous to one particular sequence present in the GenBank database (accession number AF134409). This sequence encodes a putative monomeric G-protein previously thought to be restricted in expression to the striatum (Falk *et al*., 1999). The deduced amino acid sequence of this protein suggests that it is a member of the Ras family of monomeric G-proteins and, accordingly, it was originally designated 'Rhes' (Ras Homologue Expressed in Striatum; Falk *et al*., 1999). The function of Rhes has not been defined but it was speculated that it might be involved in control of secretion since it shares regions of high homology with another G-protein, Dexras-1, that has been implicated in the regulation of exocytosis in corticotrophs (Graham *et al*., 2001). If Rhes plays an equivalent role in pancreatic endocrine cells, then it may be an ideal candidate to regulate the actions of imidazoline insulin secretagogues.

To confirm that Rhes is expressed in β-cells, oligonucleotide primers were designed to amplify the rat and human forms of the molecule and appropriate products were amplified successfully from a variety of β-cell lines (including RINm5F, BRIN-BD11 and INS-1 cells) as well as from rat and human islets. Thus, despite the initial suggestion that Rhes expression may be restricted to the striatum, these results reveal that it is also expressed in pancreatic endocrine cells.

In order to examine the possibility that Rhes may be a functional target for efaroxan in β-cells, rat islets and clonal BRIN-BD11 cells were exposed to efaroxan for 18 h. BRIN-BD11 cells were selected for these studies since, in common with normal rat islets (Chan *et al*., 1993; 2001) efaroxan has been reported to induce a state of functional desensitization in these cells during chronic exposure (Chapman *et al*., 1999; McClenaghan *et al*., 2001; see also Figure 3c). Desensitization is defined as the state in which the ability of efaroxan to promote insulin secretion is lost. This response is selective (since the effects of nutrient secretagogues are unaffected) and, although the molecular mechanisms have not been elucidated, it seems probable that it occurs by depletion of a critical component located within the efaroxan-sensitive pathway.

After exposure to efaroxan, islets and BRIN-BD11 cells were harvested and RNA extracted. The extent of Rhes expression was then examined by RT–PCR under conditions when amplification was maintained within the exponential range. The level of Rhes amplification was compared with that observed in control cells cultured in the absence of efaroxan. Amplification of β-actin transcripts was used to verify the integrity of the RNA in each sample and to allow a comparison of the extent of Rhes amplification under the various incubation conditions. Exposure of either BRIN-BD11 cells (Figure 3a,b) or normal rat islets (Figure 3a) to efaroxan for 18 h resulted in a marked reduction in Rhes expression by comparison with control, suggesting that the loss of efaroxan-induced insulin secretion seen under these conditions (Figure 3c; Chan *et al*., 1993; Chapman *et al*., 1999; McClenaghan *et al*., 2001) was correlated with a reduction in Rhes expression. No change was observed in the expression of β-actin transcripts. These results provide firm evidence that Rhes expression is regulated in an imidazoline-sensitive manner in β-cells. They also suggest that there may

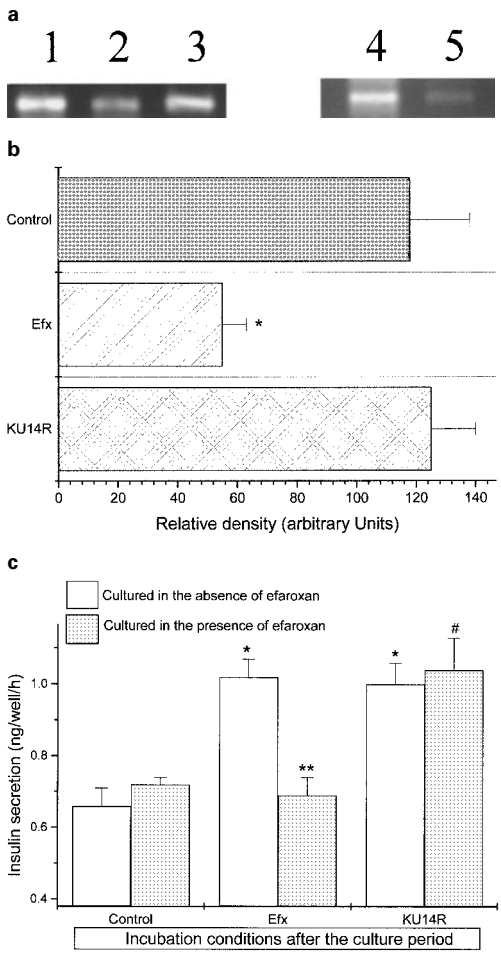

Figure 3 Effects of efaroxan and KU14R on Rhes transcript expression and insulin secretion in pancreatic β-cells. (a) BRIN-BD11 cells (lanes 1–3) or isolated rat islets (lanes 4–5) were cultured for 18 h under control conditions (lanes 1 and 4) or in the presence of efaroxan (100 μM; lanes 2 and 5) or KU14R (100 μM; lane 3). RNA was then isolated and Rhes expression analysed by RT–PCR. (b) Amplification of Rhes transcripts in BRIN-BD11 was quantified by densitometric scanning of band intensity. Data represent mean values ± s.e.mean from six (for efaroxan) or three (for KU14R) separate experiments. $*P<0.01$ relative to control (c) BRIN-BD11 cells were cultured in either the absence (open bars) or in the presence of 100 μM efaroxan (filled bars) for 18 h. They were then washed and incubated in the absence of additional reagents (control) or in the presence of efaroxan (Efx; 100 μM) or KU14R (100 μM) as shown. Results are mean values ± s.e.mean ($n=8$). $*P<0.001$ relative to control cells; $**P<0.001$ relative to cells cultured in the absence of efaroxan; $\#P<0.001$ relative to cells cultured in the presence of efaroxan and also exposed to efaroxan during the final incubation period.

be a close correlation between the ability of efaroxan to stimulate insulin secretion from β-cells and the level of Rhes expression.

Since islets and BRIN cells were cultured in standard RPMI-1640 medium containing 11.1 mM glucose, it follows that stimulation of insulin secretion *per se* was not responsible for the decline in Rhes expression (since secretion from islets is more than half-maximally stimulated at 11.1 mM glucose). Rather, the presence of an imidazoline agonist was required. In order to confirm this, BRIN-BD11 cells were also treated with a close structural analogue of

efaroxan, KU14R. In rat (and human) islets incubated *in vitro*, KU14R acts functionally as an imidazoline antagonist (Chan *et al*., 1994; 2001), since it blocks the stimulatory effect of efaroxan and certain other imidazoline reagents on insulin secretion, without affecting the response to glucose. By contrast, KU14R has been reported to directly increase insulin release from BRIN-BD11 cells (Ball *et al*., 2000). This indicates that efaroxan and KU14R must stimulate secretion by different mechanisms in BRIN-BD11 cells and that only the former activates the 'imidazoline' pathway. In confirmation of this, we observed that both efaroxan and KU14R caused a significant increase in insulin release from BRIN-BD11 cells (Figure 3b) but that, when cells were exposed to efaroxan for 18 h, they became desensitized to this agent but still responded normally to KU14R. Strikingly, these changes correlated closely with alterations in Rhes expression (Figure 3a). Exposure of cells to efaroxan resulted in down-regulation of Rhes transcript levels whereas KU14R failed to modify Rhes expression (Figure 3a, b). These results suggest very strongly that Rhes represents an imidazoline-regulated molecule that could be involved in mediating the insulin secretory response to efaroxan.

In conclusion, we present evidence that the monomeric G-protein, Rhes, is expressed in pancreatic β-cells and is involved in the mechanism by which efaroxan promotes insulin secretion. As such, Rhes represents the first protein to be implicated in controlling the distal events associated with imidazoline-induced insulin secretion. The data are consistent with the concept that changes in Rhes expression may be involved in regulation of the sensitivity of pancreatic β-cells to efaroxan.

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References

BALL, A.J, FLATT, P.R. & MCCLENAGHAN, N.H. (2000). Stimulation of insulin secretion in clonal BRIN-BD11 cells by the imidazoline derivatives KU14R and RX801080. *Pharmacol. Res.,* **42,** 575–579.

BROWN, C.A., LOWETH, A.C., SMITH, S.A. & MORGAN, N.G. (1993). Evidence that the stimulatory effects of imidazoline compounds on insulin secretion are not mediated by interaction with non-adrenergic idazoxan binding sites. *Br. J. Pharmacol.,* **108,** 312–317.

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

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

CHAN, S.L.F., MOURTADA, M. & MORGAN, N.G. (2001). Characterization of a K_{ATP} channel-independent pathway involved in potentiation of insulin secretion by efaroxan. *Diabetes,* **50,** 340–347.

CHAN, S.L.F., PALLETT, A.L., CLEWS, J., RAMSDEN, C.A., CHAPMAN, J.C., KANE, C., DUNNE, M.J. & MORGAN, N.G. (1998). Characterisation of new efaroxan derivatives for use in purification of imidazoline binding sites. *Eur. J. Pharmacol.,* **355,** 67–76.

CHAPMAN, J.C., MCCLENAGHAN, N.H., COSGROVE, K.E., HASHMI, M.N., SHEPHERD, R.M., GIESBERTS, A.N., WHITE, S.J., AMMALA, C., FLATT, P.R. & DUNNE, M.J. (1999). ATP-sensitive potassium channels and efaroxan-induced insulin release in the electrofusion-derived BRIN-BD11 beta-cell line. *Diabetes,* **48,** 2349–2357.

EFANOV, A.M., HOY, M., BRANSTROM, R., ZAITSEV, S.V., MAGNUSON, M.A., EFENDIC, S., GROMADA, J., BERGGREN, P.-O. (2001a). The imidazoline RX871024 stimulates insulin secretion in pancreatic beta-cells from mice deficient in K_{ATP} channel function. *Biochem. Biophys. Res. Commun.,* **284,** 918–922.

EFANOV, A.M., ZAITSEV, S.V., MEST, H.J., RAAP, A., APPELSKOG, I.B., LARSSON, O., BERGGREN, P.-O. & EFENDIC, S. (2001b). The novel imidazoline compound BL11282 potentiates glucose-induced insulin secretion in pancreatic beta-cells in the absence of modulation of K_{ATP} channel activity. *Diabetes,* **50,** 797–802.

EGLEN, R.M., HUDSON, A.L., KENDALL, D.A., NUTT, D.J., MORGAN, N.G., WILSON, V.G. & DILLON, M.P. (1998). Seeing through a glass darkly: casting light on the imidazoline ''I'' sites. *Trends Pharmacol. Sci.,* **19,** 381–390.

ERLANGER, B.F., CLEVELAND, W.L., WASSERMANN, N.H., KU, H.H., HILL, B.L., SARANGARAJAN, R., RAJAGOPALAN, R., CAYANIS, E., EDELMAN, I.S. & PENN, A.S. (1986). Auto-anti-idiotype: a basis for autoimmunity and a strategy for anti-receptor antibodies. *Immunol. Rev.,* **94,** 23–37.

FALK, J.D., VARGIU, P., FOYE, P.E., USUI, H., PEREZ, J., DANIELSON, P.E., LERNER, D.L., BERNAL, J. & SUTCLIFFE, J.G. (1999). Rhes: A striatal-specific Ras homolog related to Dexras1. *J. Neurosci. Res.,* **57,** 782–788.

GEY, G.O. & GEY, M.K. (1936). Maintenance of human normal cells in continuous culture: preliminary report; cultivation of mesoblastic tumors and normal cells and notes on methods of cultivation. *Am. J. Cancer,* **27,** 45–76.

GRAHAM, T.E., KEY, T.A., KILPATRICK, K. & DORIN, R.I. (2001). Dexras1/AGS-1, a steroid hormone-induced guanosine triphosphate-binding protein, inhibits 3′,5′-cyclic adenosine monophosphate-stimulated secretion in AtT-20 corticotroph cells. *Endocrinology,* **42,** 2631–2640.

JONAS, J.-C., PLANT, T.D. & HENQUIN, J.-C. (1992). Imidazoline antagonists of alpha 2-adrenoceptors increase insulin release in vitro by inhibiting ATP-sensitive K+ channels in pancreatic beta-cells. *Br. J. Pharmacol.,* **107,** 8–14.

LACEY, R.J., CABLE, H.C., JAMES, R.F., LONDON, N.J.M., SCARPELLO, J.H.B. & MORGAN, N.G. (1993). Concentration-dependent effects of adrenaline on the profile of insulin secretion from isolated human islets of Langerhans. *J. Endocrinol.,* **138,** 555–563.

MCCLENAGHAN, N.H., BALL, A.J. & FLATT, P.R. (2001). Specific desensitization of sulfonylurea- but not imidazoline-induced insulin release after prolonged tolbutamide exposure. *Biochem. Pharmacol.,* **61,** 527–536.

MEST, H.J., RAAP, A., SCHLOOS, J., TREINIES, I., PAAL, M., GIESE, U. & KOIVISTO, V. (2001). Glucose-induced insulin secretion is potentiated by a new imidazoline compound. *Naunyn Schmiedebergs Arch. Pharmacol.,* **364,** 47–52.

MONKS, L.K., COSGROVE, K.E., DUNNE, M.J., RAMSDEN, C.A., MORGAN, N.G. & CHAN, S.L.F. (1999). Affinity isolation of imidazoline binding proteins from rat brain using 5-amino-efaroxan as ligand. *FEBS Lett.,* **447,** 61–64.

MORGAN, N.G. & CHAN, S.L.F. (2001). Imidazoline binding proteins in the endocrine pancreas; can they fulfil their potential as targets for the development of new insulin secretagogues? *Curr. Pharmaceut. Des.,* **7,** 1413–1431.

MORGAN, N.G., CHAN, S.L.F., MOURTADA, M., MONKS, L.K. & RAMSDEN, C.A. (1999). Imidazolines and pancreatic hormone secretion. *Ann. N.Y. Acad. Sci.,* **881,** 217–228.

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

RUSTENBECK, I., HERRMANN, C., RATZKA, P. & HASSELBLATT, A. (1997). Imidazoline/guanidinium binding sites and their relation to inhibition of K(ATP) channels in pancreatic B-cells. *Naunyn Schmiedebergs Arch. Pharmacol.,* **356,** 410–417.

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

ZAITSEV, S.V., EFANOV, A.M., EFANOVA, I.B., LARSSON, O., OSTENSON, C.G., GOLD, G., BERGGREN, P.-O. & EFENDIC, S. (1996). Imidazoline compounds stimulate insulin release by inhibition of K_{ATP} channels and interaction with the exocytotic machinery. *Diabetes,* **45,** 1610–1618.

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