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Rhes expression in pancreatic β-cells is regulated by efaroxan in a calcium-dependent process

James P. Taylor [a,1], Darryl A. Jackson [a,2], Noel G. Morgan [b], Sue L.F. Chan [a,*]

[a] *Institute of Cell Signalling, School of Biomedical Sciences, University of Nottingham, The Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK*
[b] *Institute of Biomedical and Clinical Science, Peninsula Medical School, Research Way, Plymouth PL6 8BU, UK*

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Abstract

The monomeric G-protein Rhes has been described to be present in pancreatic β-cells, and a putative role in the control of insulin release has been proposed. Here, we show that treatment of β-cells with the imidazoline insulin secretagogue efaroxan resulted in a concentration- and time-dependent increase in the expression of Rhes, which peaked after 4 h of efaroxan exposure; thereafter, Rhes mRNA levels decreased. Marked stereoselectivity was displayed, with (−)-efaroxan (the selectively insulinotropic enantiomer) being much more effective than (+)-efaroxan at raising Rhes transcript levels. The mechanism by which Rhes gene expression is activated in β-cells appears to require the influx of extracellular calcium and *de novo* protein synthesis, and is not directly associated with the release of insulin. The present results confirm our earlier proposal that Rhes is an imidazoline-regulated transcript in pancreatic β-cells. Studies to understand the role of Rhes as a regulator of β-cell function are, thus, warranted.

Keywords: Insulin secretion; Efaroxan; Imidazoline; Rhes; β-cells; Pancreatic islet; KU14R

Efaroxan is an imidazoline insulin secretagogue representative of a group of structurally similar molecules that increase insulin release in a glucose-dependent manner [1–6]. The critical molecular target for efaroxan has not been defined, although electrophysiological [7] and biochemical studies [8] provide strong evidence that imidazolines can bind to the pore-forming subunit of the ATP-sensitive potassium (K_{ATP}) channel, Kir6.2, in the β-cell. Thus, efaroxan and related compounds may mediate some of their effects on insulin secretion by blocking K_{ATP} channels and promoting an increase in cytosolic free calcium [3,9,10]. However, we, and others, have shown that additional, potentially more important, molecular targets also exist and we have proposed that one or more of these is likely to represent the primary site of action of efaroxan in the β-cell [11–14]. These additional sites have not yet been identified in molecular terms and this remains an important objective.

Using an expression cloning approach in combination with an anti-idiotypic antiserum raised against partially purified anti-efaroxan antibodies, we identified the monomeric G-protein, Rhes (Ras Homologue Expressed in Striatum; [15]), as a potential efaroxan-regulated protein in β-cells [16]. Western blot analysis confirmed that the anti-idiotypic antibodies recognised recombinant Rhes expressed in *Escherichia coli* [16] and we also showed that the expression of Rhes was regulated in an efaroxan-sensitive manner in rodent β-cells. Thus, we suggested that Rhes should be considered as a potential target for efaroxan in β-cells.

Rhes is a 266 amino acid protein, with significant sequence homology to various members of the Ras GTP

* Corresponding author. Fax: +44 115 8230142.
E-mail address: sue.chan@nottingham.ac.uk (S.L.F. Chan).
[1] Present address: Department of Haematology, University of Liverpool, Royal Liverpool University Hospital, Liverpool L69 3GA, UK.
[2] Present address: Division of Pathology, School of Molecular and Medical Sciences, University of Nottingham, The Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK.

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binding protein family [15,17]. Such small molecular weight GTPases (SMGs) occupy central positions in many signal transduction pathways, and they are involved in the control of diverse processes including cell proliferation, differentiation, and apoptosis [18–20]. SMGs have also been implicated in intracellular vesicle trafficking, which may be important for exocytosis. Thus, it was suggested that Rhes may represent a molecule by which the direct control of insulin release by imidazolines could be mediated [16].

Recently, Zaitsev and colleagues set out to test this hypothesis and reported that neither over-expression nor down-regulation of Rhes was effective as a means to alter imidazoline-induced insulin secretion [21]. Furthermore, in contrast to our earlier study, these authors were not able to show any change in Rhes mRNA expression levels in rat islets exposed to efaroxan (or another imidazoline, BL11282) [21]. They concluded, therefore, that Rhes is not an imidazoline-regulated protein in β-cells.

Since the publication of our earlier report, we have continued to investigate the role of Rhes in efaroxan-treated β-cells and we now present further evidence that directly challenges the recent conclusions reported in [21]. We show that imidazolines alter Rhes expression dramatically and provide evidence that this is a calcium-dependent process controlled independently of insulin exocytosis. Parts of this work have been reported in abstract form [22].

Materials and methods

Materials. Collagenase (type XI), diazoxide, glibenclamide, phentolamine, nifedipine, cycloheximide, human recombinant insulin, and thyroxine were purchased from Sigma Chemical Co. (Dorset, UK). KU14R (2 (2-ethyl 2,3-dihydro-2-benzofuranyl)-2-imidazole) was synthesised in the Department of Chemistry, University of Keele, UK [23] and is available from Tocris bioscience (Bristol, UK). BayK8644, Efaroxan hydrochloride, and RX821002 were from Tocris bioscience (Bristol, UK). AR42J and αTC1-9 cell lines were kindly donated by Prof S. Watson, (Nottingham, UK) and Prof P. Jones (King's College London, UK), respectively. All other reagents were of analytical reagent grade.

Cell culture and tissue isolation. RINm5F, PC12, AR42J, AtT20, and αTC1-9 cells were cultured in RPMI-1640 medium (11.1 mM glucose), and CHO-K1 cells were cultured in DMEM-F12 medium. In all cases, the culture medium was supplemented with 10% (v/v) foetal calf serum and 2 mM glutamine, and cells were cultured at 37 °C in a humidified atmosphere of air:CO_2 (95:5). Cells were seeded (1×10^5 cells/well/4 ml) in 6-well plates. After culturing for 24 h, the medium was aspirated and fresh serum-free culture medium was added. After a pre-equilibration period of 30 min, test reagents were added and cells were cultured for a further 2 h or for the required period of time (as indicated).

Rat islets of Langerhans were isolated by collagenase digestion [16]. Islets were cultured in RPMI-1640 plus 10% (v/v) foetal calf serum before use. Other rat tissue was used immediately for RNA isolation.

RNA extraction and cDNA synthesis. Total RNA was isolated by cell lysis in TRI Reagent (Sigma), according to the manufacturer's instructions. RNA samples were treated with RQ1 RNase-free DNase (Promega) to remove any genomic DNA contamination and quantified by spectrophotometric analysis at 260 nm. First-strand cDNA synthesis was performed using 1 μg of total RNA with 200 ng random hexamer primers and Avian Myeloblastosis Virus reverse transcriptase (10 U/μl RNA, Promega).

Standard and real-time semi-quantitative PCR. For standard PCR, reactions were performed in a total volume of 25 μl using Sigma Ready-Mix PCR mix, with 1 μl cDNA product (from 20 μl RT reaction) and 1.25 μM of each primer. PCR was performed at 95 °C for 5 min, followed by 36 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The last cycle was followed by a final extension step at 72 °C for 10 min. PCR products were separated on 1% (w/v) agarose gels, with DNA staining using GelStar (BioWhittaker Molecular Applications, Rockland, ME, USA) and imaging with GelDoc software (BioRad, Hemel Hempstead, UK).

Real-time PCR was performed using 25 ng of reverse-transcribed total RNA with 300 nM of sense and anti-sense primers, 150 nM Rhes TaqMan® probe labelled at the 5′ and 3′ ends, respectively, with 6-carboxyfluorescein (FAM) and 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA), and 1 × Taqman buffer A (Applied Biosystems) in a total volume of 25 μl in an ABI PRISM 7700 Sequence Detection System Instrument (Applied Biosystems). Standard curves were constructed by amplifying serial dilutions of untreated cDNA (50 ng–0.64 pg) and plotting cycle threshold (C_T) values as a function of starting reverse-transcribed RNA, the slope of which was used to calculate relative expression of the target gene. The C_T value is the cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline, and is defined as the average intensity over a cycle range of 1–10, plus the standard deviation of this value. 18S ribosomal RNA (primers and probe, Applied Biosystems) was amplified in parallel with experimental samples and used to normalise the results in order to allow semi-quantitative analysis of Rhes expression. Reactions were performed in triplicate for each experiment.

Primers for PCR were designed using Primer Express™ (Applied Biosystems) software:

Rhes (Accession No. AF134409)
Rhes Forward primer, position 1408–1426 5′-tgttcagcatggccaagct-3′
Rhes Reverse primer, position 1458–1479 5′-caccgtactgcacggagatct-3′
Rhes probe, position 1430–1452 5′-ccacgagatgagccctgcactgc-3′

Statistical analysis. Data are presented as means ± SE for the indicated number of experiments (*n*). The statistical significance of differences between means was assessed by Student's *t*-test for unpaired data and by ANOVA where appropriate. Statistical significance was achieved when $p < 0.05$.

Results

Expression of Rhes in cells and tissues

Previous work has revealed that Rhes mRNA expression is highest in the striatum, with other parts of the brain displaying less abundant expression [15,24,25]. Low levels of Rhes mRNA have also been reported in peripheral tissue [15,24,25] and we confirm this restricted expression pattern, with low, but measurable, expression in rat adrenal, stomach, and pancreatic islets (Fig. 1A) compared to brain. Studies of alpha and beta cell lines by standard RT-PCR

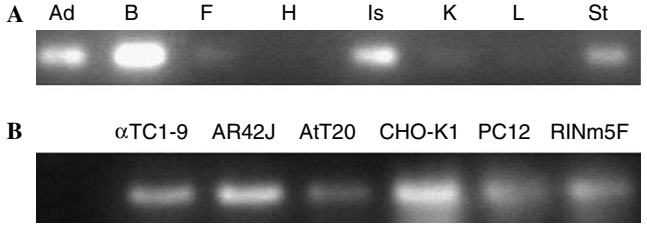

Fig. 1. Rhes Expression. RT-PCR analysis was performed on total RNA prepared from rat tissue (A) and cell lines (B), using specific primers for Rhes cDNA. (Ad, adrenal gland; B, whole brain; F, abdominal fat; H, heart; Is, islets; K, kidney; L, liver; St, stomach).