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Monomeric G-protein, Rhes, is not an imidazoline-regulated protein in pancreatic β-cells

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Abstract

The monomeric G-protein, Rhes, is a candidate imidazoline-regulated molecule involved in mediating the insulin secretory response to efaroxan [S.L. Chan, L.K. Monks, H. Gao, P. Deaville, N.G. Morgan, Identification of the monomeric G-protein, Rhes, as an efaroxan-regulated protein in the pancreatic beta-cell, Br. J. Pharmacol. 136 (1) (2002) 31–36]. This suggestion was based on observations regarding changes in Rhes mRNA expression in rat islets and pancreatic β-cells after prolonged culture with efaroxan, leading to desensitization of the insulin response to the compound. To verify this report, we have evaluated the effects of the imidazoline compounds efaroxan and BL11282 on Rhes mRNA expression in isolated rat pancreatic islets maintained in conditions identical to those used by Chan et al. The results demonstrate that desensitization of the insulin response to efaroxan, or to another imidazoline, BL11282, does not change Rhes mRNA expression levels. Transfection of MIN6 cells with plasmids containing Rhes or Rhes-antisense also does not alter efaroxan- or BL11282-induced insulin secretion. Together, these data do not support the hypothesis that Rhes is an imidazoline-regulated protein.
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It has been well established that certain imidazoline compounds possess a stimulatory activity on insulin secretion from pancreatic β-cells [1–5]. It was subsequently suggested that the insulinotropic activity of imidazolines is related to blockade of the K_{ATP} channel [6], and it was demonstrated that some of these compounds can block the pore-forming subunit of the K_{ATP} channel Kir6.2 [7]. However, our studies of the imidazoline compound RX871024 on insulin release clearly showed that in addition to an effect on the K_{ATP} channel, RX871024 also promotes K_{ATP} channel-independent (direct) effects on insulin secretion from pancreatic β-cells [8,9]. Subsequent studies in other laboratories, and with another imidazoline compound, efaroxan, confirmed our findings [10]. Moreover, we have been able to develop a pure glucose-dependent insulinotropic imidazoline compound, BL11282, which directly affects the insulin exocytotic machinery [11,12]. However, the imidazoline target yielding this direct effect is unknown. Recently, Chan et al. [13] suggested that a monomeric G-protein, Ras homologue expressed in striatum (Rhes) [14], represents an imidazoline-regulated molecule that is involved in mediating the insulin secretory response to the imidazoline efaroxan. This suggestion was based on observations regarding changes in Rhes mRNA expression in rat islets and pancreatic β-cells after prolonged culture with efaroxan. To verify this report, we have evaluated the effects of the imidazoline compounds efaroxan and BL11282 on Rhes mRNA expression in isolated rat islets maintained under conditions identical to those

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used by Chan et al. [13]. In addition, we have evaluated the effect of efaroxan and BL11282 on insulin secretion in MIN6 cells transfected with Rhes and Rhes-antisense plasmids.

Materials and methods

Isolation of rat pancreatic islets. Pancreata were removed from 2- to 3-month-old male Wistar rats, chopped, and digested at 37 °C in 5 ml Hanks' balanced salt solution containing 13 mg collagenase for 22–23 min with continuous shaking (150 strokes/min). Isolated rat pancreatic islets were incubated with or without imidazoline compounds at 37 °C for 18 h in a humidified atmosphere of 5% CO_2, in RPMI-1640 medium (5.5 mM glucose) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate, the conditions described by Chan et al. [13].

Cell culture. The β-cell line MIN6 (passages 32–38) was maintained in DMEM containing 25 mM glucose, supplemented with 10% fetal calf serum, 50 U/ml penicillin, 0.05 mg/ml streptomycin sulfate, and 50 μM β-mercaptoethanol, in a humidified atmosphere of 5% CO_2 at 37 °C. MIN6 monolayers were trypsinized (0.1% trypsin, 0.02% EDTA) at 80–90% confluency and were plated in 24-well plates 24 h before transfection.

Measurements of insulin secretion. Insulin secretion from islets was measured in Krebs–Ringer bicarbonate buffer (KRBB) containing (in mM): 115 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 20 $NaHCO_3$, 16 Hepes, and 2 mg/ml BSA; pH 7.4. Islets were preincubated in KRBB with 3.3 mM glucose at 37 °C for 1 h. Groups of three islets were incubated at 37 °C for 1 h in 300 μl of the same buffer containing 3.3 or 16.7 mM glucose, or 16.7 mM glucose and the respective test compound. Insulin secretion from MIN6 cell monolayers was measured in Earle's balanced salt solution (EBSS) containing (in mM): 115 NaCl, 5.3 KCl, 1.8 $CaCl_2$, 1.0 NaH_2PO_4, 0.8 $MgSO_4$, 26 $NaHCO_3$, and 1 mg/ml BSA; pH 7.4. MIN6 cell monolayers were preincubated in EBSS with 1 mM glucose at

37 °C for 1 h. Cells were then incubated at 37 °C for 1 h in 500 μl of the same buffer containing 1 mM or 25 mM glucose, or 25 mM glucose and the respective test compound. Supernatants from the incubations were chilled on ice and aliquoted prior to measurement of insulin by radioimmunoassay, employing rat insulin (Novo Nordisk) as standard. Stimulated insulin secretion was normalized to the level of insulin release under basal conditions, i.e., the secretion rate at 3.3 and 1 mM glucose for islets and MIN6 cells, respectively.

RNA extraction and semi-quantitative reverse-transcription (RT-PCR). At the end of the culture period, islets were collected under a stereomicroscope and employed immediately for RNA extraction, using RNeasy RNA purification kits (Qiagen), according to the manufacturer's instructions. RNA was treated with DNase I (Qiagen) for 15 min at room temperature. Reverse-transcription was carried out using SuperScript II (Invitrogen–Life Technologies) according to the manufacturer's instructions in reactions containing 1.5 μg total RNA, 150 ng random hexamer primers (Invitrogen–Life Technologies), and 40 U RNaseOut (Invitrogen–Life Technologies). Aliquots of each RT mix removed prior to the addition of reverse transcriptase served as negative controls. Semi-quantitative PCR was performed independently of cDNA samples generated from triplicate experiments. PCR was carried out in 10 μl reactions containing 0.2 μM dNTPs, 5 pmol ribosomal protein L30 (rPL30), β-actin or Rhes-specific primers, 0.4 U *Taq* polymerase (Roche), and quantities of cDNA corresponding to 10 ng (for rPL30 and β-actin) or 50 ng (Rhes) total RNA. The sequences of the primers used are shown in Table 1. PCR products were analyzed by electrophoresis on 1.5% agarose gels and documented with a digital camera (EDAS 290, Kodak) and software (1D, Kodak). All PCRs included RT-negative controls, and these reactions consistently failed to yield any amplification product. The identity of the Rhes gene amplification product was verified by DNA sequence analysis.

Plasmids. Plasmid pHG327.Rhes, which contained the cDNA for Rhes, was kindly provided by Dr. J. Gregor Sutcliffe (The Scripps Research Institute, La Jolla, CA, USA). The cleavage product of

Table 1
Primer sequences for RT-PCR

| Target gene (Accession number)	Sense primer sequence (5′ → 3′) (primer designation)	Antisense primer sequence (5′ → 3′) (primer designation)	PCR annealing temperature (°C)
rPL30 (K02932)	GGA AAG TAC GTG CTG GGG TA	CAC CTG GGT CAA TGA TAG CC	63.5
β-Actin (NM_031144)	TGT GCC CAT CTA CGA GGG GTA TGC	GGT ACA TGG TGG TGC CGC CAG ACA	59.0
Rhes (NM_133568)	GCA AGA GCT CCA TTG TCT CC	CGT GTT CTT CTT GGC TGA CA	59.0
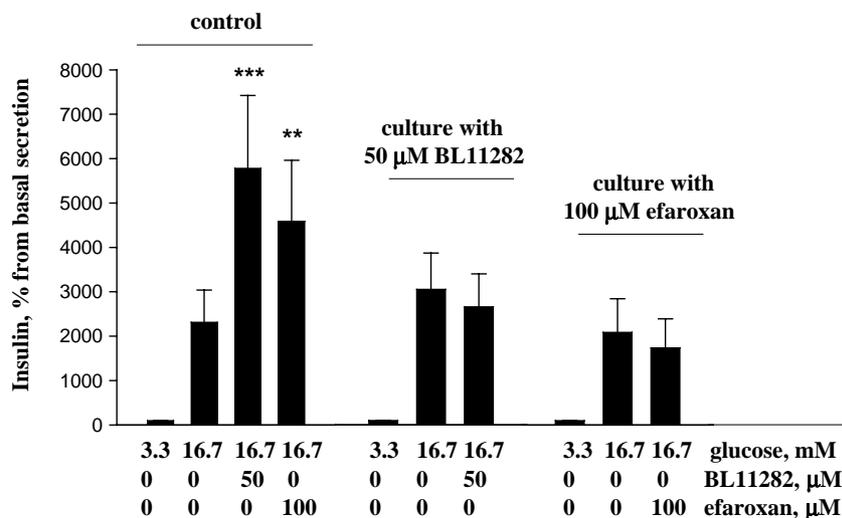
Fig. 1. Desensitization of insulin secretion by culture for 18 h in the presence of BL11282 or efaroxan. Results are expressed as means ± SE for six independent experiments. Statistical analyses were carried out by Student's *t* test (**$p < 0.01$, ***$p < 0.001$ relative to 16.7 mM glucose control).

pHG327.Rhes digestion with *Nar*I was incubated with Klenow polymerase and then digested with *Apa*I. The resulting Rhes cDNA fragment was inserted into the *Eco*RV- and *Apa*I-digested construct pRcCMVi.EGFP [15], thereby replacing the EGFP cDNA with Rhes cDNA to generate pRcCMVi.Rhes. To obtain the Rhes-antisense expression construct, an 800 bp Rhes-fragment was obtained by digesting pRcCMVi.Rhes with *Sma*I and *Apa*I. This DNA fragment was subcloned into *Hpa*I- and *Apa*I-digested pB.rIns1.DsRed [16], thereby replacing the DsRed cDNA and generating pB.rIns1.Rhes-antisense. This permitted the Rhes cDNA fragment to be placed in the antisense orientation under control of the rat insulin-1 promoter (−410/+1). All vector constructions were verified by DNA sequence analysis.

Transfection of MIN6 cells. MIN6 cells were transfected with pRcCMVi.EGFP, pRcCMVi.Rhes, pB.rIns1.EGFP, and pB.rIns1.Rhes-antisense plasmids in the presence of LipofectAMINETM 2000 (Invitrogen–Life Technologies), according to the manufacturer's instructions. Transfection efficiency was estimated by microscopic evaluation of EGFP fluorescence with an inverted microscope (Zeiss Axiovert 133TV; Carl Zeiss MicroImaging). Excitation light was obtained from a SPEX fluorolog-2 MM1T11I spectrofluorometer (Spex Industries). The following settings were used for EGFP detection: excitation at 485 nm, a 505-nm dichroic mirror, and a 505–535-nm band-pass emission filter. Measurements of insulin secretion from MIN6 cells were performed 72 h after transfection, when transfection efficiency was maximal.

Statistical analysis. Data are expressed as means ± SE for the indicated number of experiments. The difference between means was assessed with Student's *t* test.
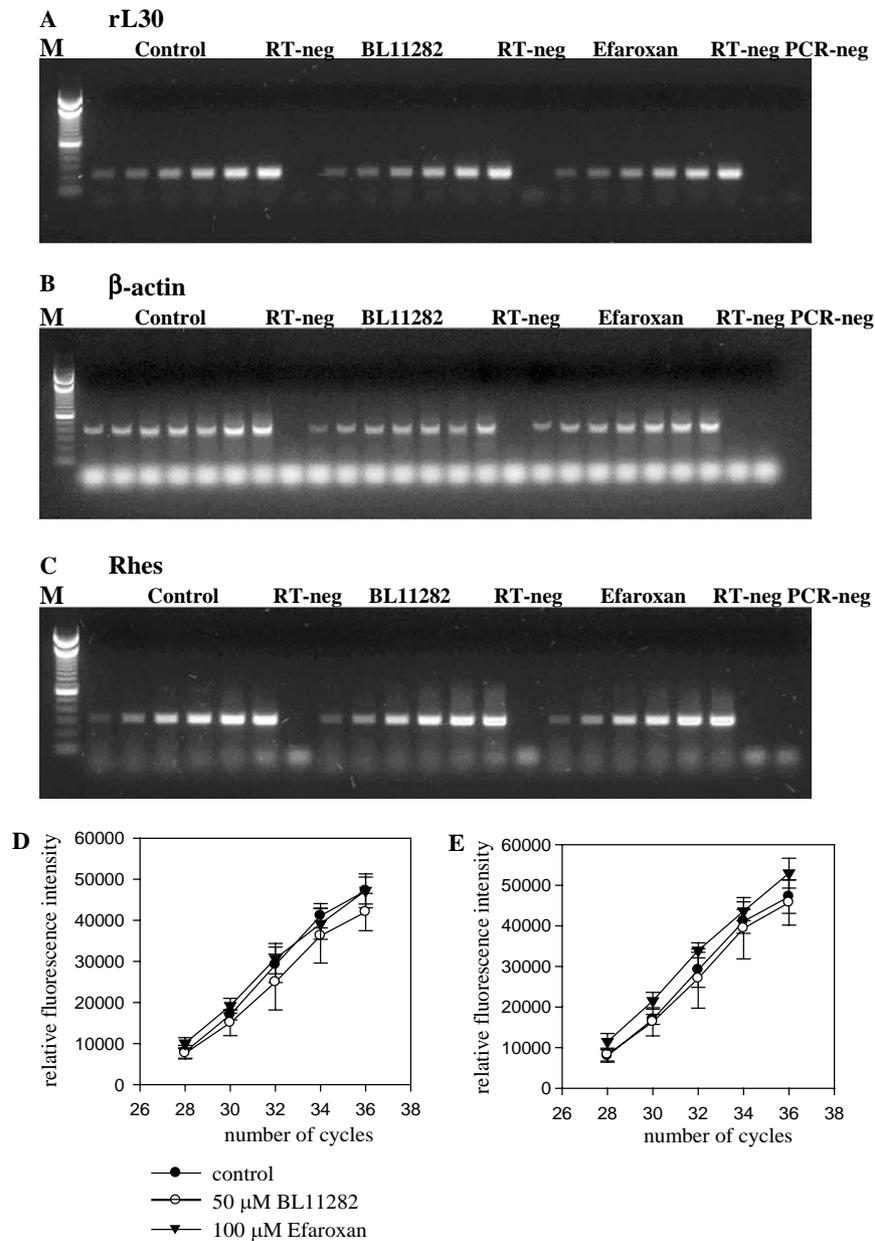

Fig. 2. Semi-quantitative RT-PCR analyses of relative mRNA levels for the rPL30 (A), β-actin (B), and Rhes (C) genes in control, BL11282- and efaroxan-desensitized rat islets. The results are presented as a digital image of an example agarose gel (A–C), and following quantitation of the fluorescence intensity of each band normalized to the band intensity for the rPL30 (D) and β-actin (E) amplifications at 25 and 26 cycles, respectively, for each respective sample. Results are expressed as means ± SE for three independent experiments.

Results

Desensitization of pancreatic islets to insulinotropic imidazoline compounds

Prolonged incubation (18 h) of pancreatic islets with 100 μM efaroxan under the same conditions as those used by Chan et al. [13] caused desensitization of the islet response to the imidazoline compound (Fig. 1), in agreement with that study. We also evaluated the effect of these desensitization conditions in the presence of the pure glucose-dependent insulinotropic imidazoline compound, BL11282. The results depicted in Fig. 1 show that incubation of rat pancreatic islets with 50 μM BL11282 for 18 h abolishes the stimulatory effect of this imidazoline at high glucose concentration.

Effect of prolonged incubation with imidazoline compounds on Rhes mRNA expression

In the study by Chan et al. [13], it was shown that desensitization of pancreatic islets to efaroxan was accompanied by a significant decrease in Rhes mRNA expression. To verify this effect, we have performed semi-quantitative RT-PCR analyses of Rhes mRNA levels in islets desensitized either to efaroxan or BL11282, in comparison to control islets. For each experiment, input quantities of each cDNA were first normalized to give comparable amplification of two different endogenous standard genes, rPL30 (cycles 23–28; see Fig. 2A) and β-actin (cycles 23–29; see Fig. 2B). At these template concentrations, comparable amplifications were obtained for both rPL30 and β-actin from cDNA prepared from control, efaroxan- and BL11282-treated islets. As shown in Fig. 2C, Rhes mRNA is expressed in rat pancreatic islets. Rhes amplification was then carried out for 28–36 cycles at the template inputs shown to yield equivalent rPL30 or β-actin amplifications, and was quantitated by normalization to the product abun-

dances of either rPL30 at 25 cycles or β-actin at 26 cycles (Figs. 2D and E). No alterations in Rhes gene expression could be detected in either efaroxan- or BL11282-treated islets, compared to controls.

Effect of Rhes over-expression or down-regulation in MIN6 cells on insulinotropic activity of imidazolines

For a final verification of the putative role for Rhes in the insulinotropic activity of imidazolines, we have employed an alternative approach involving either over-expression of Rhes or its down-regulation by a Rhes-antisense construct. The results depicted in Fig. 3 show that transfection of MIN6 cells with plasmids containing Rhes or Rhes-antisense does not affect either efaroxan- or BL11282-induced insulin release. In addition, over-expression or down-regulation of Rhes has no effect on glucose-induced insulin secretion.

Discussion

In our previous studies [9,10], it was shown that insulinotropic imidazoline compounds, in addition to their action on the K_{ATP} channel, directly promote insulin exocytosis. We subsequently developed the imidazoline compound BL11282, which stimulates exocytosis in the absence of modulation of K_{ATP} channel activity [11,12]. However, the mechanism of this K_{ATP} channel-independent effect of imidazolines on insulin exocytosis is currently unknown. Chan et al. [13] have suggested that a monomeric G-protein, Ras homologue expressed in striatum (Rhes) [14], represents an imidazoline-regulated molecule that is involved in mediating the insulin secretory response to the imidazoline efaroxan. This suggestion was based on their finding that desensitization of the insulin response to efaroxan was accompanied by decreases in expression of the Rhes gene [13]. To verify whether Rhes is indeed involved in the direct effect of imidazolines on
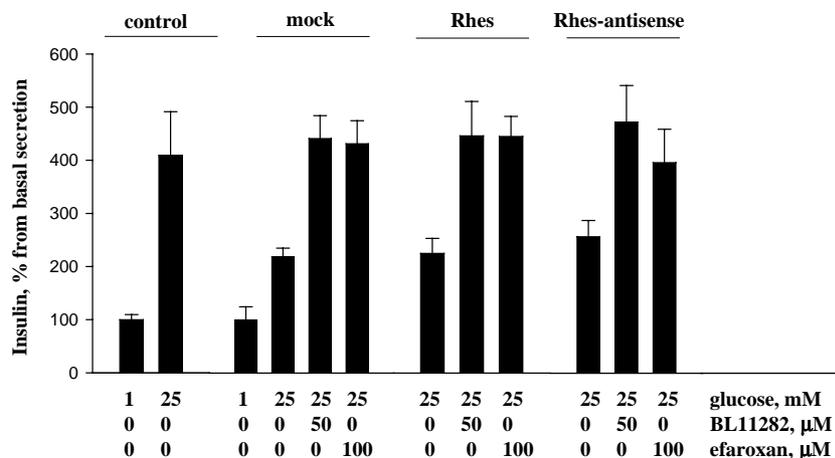

Fig. 3. Insulin secretion from MIN6 cells transfected with EGFP (mock), Rhes or Rhes-antisense constructs. Results are expressed as means ± SE for four to seven independent experiments.

insulin exocytosis, we performed desensitization experiments with efaroxan under conditions identical to those employed by Chan et al. and analyzed the effects of desensitization on Rhes mRNA levels by RT-PCR. The results of our studies confirm expression of the Rhes gene in pancreatic islets, as shown by Chan et al. [13]. However, our measurements of Rhes mRNA expression levels, followed by data analysis involving normalization to the housekeeping genes rPL30 or β-actin, did not show any significant changes in Rhes transcript expression subsequent to islet desensitization to efaroxan. Similarly, we were not able to show changes in Rhes transcript expression in islets desensitized to another imidazoline compound, BL11282, which has been shown to directly stimulate insulin exocytosis [11].

To elaborate a putative role for Rhes in the insulinotropic activity of imidazolines, we have used another approach involving either over-expression of Rhes in insulin-producing MIN6 cells or its down-regulation by a Rhes-antisense construct. The data obtained demonstrate that transfection of MIN6 cells with plasmids containing Rhes or Rhes-antisense does not affect either efaroxan- or BL11282-induced insulin secretion.

Taken together, the data obtained do not support the hypothesis that Rhes is an imidazoline-regulated protein and are not consistent with the proposal that this protein is responsible for the direct effect of imidazoline compounds on insulin exocytosis.

Acknowledgments

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