Neuronal traits are required for glucose-induced insulin secretion

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Abstract The transcriptional repressor RE1 silencer transcription factor (REST) is an important factor that restricts some neuronal traits to neurons. Since these traits are also present in pancreatic β-cells, we evaluated their role by generating a model of insulin-secreting cells that express REST. The presence of REST led to a decrease in expression of its known target genes, whereas insulin expression and its cellular content were conserved. As a consequence of REST expression, the capacity to secrete insulin in response to mitochondrial fuels, a particularity of mature β-cells, was impaired. These data provide evidence that REST target genes are required for an appropriate glucose-induced insulin secretion.

Keywords: Transcriptional repressor protein; Insulin secretion; Neuronal expression; β-Cell

1. Introduction

RE1 silencer transcription factor (REST), also named neuron restrictive silencer factor (NRSF), is a nine-zinc finger protein, which is widely expressed in all tissues with the exception of mature neuronal cells [1,2]. REST has been identified as a transcriptional repressor that negatively regulates expression of some specific neuronal genes in non-neuronal cells and in neuronal precursor cells [3–6]. This transcription factor binds to a *cis* repressor element of 23 bp termed RE1 or neuron restrictive silencer element (NRSE) and recruits some co-repressors such as mSIN3, coREST and histone deacetylases [7–10]. By this mechanism, a large number of genes are directly repressed by REST [4,11–14]. Several studies, including ours, have shown that some of these genes are also expressed in pancreatic β-cell lines where REST is absent [1,15]. These include islet-brain1/c-Jun N-terminal kinase interacting protein-1 (IB1/JIP-1) [15], connexin36 [16] and complexin I [17]. The aim of this study was to evaluate the role of REST target genes in β-cell function. For this purpose, REST was stably introduced in insulin-secreting cells. In these cells, expression of REST led to repression of its target genes. Moreover, these cells displayed an impairment of insulin secretion in response to glucose and leucine, whereas the secretory response to KCl was unchanged. This loss of function was confirmed by transient transfection of REST in different insulin-secreting cell lines. These data demonstrate that the REST target genes are required for glucose-induced insulin secretion.

2. Materials and methods

2.1. Establishment of stable cell lines

The mouse pancreatic βTC3-cell line was cultured as previously described [15]. Stable transfection was performed in 10-cm dishes using 45 μl of DOTAP solution (Roche Diagnostics) mixed with 7 μg of the plasmid encoding the transcriptional repressor REST (RIP REST) [15] and the plasmid encoding the hygromycin resistance cassette as selection marker, in molecular ratio (10:1). Transfected cells were cultured in the presence of 500 μg/ml hygromycin.

2.2. Total RNA preparation, Northern blot analysis, nuclear protein extract preparation and electromobility shift assays (EMSA)

Nuclear extracts and EMSAs were performed as previously reported [15,18]. Oligonucleotides used as labeled probes were as follows: NRSE IB1 (forward: 5′-GGCTTCAGCACCGCGGAGAGCGCCATCTCC-3′ and reverse: 5′-CCGGAGATGGCGCTCTCCGCGGTGCTGAAG-3′). Total RNA preparation and Northern blot analysis were performed as described [15].

2.3. Measurement of insulin secretion and content

Cells (5×10^5) were plated in 48-well dishes for 48 h and were cultured in 2 mM of glucose for 24 h. Cells were washed three times with a modified Krebs–Ringer/bicarbonate–HEPES buffer, pH 7.4, as described [19]. The cells were pre-incubated with this buffer containing 2 mM of glucose for 2 h at 37 °C before incubation in KRBH containing either 2 mM glucose (basal condition) or 20 mM of glucose (including 10 μM of forskolin and 100 μM of IBMX) or 20 mM leucine or 24 mM KCl for 60 min. The supernatant was removed for the measurement of secreted insulin and cells were extracted with acid/ethanol for insulin content. These measurements were determined by radioimmunoassay using rat insulin [20]. For exocytosis experiments, INS1 and βTC3 cells (5×10^5) were seeded in 24-well plates and were transiently transfected with 0.2 μg of the plasmid encoding the human growth hormone (hGH) with the effectene reagent (Qiagen) as previously described [17].

2.4. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

To measure the cellular metabolic rate, cells were plated and preincubated in KRB for 60 min. After pre-incubation, the cells were replaced with KRB under stimulatory conditions containing 0.1 mg/ml MTT (Sigma) for 30-min incubation. The supernatants were removed and the insoluble MTT metabolite within the cell monolayer was extracted with the SDS–HCl solution (0.01 M HCl and 10% SDS). The absorbance of the extractants at 570 nm was recorded using arbitrary OD units.

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3. Results

3.1. Establishment of an insulinoma cell line expressing the transcriptional repressor REST

Mouse insulin-secreting βTC3 cells were stably transfected with a plasmid encoding REST under the control of the rat insulin promoter (or the empty vector as control). Hygromycin-resistant clones were assessed for the expression of REST by Northern blot analysis. Two resistant clones, R1 and R2 (R1/R2), were found to express the REST transcript unlike the randomly selected C1 and C2 control clones (C1/C2) that do not contain endogenous REST factor (Fig. 1A). The REST-

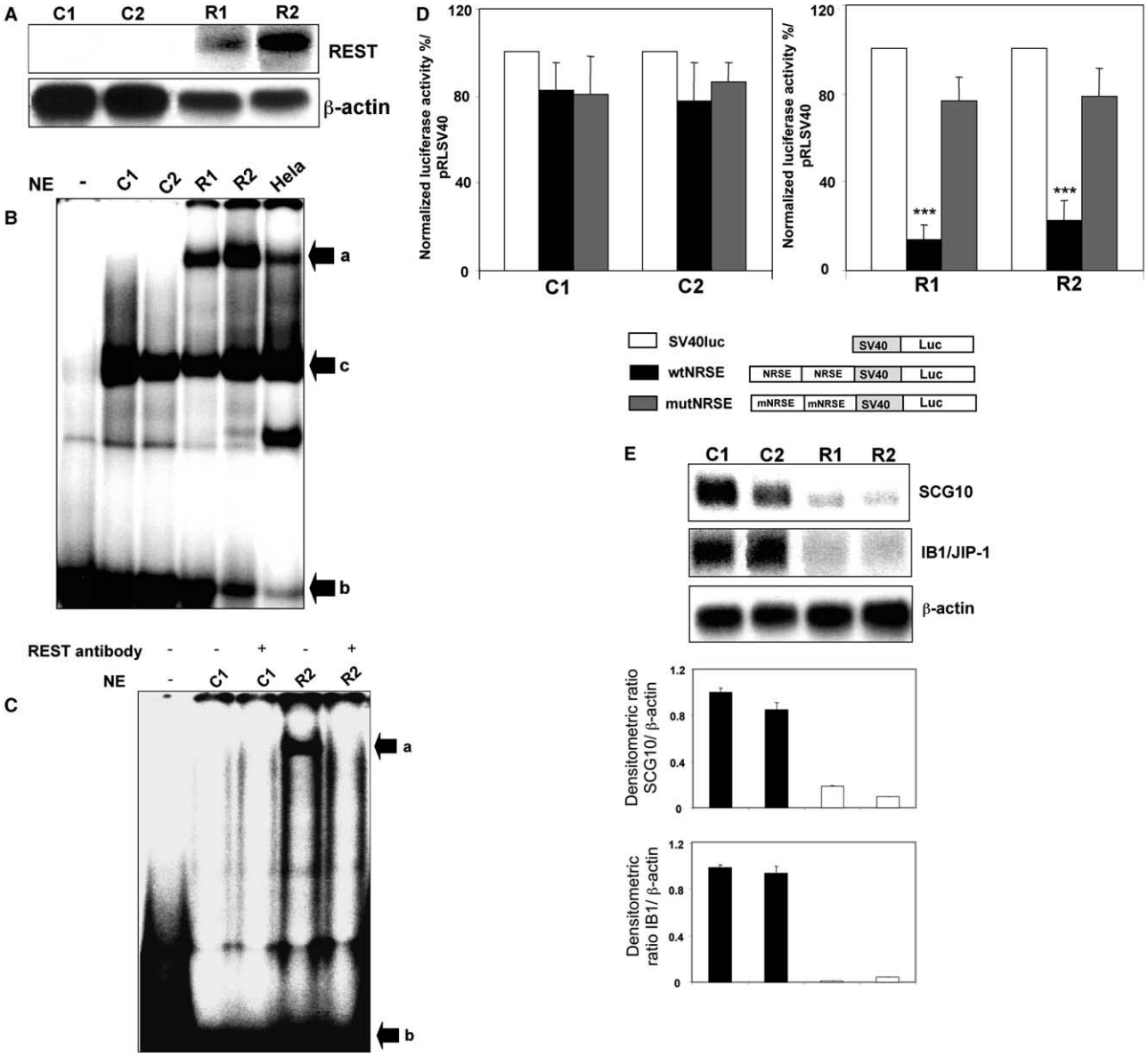

Fig. 1. (A) Northern blot analysis of exogenous REST and β-actin mRNA in βTC3 cells. Total RNA (15 μg) was isolated from two hygromycin-resistant clones transfected with RIP REST (R1 and R2) and two randomly selected controls (C1 and C2). RNA was then hybridized with the human REST cDNA probe as previously described [15]. The β-actin probe is used to evaluate the quantity of total RNA loaded. A transcript was detected only in R1 and R2 but not in control C1 and C2 cells. (B) REST binding activity is detected in REST-expressing clones R1 and R2. A similar pattern of binding activity (a) was detected with R1 and R2 cells compared to free probe migration (b). The arrow c corresponds to an unspecific band that was not competed by adding a 100- or 600-fold molar excess of unlabeled wild type NRSE (data not shown). NE, nuclear extract. (C) This DNA-binding activity with IB1NRSE was disrupted in REST-expressing clones by using REST antibodies. NE, nuclear extract. (D) The activity containing NRSE was silenced in R1 and R2 clones. Heterologous promoter constructs were then transiently transfected into control C1/C2 and R1/R2 clones. The wtNRSE led to a marked decrease in both R1 and R2 compared to the basal activity of the SV40luc. The mutation in NRSE restored the activity of mutNRSE promoter. Luciferase activities were normalized using pRLSV40 renilla. Each experiment was performed at least three times in triplicate. All values are expressed as percent of the SV40luc activity. Results are expressed as means ± S.E.M. ($^{***}P < 0.001$). (E) Expression of REST target genes such as SCG10 and IB1/JIP-1 is decreased in R1/R2 clones. Northern blot of REST target genes such as SCG10, IB1/JIP-1 and β-actin was performed as described [15]. The SCG10 and IB1/JIP-1 mRNA levels were measured by densitometric scanning. Normalized to β-actin, SCG10 mRNA levels decreased by 5- and 7-fold in R1 and R2 clones, respectively, compared to C1/C2 cells. The IB1/JIP-1 mRNA levels were almost undetectable in R1 and R2.

binding activity was evaluated using nuclear extracts prepared from R1/R2 and C1/C2 control cells. The labeled probe used was the NRSE previously identified within the IB1 promoter [15]. The REST binding activity was detected in nuclear extracts prepared from R1/R2 but not from control cells (Fig. 1B). This binding activity was disrupted by addition of a REST antibody, indicating that the observed pattern is specific to REST (Fig. 1C). The transcriptional repressive effect of REST in R1/R2 clones was functionally confirmed by measuring the activity of the luciferase reporter gene under the control of NRSE. We used a construct containing two wild-type (wtNRSE) or mutated NRSE motifs (mutNRSE) cloned upstream of the SV40 promoter linked to the luciferase gene reporter as previously described [15]. Since NRSE is a negative element, its active form should reduce expression of the reporter. Control C1/C2 cells and R1/R2 cells were transiently transfected with these different constructs. In control cells, luciferase activities of wtNRSE and mutNRSE constructs were not different from SV40luc (Fig. 1D). In contrast, in both R1/R2 cells expressing REST, the activity of constructs containing wtNRSE was reduced by 75% and 80% compared to SV40luc. As expected, this activity was restored when the NRSE motif was mutated, indicating that REST was unable to bind to the mutated NRSE. As expected, the luciferase activity was likewise restored when R1/R2 cells were treated with trichostatin, an inhibitor of histone deacetylase (data not shown). This result is consistent with previous reports showing that REST-mediated transcriptional repression requires histone deacetylase activity [15]. The transcriptional effect of REST on its endogenous target genes was then investigated. IB1/JIP-1- and SCG10-encoding genes, known to be negatively modulated by REST, were then selected [7,15]. Expression of both IB1/JIP-1 and SCG10 in R1/R2 was virtually abolished compared to control cells (Fig. 1E). Taken together, these data confirm the generation of an insulin-secreting cell model expressing the functional transcriptional repressor REST.

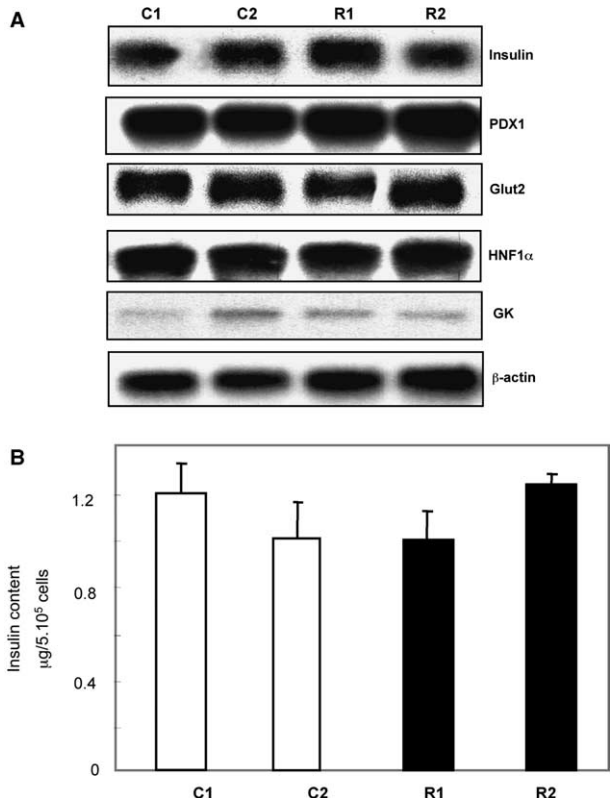

Fig. 2. Insulin expression and content of R1/R2 and C1/C2 cells. (A) Northern blot analysis of mRNA levels for insulin and several important genes controlling insulin expression and secretion [PDX1, Glut2, GK and HNF1α]. (B) Insulin content by radioimmunoassay. Similar insulin mRNA levels and content were observed in R1/R2 and C1/C2 cells. Data are means ± S.E.M. of six independent experiments.

3.2. Glucose-induced insulin secretion is impaired in REST-expressing clones

We next evaluated expression and secretion of insulin in cells stably expressing REST. R1/R2 cells and control C1/C2 cells have similar levels of insulin mRNA and protein contents (Fig. 2A and B). Northern blot analysis showed that mRNA levels of several diabetogenic genes known to regulate insulin expression and secretion in response to glucose, such as those encoding glucokinase (GK), the glucose transporter Glut2, the transcription factors PDX1 and HNF1α, were similar in R1/R2 and C1/C2 cells (Fig. 2A). Consistent with this result, computer-assisted searches failed to find any NRSEs in the genomic sequence of these genes. NRSEs were also not found in other genes important for insulin secretion including those encoding pyruvate kinase, Kir6.1 and transcription factors such as HNF4α, Nkx6.1, Nkx2.2, Pax6 and Isl1, indicating that they are not targets of REST. We next investigated the impact of the presence of REST on secretagogue-induced insulin secretion. In control cells, insulin secretion was increased by about 3-fold in response to glucose, whereas in the REST-expressing clones (R1/R2) glucose was unable to elicit the release of insulin (Fig. 3A). Leucine specifically activates the mitochondrial metabolism and acts downstream of glycolysis; K^+ depolarizes the β-cell membrane and triggers exocytosis independently of mitochondrial metabolism. In control cells, leucine and KCl stimulated insulin secretion by about 3-fold (Fig. 3B). In R1/R2 cells, direct stimulation of the secretory machinery of R1/R2 cells following K^+ depolarization and Ca^{2+} entry led to an increase in insulin release comparable to that obtained in control cells. In contrast, leucine did not stimulate insulin secretion. To confirm that impaired insulin secretion in response to glucose is not related to clonal selection, we measured secretagogue-induced secretion in two different insulin-secreting cell lines, βTC3 and INS1, in which we transiently re-expressed REST. To monitor exocytosis of the cells, we co-transfected the cells with a plasmid encoding hGH. In insulin-secreting cells, hGH is targeted to secretory granules and is co-released with insulin during the exocytotic event [19]. hGH release can therefore be used as a reporter to measure secretion from transiently transfected cells. In the absence of REST, glucose, leucine and K^+ elicited an increase of about 3-fold of hGH release both in βTC3 and INS1 cells (Fig. 4A and B). When REST was expressed, the secretory response to glucose and leucine was either diminished (INS1 cells) or abolished (βTC3 cells). However, in all cases KCl-induced secretion was preserved. These data, together with the findings obtained with stably transfected cell lines, demonstrate that the exocytotic apparatus of REST-expressing cells is still functional but the coupling of the secretory machinery to mitochondrial metabolism is missing.

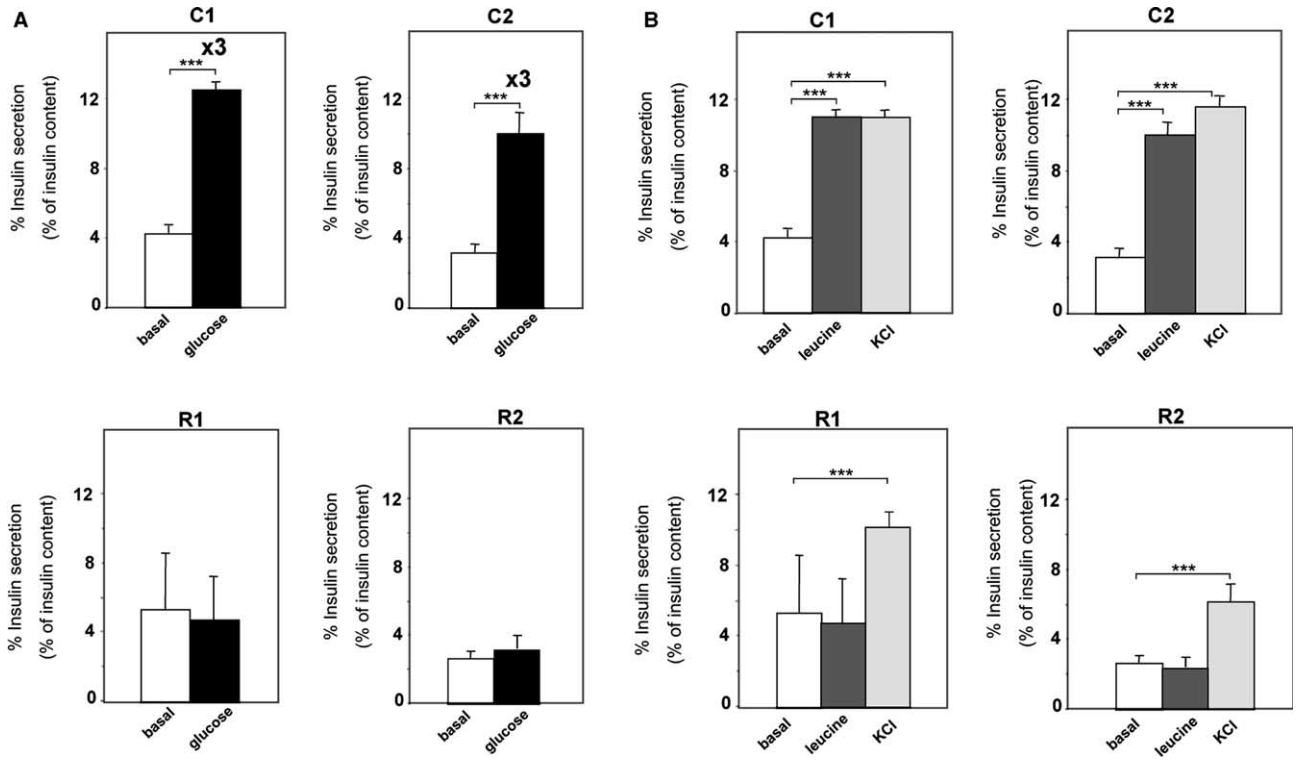

Fig. 3. (A) Glucose-induced insulin secretion of R1/R2 and C1/C2 cells. Cells in (5×10^5) were seeded in 24-well plates, cultured for 48 h and pre-incubated in KRBH buffer under basal condition (2 mM of glucose) for 60 min. Cells were incubated in the same buffer for 60 min in 2 mM (basal) or 20 mM of glucose plus 100 μM of IBMX and 10 μM of forskolin. The supernatant was recovered for insulin measurement. Results are expressed as a percentage of insulin content. Control C1 and C2 cells were able to stimulate the secretion by 3-fold, whereas R1 and R2 cells did not stimulate insulin secretion. (B) Insulin secretion in response to non-glucose secretagogue. C1 and C2 cells stimulated insulin secretion by 3-fold in response to leucine and KCl. R1 and R2 cells did not stimulate insulin secretion in response to leucine, whereas the response to KCl was similar to control cells. Results are expressed as means ± S.E.M. of four independent experiments in triplicate (***$P < 0.001$).

3.3. The cellular metabolic rate is not altered by REST

Glucose-stimulated insulin secretion requires glycolysis and mitochondrial oxidation [21]. Leucine acts downstream of glycolysis and depends exclusively on mitochondrial oxidation for its signal generation [21]. The reduction of the tetrazolium salt (MTT) is regarded as an indicator of cell redox activity, which is attributed mainly to mitochondrial enzymes and electron carriers. Therefore, the MTT reduction assay can be used to evaluate the mitochondrial metabolic rate in β-cells under stimulatory conditions such as glucose or leucine [22]. We performed the MTT reduction assay in R1/R2 and control cells over a period of 30 min. R1/R2 and control C1/C2 cells exhibited a similar increase of MTT reduction under glucose and leucine stimulation, indicating that the REST-expressing cells had a normal metabolic rate (Fig. 5).

4. Discussion

The transcriptional repressor REST is not expressed in insulin-secreting cell lines [1,15]. By reintroducing REST in insulin-secreting cell lines, we found that expression of its known target genes such as IB1/JIP-1 and SCG10 was repressed, whereas insulin production was unchanged. Because the major function of pancreatic β-cells is to secrete insulin in response to glucose, we measured the capability of REST-expressing cells to accomplish this task. In REST-expressing cells, basal insulin secretion was unaffected whereas glucose-induced insulin secretion was impaired. Similar results were obtained after transient expression of REST in two different insulin-secreting βTC3 and INS1 cell lines, confirming that the impaired insulin secretion in response to glucose observed in R1/R2 cells expressing REST cannot be attributed to clonal selection. Since leucine is an exclusive metabolic activator of mitochondria, the loss of glucose- and leucine-induced insulin secretion could be caused by a reduction of mitochondrial activity. However, the MTT reduction in response to glucose and leucine was similar in REST-expressing cells and control cells, suggesting a normal cellular metabolic rate from mitochondria oxidation, which may reflect a normal ATP production. In principle, the secretory defect observed in REST-expressing cells could result from a defective coupling between glucose metabolism and insulin exocytosis and/or from a generalized impairment in the process of insulin exocytosis. The former hypothesis appears very unlikely because the secretory response of REST-expressing cells to depolarizing K^+ concentrations, which triggers insulin release independently from mitochondrial metabolism, was unaffected. In this report, we show that the loss of insulin secretion is not associated with any change in the expression of genes known to control insulin production and secretion. Consistent with this observation, these genes were not identified as targets of REST. The impairment of glucose-induced insulin secretion is most probably the result of alterations affecting multiple REST target genes. A battery

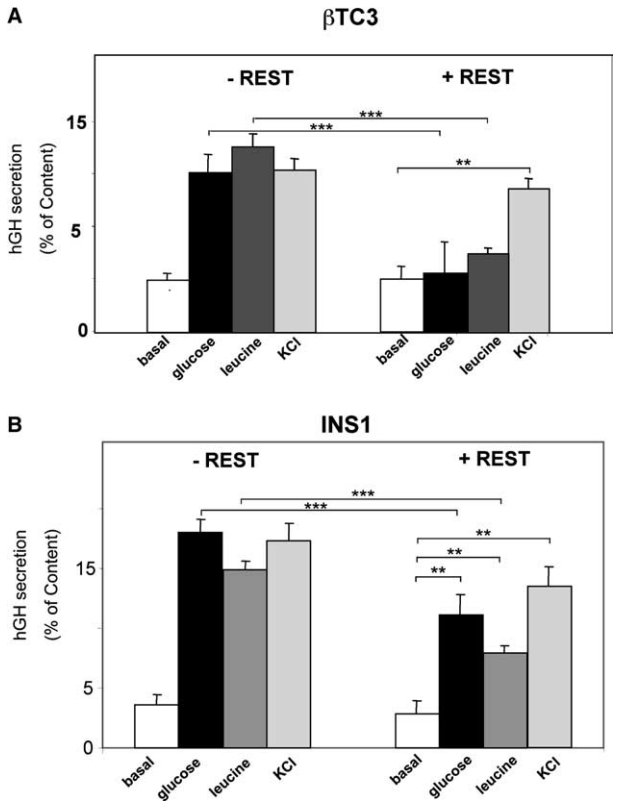

Fig. 4. Effects of transient expression of REST on secretagogue-induced secretion in insulin-secreting βTC3 and INS1 cells. (A) βTC3 and (B) INS1 cells were transiently co-transfected with a plasmid encoding hGH as a reporter gene for secretion and with the REST expression vector (under the control of the rat insulin promoter RIP, +REST) or the empty RIP vector as control (−REST). The figure shows means ± S.E.M. of at least three independent experiments measured in triplicate ($^{**}P < 0.01$, $^{***}P < 0.001$).

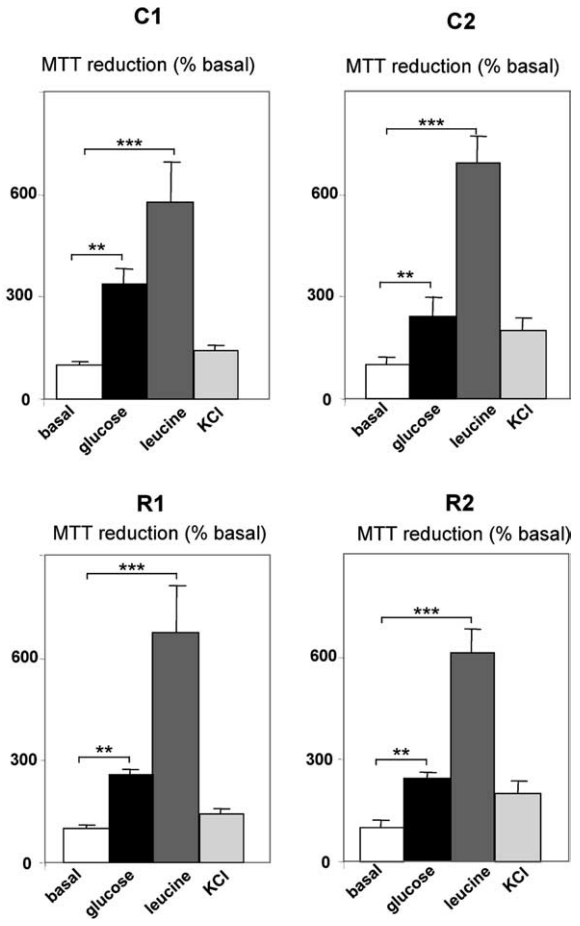

Fig. 5. MTT reduction assays. Control C1/C2 and R1/R2 cells were cultured in a 24-well plate for 2 days and pre-incubated for 60 min in KRBH buffer under basal condition. Cells were then incubated for 60 min under basal condition or stimulatory conditions (glucose, leucine and KCl) in the presence of MTT. Numbers are means ± S.E.M. of four independent experiments in triplicate ($^{**}P < 0.01$, $^{***}P < 0.001$).

of REST target genes has already been shown to be expressed in β-cells [1,15–17]. Computer-assisted searches for NRSE in the whole genomic sequence predict the existence of a large number of additional putative REST target genes that have so far not been experimentally validated [23]. Any of these genes might also be expressed in insulin-secreting cells and be necessary for the secretory phenotype of β-cells.

In the future, detailed analysis of the gene pattern in our β-cell model expressing REST will be very useful for the identification of all these genes. In view of the phenotype observed in β-cells expressing REST, the functional characterization of its target genes will contribute to clarify the mechanisms coupling glucose metabolism to insulin secretion. In this report, we demonstrate that REST target genes are necessary for glucose-induced insulin secretion. As a consequence, a loss of function or a decrease of expression of its target genes could lead to impaired glucose-induced insulin secretion and potentially to diabetes. In support of this, a mutation in the human *MAPK8IP1* gene was already found to be associated with a rare and monogenic form of type 2 diabetes in a French diabetic family [18]. Type 1 diabetes results from an autoimmune attack that may be directed at molecular targets specifically expressed by β-cells and neurons, such as the autoantigen (ICA) 512, a receptor tyrosine phosphatase-like protein [24]. Thus, the identification of additional REST target genes will highlight new candidate genes potentially involved in both forms of diabetes.

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