Glucagon Stimulates Expression of the Inducible cAMP Early Repressor and Suppresses Insulin Gene Expression in Pancreatic β-Cells

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The hormone glucagon is secreted by the α-cells of the endocrine pancreas (islets of Langerhans) during fasting and is essential for the maintenance of blood glucose levels by stimulation of hepatic glucose output. Excessive production and secretion of glucagon by the α-cells of the islets is a common accompaniment to diabetes. The resulting hyperglucagonemia stimulates hepatic glucose production, thereby contributing to hyperglycemia of diabetes. The reduced insulin secretion in diabetes and resultant failure to suppress glucagon secretion by intra-islet paracrine mechanisms is believed to cause the hypersecretion of glucagon. Here, we report the discovery of a new mechanism by which glucagon suppresses insulin secretion. We show that glucagon, but not glucagon-like peptide 1 (GLP-1), or pituitary adenylyl cyclase–activating peptide (PACAP) specifically induces the expression of the transcriptional repressor inducible cAMP early repressor (ICER) in pancreatic β-cells, resulting in a repression of the transcriptional expression of the insulin gene. Remarkably, glucagon, GLP-1, and PACAP all stimulate the formation of cAMP to a comparable extent in rat pancreatic islets, but only glucagon activates the expression of ICER and represses insulin gene transcription in β-cells. These findings lead us to propose that hyperglucagonemia may additionally aggravate the diabetic phenotype via a suppression of insulin gene expression mediated by the transcriptional repressor ICER. *Diabetes* 49:1681–1690, 2000

The β-cells of the pancreases of individuals with type 2 diabetes fail to produce insulin in the amounts required to maintain glucose levels in the physiological range. This circumstance is due to impairments at various levels in the pathways of the metabolism of glucose by pancreatic β-cells and in the actions of insulin on peripheral tissues such as skeletal muscle, liver, and adipose tissue (insulin resistance). The β-cells of individuals with type 2 diabetes fail to sense glucose as a signal for insulin secretion and insulin gene transcription. At the physiological level, glucagon produced by the α-cells of the pancreatic islets acts as an antagonist of insulin actions inasmuch as glucagon stimulates hepatic glucose production as well as lipolysis and glycogenolysis by fat and skeletal muscle, respectively. Although glucagon is essential for the maintenance of plasma glucose levels during periods of fasting, elevated plasma glucagon levels in the presence of hyperglycemia are counterproductive. Plasma glucagon concentrations are elevated in type 2 diabetic individuals. The secretion of glucagon is controlled by multiple factors, including circulating intermediary metabolites, neurotransmitters, and hormones. Glucagon secretion is normally suppressed by hyperglycemia. However, this normal homeostatic suppression is lost in individuals with diabetes, inasmuch as hyperglycemia is accompanied by hyperglucagonemia, which in turn perpetuates hyperglycemia by stimulating hepatic glucose output (1,2).

Pancreatic β-cells express glucagon receptors (3) as well as receptors for the hormones glucagon-like peptide 1 (GLP-1) (4) and pituitary adenylyl cyclase–activating peptide (PACAP) (5). Upon binding to their receptors, glucagon, GLP-1, and PACAP stimulate the formation of cAMP, resulting in an activation of protein kinase A (PKA) that phosphorylates the cAMP response element binding protein (CREB), thereby inducing the transcriptional activity of CREB. CREB is known to stimulate insulin gene transcription by binding to a cAMP response element (CRE) in the promoter of the insulin gene (6,7). Activation of CREB also leads to a stimulation of the expression of a repressor of cAMP-mediated gene transcription, the inducible cAMP early repressor (ICER). ICER is formed by the cAMP-mediated activation of an internal promoter (P2) within the gene-encoding isoforms of the CRE modulator (CREM) (8). As a consequence of the internal transcription driven by P2, ICER is synthesized and consists of the DNA binding domain (bZIP) of CREM devoid of the transactivation domain and thereby acts as a repressor of cAMP-activated gene transcription. The ICER repressor binds CREs in the promoters of

numerous genes and inhibits transcriptional activation by CREB (9). Recently, ICER has been reported to reduce transcription of the human insulin gene (10).

In this article, we show that glucagon induces the expression of ICER, which interacts with the CRE in the insulin promoter and suppresses insulin gene transcription in pancreatic β-cells. The induction of ICER is not seen with the hormones GLP-1 and PACAP that have receptors on β-cells and that also activate the formation of cAMP levels in islet cells comparable to that of glucagon. These observations suggest that glucagon specifically induces the expression of ICER in β-cells and that chronic hyperglucagonemia and consequent induction of ICER may represent an additional mechanism by which the production of insulin is impaired in diabetes.

RESEARCH DESIGN AND METHODS

Preparation of rat pancreatic islets. Rat pancreatic islets were isolated from adult male and female Sprague-Dawley rats weighing between 150 and 300 g (11). The islets were incubated overnight in RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 5.5 mmol/l glucose, 1% penicillin and streptomycin (Gibco-BRL), and 10% heat-inactivated fetal calf serum (Gibco-BRL). The islets were then treated with one of the following vehicles: phosphate-buffered saline (PBS); 0.5 mmol/l isobutylmethylxanthene (IBMX) (Sigma, St. Louis, MO), an inhibitor of phosphodiesterase; 10 μmol/l forskolin (Sigma), an activator of adenyl cyclase (12), plus 0.5 mmol/l IBMX; or 10 nmol/l glucagon, 10 nmol/l GLP-1, or 10 nmol/l PACAP (Peninsula Laboratories, Belmont, CA) plus 0.5 mmol/l IBMX. To determine whether the effects of GLP-1 on the induction of ICER are glucose dependent (13), islets were also cultured at 11.1 mmol/l glucose.

Reverse transcription–polymerase chain reaction. RNA was extracted from rat islets (after treatment with IBMX and/or forskolin, glucagon, GLP-1, or PACAP) with Trizol reagent (Gibco-BRL). Reverse transcription (RT) was performed with oligo (dT) 18 and RNase H–reverse transcriptase according to instructions of the manufacturer (Superscript II; Life Technologies, Grand Island, NY). Negative controls for the RT procedure and control for DNA contamination were performed by omitting the reverse transcriptase in parallel samples. Primers for ICER were forward (fw), 5′-CAAAAGCCCAACATG GCTG-3′, and reverse (rv), 5′-CCAATTCACACTCTACAGCAG-3′. The forward primer is specific for the ICER transcript. The reverse primer is specific for 3′ transcripts of the CREM gene. Primers for ICER RT–polymerase chain reaction (PCR) were chosen to amplify DNA binding domain (DBD)-I isoforms of ICER. PCR conditions were 25–30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and, finally, 72°C for 5 min; viability of the RT product was controlled by a separate PCR with primers specific for the housekeeping mRNA adenine-phosphoribosyl transferase (APRT) (fw: 5′-TCCGAATCTG AGTTGCAGC-3′; rv: 5′-CTGCACACATGGTTCCTC-3′). Rat insulin-1 primers were as follows: fw: 5′-TCAGCAAGCAGGTCATTGTTCC-3′; rv: 5′-GGTGCAGCCTGATCCACAATG-3′. PCR conditions were similar to those mentioned above for 35 cycles. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Southern blot analysis. The ICER PCR products were transferred to a Magnacharge nylon membrane (Micron Separations, Westboro, MA). The membrane was then hybridized with a ^{32}P-labeled oligonucleotide (5′-ATCA GTTTCATCTCCAGTTACAGCCATGTT-3′) complementary to the sequence of the ICER PCR products. Of the 3′ ends of the ICER forward primer and the Southern blot probe, 10 bp is complementary. Random samples of ICER PCR products were verified by sequencing.

Western immunoblot analysis. Nuclear extracts were prepared from forskolin-stimulated rat islets according to the method of Schreiber et al. (14). Equal amounts of protein were fractionated by electrophoresis on SDS–12% polyacrylamide gels (PAGE) and electrophoretically transferred onto a nitrocellulose filter. Immunoreactive ICER was detected with the ECL Analysis System (Amersham Life Sciences, Buckinghamshire, U.K.) with peroxidase-linked anti-rabbit immunoglobulin as the second antibody. Antiserum α-CREM S4 (15) was used for immunodetection of ICER at a dilution of 1:500. This antiserum detects two closely migrating ICER proteins, ICER and ICER-γ, and also longer CREM isoforms if present. No longer CREM isoforms were detected by RT-PCR in rat islets (data not shown).

Immunocytochemistry. Rat islets were isolated and dispersed into single cells by incubation with trypsin/EDTA (Gibco-BRL). After washing several times in incubation media, cells were plated in cell culture chambers coated with poly-L-lysine (Sigma) in RPMI (Gibco-BRL) with 10% fetal calf serum and 5.5 mmol/l glucose. Cells were treated with IBMX (0.5 mmol/l) alone or IBMX

and forskolin (10 μmol/l). After fixation in 4% paraformaldehyde in PBS for 10 min at room temperature and several rinses in PBS, samples were permeabilized with 100% methanol for 10 min at –20°C and then blocked with 3% normal donkey serum for 10 min at room temperature. The samples were then incubated at 4°C overnight with a nonimmune serum or a guinea pig antiserum raised against insulin (Linco Research, St. Louis, MO) or the αCREM S4 antiserum (16,17) at dilutions of 1:1,000 and 1:500, respectively. After rinsing with PBS, slides were again blocked with 3% normal donkey serum for 10 min at room temperature before being localized with donkey anti–guinea pig indo-carbocyanine (1:500) (Jackson Immunoresearch Laboratories, West Grove, CA) and goat anti-rabbit dichloroazinylamino fluorescein–conjugated secondary antibodies (1:200) for 30 min at room temperature. Slides were rinsed with PBS, mounted in fluorescent mounting medium (Kierkegaard & Perry Laboratories, Gaithersburg, MD), and stored in the dark. Images were obtained using a Zeiss Epi-fluorescence microscope equipped with an Optronics TEC-470 charge-coupled device camera (Optronics Engineering, Goleta, CA) interfaced with a Power Macintosh 7100 installed with IP Lab Spectrum analysis software (Signal Analytics, Vienna, VA).

cAMP formation by isolated rat pancreatic islets. Isolated rat islets were treated with 0.5 mmol/l IBMX and either glucagon, GLP-1, or PACAP (10 nmol/l each) for 0 and 30 min. Thereafter, cells including media were taken up in 100% ice-cold ethanol and frozen at –80°C overnight. After thawing and lyophilization, samples were dissolved in a buffer of a cAMP enzyme-linked immunoassay kit (Assay Designs, Ann Arbor, MI). Measurements were performed according to instruction of the manufacturer. Results are given in Fig. 3C as fold-induction (mean ± SE) of cAMP between 0 and 30 min of treatment.

Plasmids and cell transfection. Expression plasmids were all driven with the cytomegalovirus (CMV) promoter in the plasmid pCDNA3.1 vector (Invitrogen, La Jolla, CA). The insulin-1 gene 5′ flanking sequences from bp –410 to +1 inserted adjacent to a firefly luciferase reporter have been described (18). A deletion of the four central nucleotides in the CRE of the insulin promoter (Fig. 4A) was introduced by standard methods (19).

INS-1 immortalized pancreatic β-cells (provided by C. Wolheim, University of Geneva, Switzerland, passages 99–104) were grown at 37°C in humidified 5% CO_2–95% O_2 in RPMI (Life Technologies, Grand Island, NY) at 5.5 mmol/l glucose per liter supplemented with 10% heat-inactivated fetal calf serum, 1% HEPES buffer, pH 7.6, 1% sodium-pyruvate (Gibco-BRL), 5 μl/l β-mercaptoethanol (Sigma), and 100 U penicillin and 100 μg streptomycin per milliliter. Transfections were performed with Lipofectamine 2000 reagent (Gibco-BRL) according to the instructions of the manufacturer. Transfections for luciferase assays were performed in 12-well plates. There was 1 μg of the promoter/reporter construct transfected per well. A Rous sarcoma virus (RSV)-β-galactosidase vector was cotransfected for normalization of luciferase assays for transfection efficiency. Luciferase activity obtained after transfection was normalized to the luciferase activity obtained after transfection of the promoterless luciferase plasmid in the same experiment.

Dispersed primary rat islets were cultured in 12-well dishes in RPMI 1640 (Gibco-BRL) at 5.5 mmol glucose per liter supplemented with 10% heat-inactivated fetal calf serum and 100 U penicillin and 100 μg streptomycin per milliliter at 37°C in humidified 5% CO_2–95% O_2. Transfections were performed with GeneFector (Venn-Nova, Pompano Beach, FL) according to the instructions of the manufacturer using 5 μl of reagent per transfection reaction. There was 5 μg of the promoter/reporter construct transfected per well. An RSV-β-galactosidase vector (1 μg) was cotransfected for normalization of luciferase assays for transfection efficiency. Transfection was performed in culture medium lacking serum and antibiotics/antimycotics for 4 h. Thereafter, an equal amount of medium with twice the concentration of serum and antimicrobial agents was added to each culture well. At this time, cells were treated with GLP-1, PACAP, or glucagon at a concentration of 10 nmol/l each. Cells were harvested after 12 h for measurements of reporter activities. Luciferase activity obtained after transfection was normalized to the background luciferase activity obtained after transfection of the promoterless luciferase plasmid in the same experiment.

ICER antisense RNA experiments. INS-1 cells were transfected with full-length ICER cDNA inserted in the antisense (AS) orientation into plasmid pcDNA 3.1 (pcDNA ICER-AS) together with the –410-bp insulin promoter/luciferase reporter plasmid using Lipofectamine 2000. Transfection efficiency, as assessed by transfecting a CMV-EGFP (enhanced green fluorescent protein; Clontech, La Jolla, CA) vector and visualizing under a microscope equipped with an appropriate fluorescent light source, was 80% (data not shown). At 48 h after cotransfection of pcDNA ICER-AS, cells were harvested and extracted, ICER and CREB were assessed by Western immunoblot, and insulin promoter activity was measured by luciferase assays.

Electrophoretic mobility shift assays. Synthetic oligonucleotides corresponding to the CRE within the rat insulin-1 gene promoter were annealed, end-labeled with ^{32}P with T4 polynucleotide kinase, and purified over a Sephadex
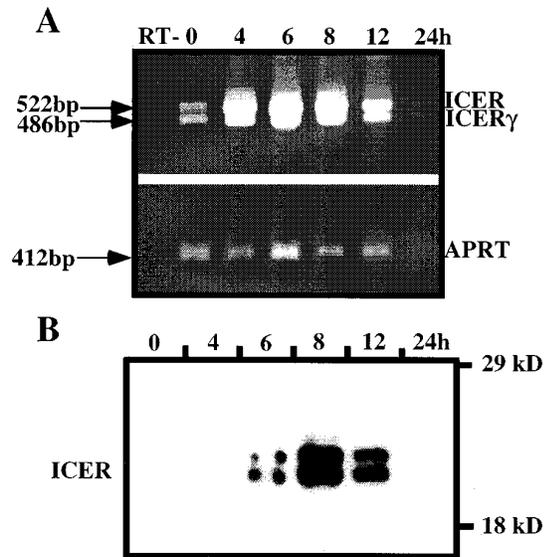
A

B

FIG. 1. Induction of ICER mRNAs in primary rat islets by cAMP production. *A*: Cultured rat pancreatic islets were incubated in 10 μmol/l forskolin and 0.5 mmol/l IBMX for 24 h. At the times indicated, RT-PCR was performed on aliquots of islets to assess amounts of ICER mRNA. Primers for ICER RT-PCR were chosen to amplify DBD-I isoforms of ICER. RT-PCR of APRT mRNA was performed as a control to assess the integrity and input of cDNA. *B*: Induction of ICER protein in cultured rat islets by cAMP. Rat islets were incubated in 10 μmol/l forskolin and 0.5 mmol/l IBMX for different times as indicated in the figure. Nuclear extracts were prepared and extracted for Western immunoblot to detect ICER. Induction of cAMP formation by forskolin and IBMX results in elevated ICER protein levels in the nuclei of the rat islets. The effects of forskolin and IBMX on ICER levels reaches a maximum at 8 h of induction, after which ICER protein levels diminish.

G-50 spin column (Boehringer Mannheim, Nutley, NJ). Electrophoretic mobility shift assays (EMSAs) were performed by incubating ~5 × 10⁴ cpm of end-labeled DNA probe (0.02–0.03 ng DNA) with glutathione S-transferase (GST) fusion proteins in a binding buffer (10 mmol/l Tris-HCl [pH 8.0], 40 mmol/l KCl, 6% glycerol, 1 mmol/l dithiothreitol, and 0.05% Nonidet P-40) for 20–30 min at room temperature. The reaction mixture was analyzed by electrophoresis on a 6% nondenaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film for 6–72 h. The recombinant GST-CREB and GST-ICER plasmids have been described previously (17).

Statistical analysis. Student's t test was performed with StatView software. A P value of <0.05 was considered statistically significant.

RESULTS

Induction of ICER mRNA and protein by forskolin and IBMX in rat islets. After culture overnight, isolated rat islets were incubated with IBMX and forskolin and harvested for analyses of amounts of ICER mRNA by RT-PCR at different time points. ICER mRNA is detected at basal conditions and is induced with IBMX and forskolin with a maximum induction at 4–8 h, after which ICER mRNA levels decrease (Fig. 1*A*). These findings of a biphasic expression of ICER mRNA in response to elevations in cAMP induced by IBMX plus forskolin are in accordance with results observed earlier in studies of rat testes and hypothalamus (15,20–22). Changes in insulin mRNA levels by RT-PCR were not detectable in any of the experiments, likely owing to the long half-life of pro-insulin mRNA of >24 h (23) (data not shown).

To determine whether the induction of ICER mRNA also results in a change in ICER protein levels in pancreatic islets, rat islets were incubated with forskolin plus IBMX for various times. Protein extracts were prepared from the islets and were examined by Western immunoblots. The results show that ICER protein is induced with a time course similar to the induction of ICER mRNA. Furthermore, the induction of ICER protein in response to elevated cAMP induced by IBMX plus forskolin reaches a maximum concentration at 8 h; thereafter, levels of ICER protein diminish (Fig. 1*B*).

To assess whether ICER is induced in the pancreatic β-cells of the rat islets that produce insulin, dispersed rat pancreatic islet cells were prepared, treated with ICER, and subjected to in situ double immunostaining for insulin and ICER. The forskolin treatment of dispersed islet cells results in a marked induction of ICER in the nuclei of β-cells (Fig. 2). The antiserum used to detect ICER would also detect longer CREM isoforms, if present. However, we have not been able to detect any longer CREM isoforms in rat islets by either RT-PCR (data not shown) or Western immunoblot (Fig. 1). Double immunostaining with anti-insulin and anti-ICER sera shows that the majority of cells that express ICER immunoreactivity in their nuclei after treatment with forskolin are insulin-producing
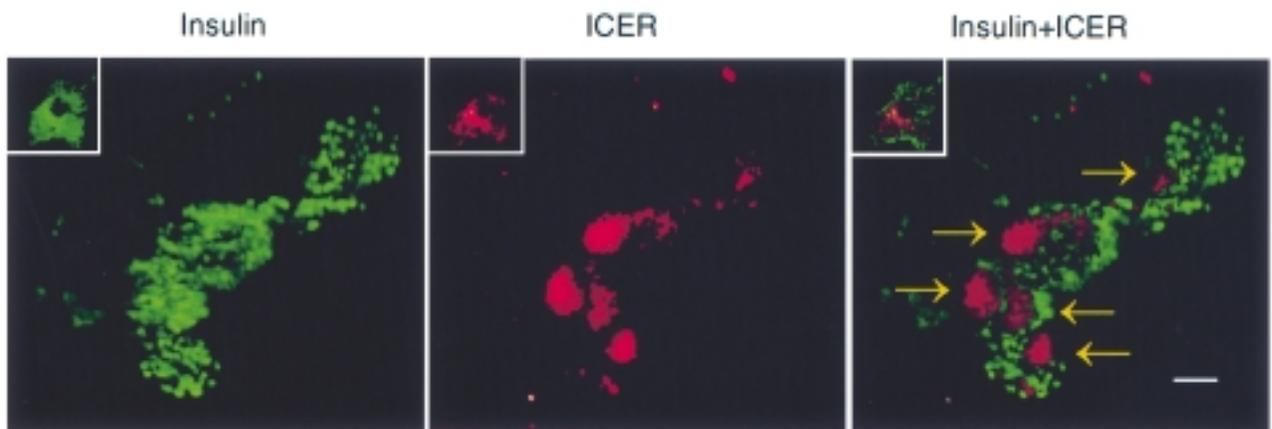

FIG. 2. Immunocytochemistry for ICER and insulin in dispersed rat islet cells. Rat islets cells were dispersed and plated on glass coverslips. After treatment with forskolin/IBMX for 8 h, cells were fixed in situ and immunostained with anti-insulin and anti-ICER sera. Most insulin-producing cells show induction of ICER protein in their nuclei (arrows). The inset in the top left corner shows the control experiment without forskolin/IBMX stimulation. The bar in the right panel represents 10 μm.

β-cells. However, a small number of cells that do not stain for insulin do stain for ICER (data not shown), suggesting that the expression of ICER in pancreatic islets is not restricted to pancreatic β-cells (Fig. 2).

Stimulation of the induction of ICER mRNA in rat islets by glucagon but not by GLP-1 or PACAP. Having demonstrated that ICER is expressed in rat islet β-cells in response to forskolin, we sought to determine the physiologic relevance of this finding. At least three hormones that increase cellular levels of cAMP have $G_{s\alpha}$-coupled receptors on β-cells, glucagon, GLP-1, and PACAP and are known to be important physiological regulators of β-cell functions (5,13). Therefore, aliquots of rat islets were individually incubated with IBMX and with these hormones at concentrations of 10 nmol/l. Remarkably, only glucagon, and neither GLP-1 nor PACAP, induced ICER mRNA (Fig. 3*A*). This effect of glucagon to induce ICER expression was also seen at ambient glucose levels of 5.5 mmol/l (data not shown) as well as 11.1 mmol/l. This latter glucose concentration was chosen to unmask possible actions of GLP-1 at higher glucose levels (glucose-dependent effects of GLP-1) (13). A time course experiment showed induction of ICER mRNA as early as 6 h after incubation of rat islets with glucagon, and increased ICER mRNA was detected by RT-PCR until 12 h (data not shown). This unexpected finding of the induction of ICER mRNA by glucagon but not GLP-1 or PACAP prompted an examination of cAMP formation in rat islets in response to these hormones. Surprisingly, glucagon, GLP-1, and PACAP all activated the formation of cAMP equivalently by 30- to 40-fold (Fig. 3*C*). Thus, glucagon, GLP-1, and PACAP all elevate cAMP levels, and GLP-1 and PACAP stimulate insulin gene transcription in rat islets, as has been shown before (5), but glucagon is unique among these three hormones in the activation of ICER expression.

Induction of ICER inhibits insulin promoter activity in INS-1 cells. Because ICER is a known repressor of cAMP-mediated gene transcription (8) and the rat insulin-1 gene contains a CRE in its promoter (6,7), we carried out experiments to determine whether ICER represses the cAMP-activated transcription of the rat insulin gene. The actions of ICER on the transcriptional expression of the insulin gene were examined in transient cotransfection experiments of INS-1 cells with a recombinant insulin promoter/reporter and a PKA expression plasmid vector (Fig. 4). Cotransfection of the expression vector encoding the catalytic subunit of PKA (PKAc) with the –410-bp insulin promoter/luciferase vector reporter shows that PKA stimulates transcription from the –410-bp insulin promoter (Fig. 4*B*). Deletion of the central four nucleotides (ACGT) of the insulin promoter CRE results in a reduction in basal luciferase activity by ~40%, as shown previously (24) (data not shown). Cotransfection of the PKAc expression vector with the CRE-mutated promoter/reporter vector did not activate the –410-bp insulin promoter/reporter gene. Cotransfection of CMV-CREB and PKAc in combination further increases insulin gene transcription (Fig. 4*C*).

It is important to note the dose-dependency of the PKAc expression vector on stimulation of the –410-bp rat insulin promoter/reporter (Fig. 4*B*). For the experiments in Fig. 4*C*, PKAc was transfected at a dose that only led to a significant activation of the insulin reporter when an expression vector for CREB was cotransfected. Coexpression of ICER with the –410-bp insulin-1 promoter/luciferase reporter inhibited the

enhancement of insulin gene expression mediated by CREB plus PKAc (Fig. 4*C*). Moreover, overexpression of ICER in pancreatic β-cells leads to a decrease in the basal insulin promoter activity reported by the –410-bp insulin/luciferase plasmid vector (Fig. 4*D*).

RT-PCR analysis of several pancreatic insulin-producing β-cell lines (INS-1, RIN, and βTC-3) revealed a constitutive expression of ICER that could not be further stimulated by forskolin/IBMX (data not shown). This finding may explain why such a large dose of ICER expression plasmid was needed to reduce activity of the –410-bp insulin/luciferase plasmid reporter in INS-1 cells (Fig. 4*D*).

ICER AS-RNA expression inhibits ICER production and increases insulin promoter activity. We next sought to investigate the effects of reducing endogenous ICER protein levels in INS-1 cells on insulin/luciferase plasmid reporter activity. We used an AS-RNA approach to knock down ICER production in INS-1 cells. Full-length ICER AS-RNA expression by transfection in INS-1 cells reduced ICER but not CREB to undetectable levels, as assessed by Western immunoblot (Fig. 5). This result was accompanied by a statistically significant increase in the activity of the –410-bp insulin promoter/luciferase reporter (Fig. 5*A*). The same experiment conducted with a reporter plasmid with a mutated CRE within the insulin promoter showed no effect of reduced ICER protein. A cotransfected RSV-β-galactosidase vector was used to normalize the luciferase levels but also to control for nonspecific effects of the ICER-AS plasmid on generalized gene expression. Neither the level of CREB (Fig. 5) nor the β-galactosidase activity (data not shown) was affected by the ICER-AS plasmid, indicating a specific effect of the ICER-AS approach.

Glucagon inhibits insulin promoter activity in rat islet cells. To determine whether glucagon, which induces ICER production in rat islets, also leads to a downregulation of the insulin transcription rate (as found in the transient transfection experiments in INS-1 cells), dispersed primary rat islet cells were transfected with the –410 rat insulin promoter/reporter plasmid and treated with one of the following solutions for 12 h: vehicle alone, GLP-1, PACAP, or glucagon (all at 10 nmol/l concentrations). Using the rat insulin promoter ensured that the effects being measured would be specific to β-cells in the dispersed islet cell culture. Whereas both GLP-1 ($P < 0.05$) and PACAP (NS) increased insulin transcription rates as reflected by increased relative luciferase activity, glucagon treatment led to a reduced insulin transcription rate ($P < 0.05$) (Fig. 6).

ICER and CREB bind to the insulin promoter CRE. To determine whether ICER interacts with the insulin CRE directly, EMSAs were performed on the labeled rat insulin CRE and GST-ICER and GST-CREB. The results of the EMSAs show that both CREB and ICER bind to the insulin promoter CRE (Fig. 7).

DISCUSSION

Here we demonstrate the induction of ICER by glucagon and forskolin in pancreatic islets (β-cells). Further, we show that ICER—by binding to the CRE—reduces the activation of the insulin gene promoter in the clonal pancreatic β-cell line INS-1 (10), and reduction of ICER expression in INS-1 cells increases insulin promoter/reporter activity. Using a rat insulin-I luciferase reporter, we demonstrate that glucagon,

A

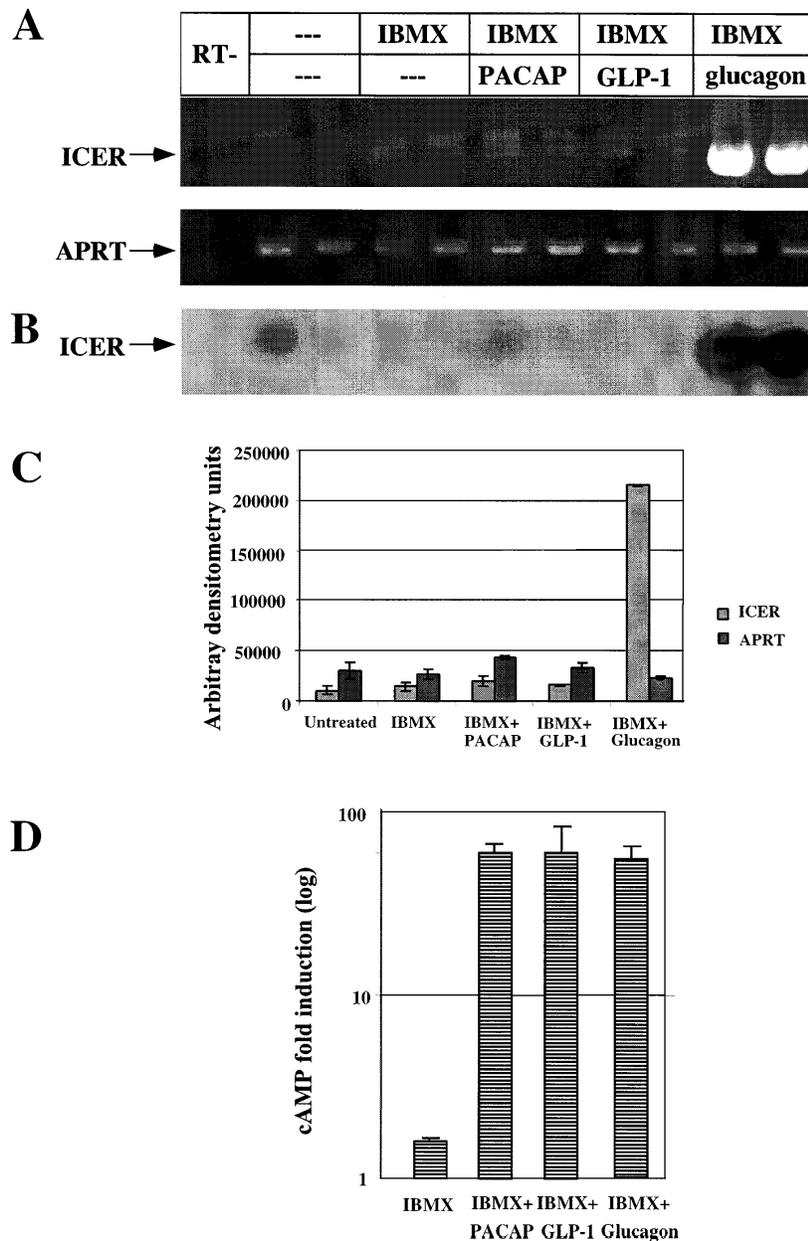| RT- | --- | IBMX | IBMX | IBMX | IBMX |
| | --- | --- | PACAP | GLP-1 | glucagon |

ICER →

APRT →

B ICER →

C

D

FIG. 3. Glucagon, but not PACAP or GLP-1, induces ICER mRNA in cultured rat islets. *A*: Islets were treated with 0.5 mmol/l IBMX and glucagon, GLP-1, or PACAP (10 nmol/l each) for 8 h. ICER mRNA was assayed by RT-PCR, and PCR products were displayed on an agarose gel (see RESEARCH DESIGN AND METHODS). Glucagon, but not GLP-1 or PACAP, induces ICER in rat islets cultured in 11.1 mmol/l glucose. RT-PCR of rat APRT mRNA was performed to assess the integrity and input of cDNA. *B*: Southern blot of ICER RT-PCR products shown in *A*. After transfer to a membrane, hybridization with a ^{32}P-labeled oligonucleotide specific for the ICER PCR product sequence and autoradiography were performed. *C*: Graphical depiction of densitometric analysis of RT-PCR bands shown in *A*. *D*: cAMP production in rat islets. Rat islets were exposed to 0.5 mmol/l IBMX and glucagon, GLP-1, or PACAP (10 nmol/l each) for 0 and 30 min. Total cAMP was measured thereafter in extracts of cells and culture media. Glucagon, GLP-1, and PACAP stimulate cAMP formation to a comparable degree.

but not GLP-1 or PACAP, reduces insulin gene transcription in dispersed primary rat β-cells.

Hyperglucagonemia, which characteristically occurs in diabetes, is considered to constitute a major factor contributing to the metabolic disturbances in both types 1 and 2 diabetes (1,2). The elevated plasma levels of glucagon increase hepatic glucose output resulting in further elevated fasting glucose levels and a resistance to the actions of insulin (2). Diabetes

manifests by a deficiency of the pancreatic β-cells in producing insulin in the amounts required to meet the body's needs. The reduced insulin production characteristic of diabetes is believed to be due to impaired expression of the insulin gene. Such impairment of insulin gene expression can come about by one or more of several mechanisms. Mutations in transcription factors involved in the transcription of the insulin gene, e.g., insulin promoter factor (IPF)-1, hepatic nuclear
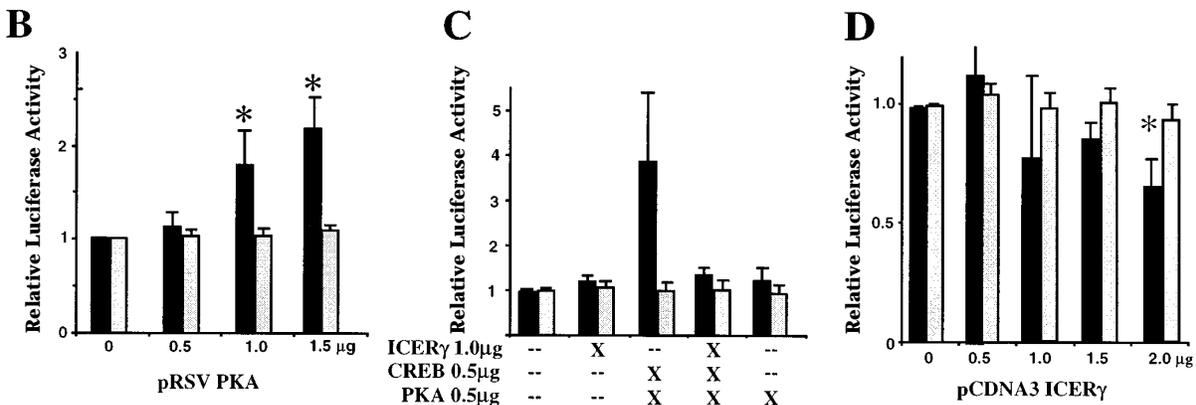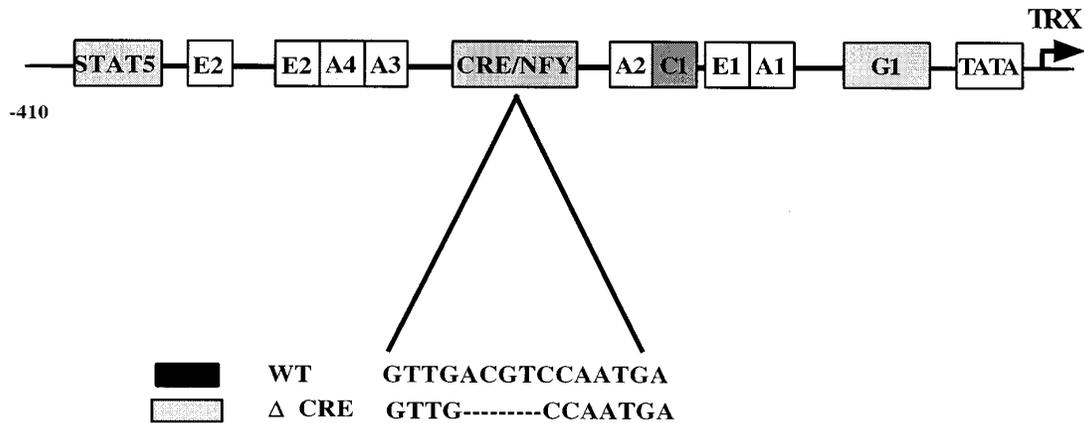
FIG. 4. Transient cotransfection experiments in INS-1 cells. All transfections were done with equal amounts of transfected DNA by addition of appropriate empty vector plasmid DNA when required. The mean ± SE of three independent experiments are given. A: Schematic of the wild-type (WT) –410-bp rat insulin promoter/luciferase reporter and the reporter with the mutated CRE site. Deletion of the central four nucleotides (ACGT) of the insulin promoter CRE results in a reduction in basal luciferase activity by ~40%, as previously shown (24) (data not shown). B: Cotransfection of the catalytic subunit of PKA induces –410-bp rat insulin-1 insulin promoter/luciferase expression. This effect is attenuated when the CRE is mutated. C: ICER inhibits stimulation of the –410-bp rat insulin-1 promoter/luciferase plasmid by CREB and PKA (to enhance phosphorylation of CREB). D: Cotransfection of ICER suppresses basal expression of the –410-bp rat insulin-1 reporter/luciferase vector. The effect of ICER is abolished in the CRE mutated promoter vector. In B–D, the intact CRE (□) and the mutated CRE designated in A (■) are indicated. Statistical analysis was performed with a Student's t test. $*P < 0.05$. TRX, start of gene transcription.

factor (HNF)-1α, HNF-1β, HNF-4α, and β2/NeuroD, result in impaired insulin production in early-onset forms of diabetes known as maturity-onset diabetes of the young (MODY) (25). Prolonged hyperglycemia exerts toxic effects on β-cells (so-called glucose toxicity) and has been shown to reduce the expression of the transcription factors RIPE3b1 and IPF-1 (26,27), which are required for proper regulation of insulin gene transcription and inducement of the expression of the transcription factor CCAAT/enhancer binding protein β, an inhibitor of insulin gene transcription (18,28).

The results of our study support the idea that the induction of the cAMP-inducible transcriptional repressor ICER may be an additional mechanism by which insulin gene transcription is impaired in the context of hyperglucagonemia, as occurs in diabetes. Glucagon appeared to be relatively specific in the induction of ICER in β-cells. The hormones GLP-1 and PACAP also stimulated cAMP formation in pancreatic β-cells (29,30) but failed to stimulate ICER gene expression. Our

findings suggest that glucagon activates signaling pathways in pancreatic β-cells that are distinct from the cAMP signaling pathway because glucagon, GLP-1, and PACAP all increase β-cell levels of cAMP to comparable levels. Yet only glucagon, and not GLP-1 or PACAP, induces the expression of the ICER repressor, which downregulates insulin promoter activity. Alternatively, therefore, GLP-1 and PACAP may activate additional signal pathways that inhibit cAMP-mediated induction of ICER expression. Thus, it appears that the intracellular effects of glucagon, GLP-1, and PACAP differ with regard to the formation of ICER and consequent inhibition of insulin gene transcription.

Several caveats regarding our findings are worth noting. Glucagon (31,32), GLP-1 (33–36), and PACAP (5) are reported to activate signal transduction pathways apart from the cAMP pathway. Furthermore, a compartmentalization of the cAMP signaling pathway within cells (37) may account for diverging intracellular effects of different hormone recep-

FIG. 6. Transient cotransfection experiments in dispersed rat islets. All transfections were done with equal amounts of DNA by addition of the appropriate empty vector. Means ± SE of three independent experiments are given. GLP-1 and PACAP increase, whereas glucagon decreases the transcription rate of the –410 rat insulin-1 reporter/luciferase vector. Statistical analysis was performed with a Student's *t* test. *$P < 0.05$.
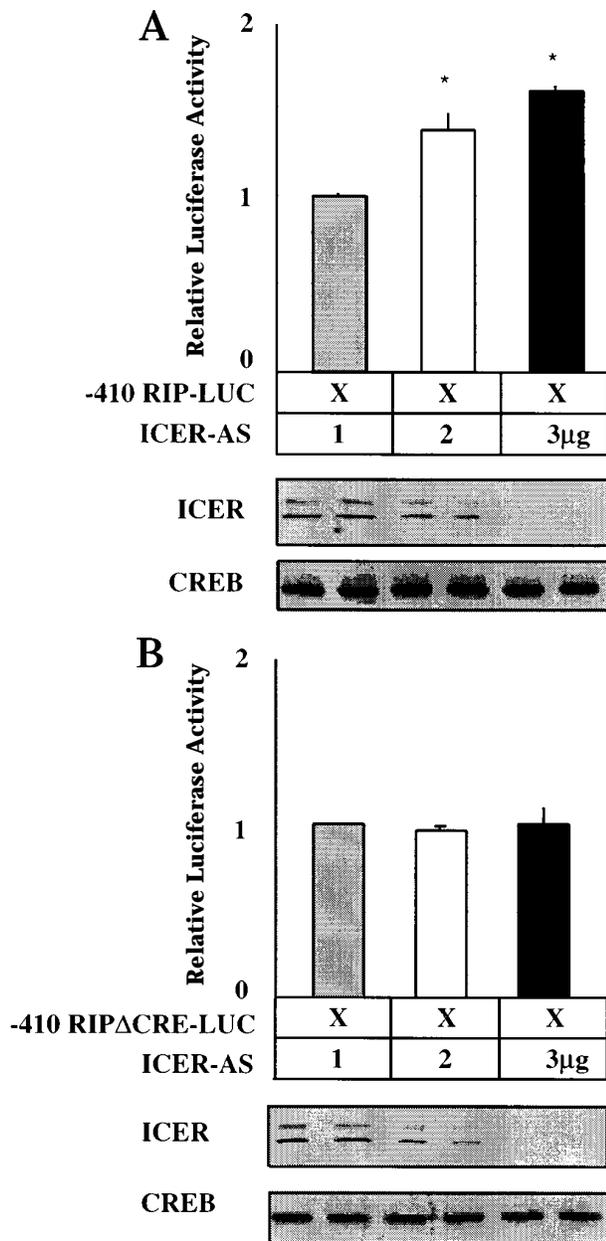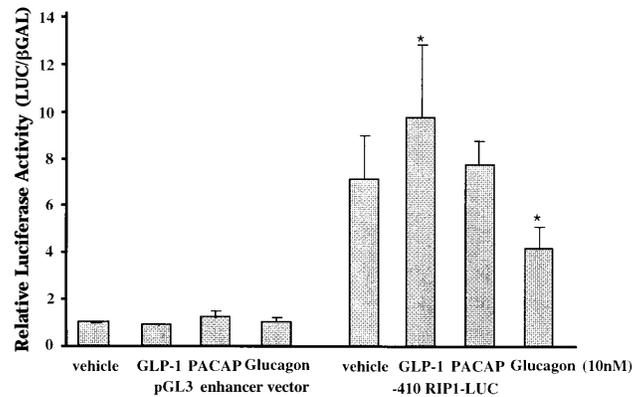
FIG. 5. Antisense inhibition of ICER production in INS-1 cells results in increased insulin gene expression. Transient cotransfection experiments in INS-1 cells are shown. All transfections were done with equal amounts of transfected DNA by addition of appropriate empty vector plasmid DNA when required. The means ± SE of three independent experiments are given. Transfection of a full-length AS ICER expression vector (ICER-AS) reduces ICER but not CREB protein levels in INS-1 cell nuclear extracts. *A*: Expression of a cotransfected wild-type –410 insulin promoter/reporter increases with diminishing ICER levels. *B*: Expression of a cotransfected –410 insulin promoter/reporter with a mutated CRE (see Fig. 4*A*) remains unchanged with diminishing ICER levels. Statistical analysis was performed with a Student's *t* test. *$P < 0.05$.

tors, even in a situation in which cAMP may be generated by each receptor upon binding of its cognate ligand. Thus, it is unclear which intracellular pathway is involved in the apparent selective effect of glucagon on ICER expression in pancreatic β-cells. To this end, further studies will be necessary

to elucidate the differences in intracellular signaling in pancreatic β-cells.

To assess the functional significance of ICER on insulin gene expression, we conducted transient experiments in INS-1 cells (Fig. 3). It is important to note here that the pathway we used to stimulate the insulin reporter gene with PKA may not necessarily reflect the physiological signal transduction pathway of glucagon in pancreatic β-cells. Rather, we have sought to use a system that, in vitro, reflects enhanced insulin gene transcription through activation by phosphorylated CREB to then study the effect of superimposed overexpression of ICER. Because *1*) glucagon treatment increases ICER expression in pancreatic β-cells and *2*) ICER downregulates insulin promoter activity in INS-1 cells, we next determined whether glucagon treatment reduces insulin transcription in primary rat β-cells and knockdown of ICER increases insulin promoter/reporter activity in INS-1 cells. Indeed, knockdown of ICER by way of the AS approach led to increased insulin promoter/reporter activity in INS-1 cells (Fig. 5). Further, dispersed rat islet cells treated with glucagon have a reduced rate of insulin transcription, as assessed by a rat insulin-I promoter/luciferase reporter plasmid (Fig. 6).

CREB and/or CREB-like factors have been proposed to activate the cAMP-activated response elements (CAREs) of the ICER promoter, but a direct demonstration of such activation has not been accomplished. CREB is known to be activated by phosphorylation of serine-133 not only by PKA (38) but by several other protein kinases, e.g., CREB kinase (39), calcium-calmodulin kinase (40,41), mitogen-activated protein kinase (42–44), and Akt/PKB kinase (45). Furthermore, GLP-1 and PACAP are hormones that are known to augment insulin secretion from β-cells during periods of nutrient intake (feeding) (5,13), whereas glucagon secretion is suppressed during feeding and acts during periods of nutrient deprivation to mobilize glucose from stores in liver, muscle, and fat essential to maintain blood glucose levels (1,2). Thus, in a physiological sense, glucagon should suppress the secretion and production of insulin during fasting to
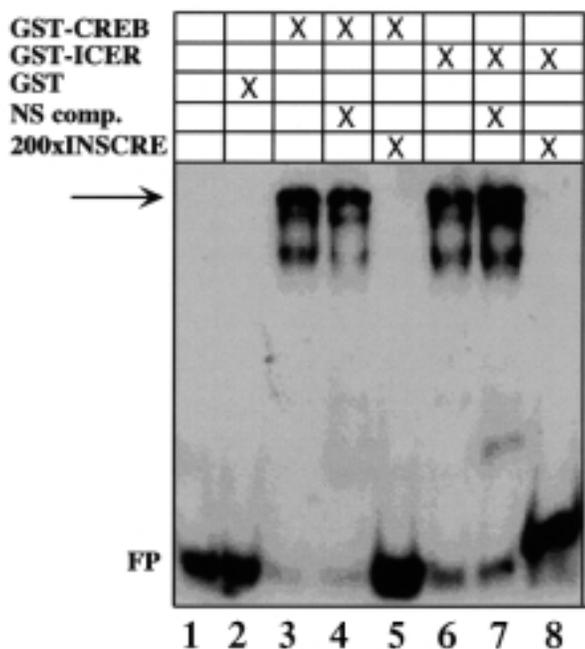
	1	2	3	4	5	6	7	8
GST-CREB			X	X	X			
GST-ICER						X	X	X
GST		X						
NS comp.				X			X	
200xINSCRE					X			X

FIG. 7. EMSA with recombinant GST-CREB and GST-ICER interaction with the rat insulin-I promoter CRE. GST fusion proteins were incubated with a ^{32}P-labeled insulin CRE DNA probe and analyzed by electrophoresis on a 6% PAGE gel. Both GST-CREB and GST-ICER interact with the insulin CRE. The interaction is competed by unlabeled insulin CRE but not by a nonspecific competitor (NS comp.). Lane 1, no protein; lane 2, GST alone; lane 3, GST-CREB alone; lane 4, GST-CREB and NS comp.; lane 5, GST-CREB and 200-fold excess cold INSCRE probe competitor; lane 6, GST-ICER alone; lane 7, GST-ICER and NS-comp.; lane 8, GST-ICER and 200-fold excess cold INSCRE probe. FP, free probe. INSCRE, oligonucleotide consisting of the CRE and flanking base pairs of the rat insulin-1 gene promoter (see RESEARCH DESIGN AND METHODS).

prevent hypoglycemia due to unwanted hypoglycemic actions of insulin during fasting.

ICER is expressed by the CREM gene, and its expression is believed to be induced by the binding of phosphorylated CREB and/or CREB-like proteins to a cassette of four CAREs that reside within an internal promoter (P2) within the CREM gene (8,9,46). ICER binds to and represses transcription from CREs of target genes and thus reduces expression of genes that are initially positively transactivated by CREB. ICER feeds back and represses its own induction through interaction with the CAREs within its own promoter. Under conditions of sustained activation of CREB, ICER mRNA and protein cycle through phases of expression and self-repression. The observations of our study are in accord with this concept of self-limited expression of ICER mRNA and protein.

Recently, Inada et al. (10) have demonstrated that CREM isoforms are also present in rat islet cells and that certain CREM isoforms positively transactivate insulin gene transcription, whereas ICER, another product of the CREM gene, inhibits the transactivation. By RT-PCR, we attempted to detect longer CREM isoforms in primary rat islets and failed to detect any product. We were able to detect only ICER consistently as a product of the CREM gene in rat islets. This result is in accord with the Western immunoblot of rat islet protein extracts, which shows only ICER immunoreactivity and no higher molecular weight immunoreactivity, suggesting the presence

of longer CREM isoforms. One reason for the discrepancy between our data and those of Inada et al. is that Inada et al. may have detected CREM isoforms in immortalized rat insulinoma cells (RIN), which may express a wider variety of CREM isoforms not present at significant levels in rat islets.

In the context of diabetes, it seems unlikely that sustained hyperglucagonemia, by virtue of inducing a cyclical expression of ICER, may by itself lead to a reduced expression of the insulin gene. But together with additional factors, such as mutations in transcription factors regulating insulin gene expression or the consequences of glucose toxicity on pancreatic β-cells, the induction of the cAMP-inducible repressor ICER in response to hyperglucagonemia may present an additional mechanism contributing to dysregulated insulin gene expression and synthesis.

The CRE of the insulin gene binds CREB (6,7,47) and confers basal activity but has relatively weak cAMP responsiveness compared with the CREs of the promoters of the glucagon and somatostatin genes (48). In addition, the CRE of the insulin gene is not activated, as in pancreatic α-cells, by membrane depolarization or calcium influx (49–51). Sequence comparison of the CREs of the insulin gene promoter with those of somatostatin and glucagon gene promoters reveals an atypical sequence at the 3′ end of TGACGTCC (the C at the 3′ end is atypical for the conserved CRE sequence TGACGTCA). Recently, Oetjen et al. (7) showed that at its 3′ end, the insulin CRE overlaps with a CAAT motif, which binds the ubiquitous transcription factor NF-Y. NF-Y confers basal activity of the insulin gene promoter, and presence of NF-Y attenuates the cAMP response of the insulin promoter CRE. This attenuation of the cAMP response of the insulin gene promoter is further supported by the observation that when the insulin CRE is mutated into a classic CRE and the NF-Y binding site is abolished, the cAMP response of the insulin gene is markedly enhanced (7). In this regard, the weak effect of CREB on activating the rat insulin-1 gene promoter further allows the effects of ICER as a suppressor to have a discernible effect on insulin expression. An additional possibility for ICER to have an inhibitory effect on CREB-mediated insulin gene transactivation would be by a higher affinity of ICER to the insulin CRE than CREB. However, by using off-rate analyses, we have been unable to demonstrate a difference in binding affinities of CREB and ICER on the insulin CRE (data not shown).

As part of the CREM gene, ICER may have additional effects on pancreatic β-cell biology. The CREM gene has been attributed a role in the regulation of cell proliferation and regeneration (52–54). In the homozygous CREM-null mouse, liver regeneration is impaired, implicating CREM gene products in the regulation of tissue regeneration (52). In drawing analogies to this concept, it is conceivable that the induction of ICER by glucagon or other factors within the β-cell may be part of a genetic program enabling proliferation of pancreatic β-cells (or precursors) in an attempt to augment β-cell mass to compensate for the relative insulin deficiency in type 2 diabetes (55).

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