Glucose represses connexin36 in insulin-secreting cells

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Summary

The gap-junction protein connexin36 (Cx36) contributes to control the functions of insulin-producing cells. In this study, we investigated whether the expression of Cx36 is regulated by glucose in insulin-producing cells. Glucose caused a significant reduction of Cx36 in insulin-secreting cell lines and freshly isolated pancreatic rat islets. This decrease appeared at the mRNA and the protein levels in a dose- and time-dependent manner. 2-Deoxyglucose partially reproduced the effect of glucose, whereas glucosamine, 3-*O*-methyl-D-glucose and leucine were ineffective. Moreover, KCl-induced depolarization of β-cells had no effect on Cx36 expression, indicating that glucose metabolism and ATP production are not mandatory for glucose-induced Cx36 downregulation. Forskolin mimicked the repression of Cx36 by glucose. Glucose or forskolin effects on Cx36 expression were not suppressed by the L-type Ca^{2+}-channel blocker nifedipine but were fully blunted by the cAMP-dependent protein kinase (PKA) inhibitor H89. A 4 kb fragment of the human *Cx36* promoter was identified and sequenced. Reporter-gene activity driven by various *Cx36* promoter fragments indicated that *Cx36* repression requires the presence of a highly conserved cAMP responsive element (CRE). Electrophoretic-mobility-shift assays revealed that, in the presence of a high glucose concentration, the binding activity of the repressor CRE-modulator 1 (CREM-1) is enhanced. Taken together, these data provide evidence that glucose represses the expression of Cx36 through the cAMP-PKA pathway, which activates a member of the CRE binding protein family.

Key words: Gap junctions, Connexin36, Glucose, Transcription, cAMP

Introduction

The homeostasis of multicellular organisms depends on many systems allowing the cells to review the functional state of their neighbors (Spray, 1998). Channels located at gap junctions are one way by which vertebrate cells communicate (Meda, 2000). It has been established that this type of intercellular communication permits coordinated cellular activity, including secretion (Meda, 1996). Gap junctions are specific membrane structures consisting of aggregates of intercellular channels that enable the direct exchange of ions and small metabolites such as second messengers. Intercellular channels result from the association of two hemichannels, named connexons, which are separately contributed by two adjacent cells. Each connexon is an assembly of six transmembrane connexins, encoded by a family of genes with more than 20 members (Sohl and Willecke, 2004).

We and others have demonstrated that connexin36 (Cx36; 36 kDa) is the sole connexin isoform expressed in insulin-producing β-cells of rat and mouse pancreatic islets (Serre-Beinier et al., 2000; Theis et al., 2004). In order to evaluate the contribution of Cx36 to the control of insulin secretion, the Cx36 content has been artificially modified in insulin-secreting cells (Caton et al., 2003; Le Gurun et al., 2003). These experiments provided evidence that Cx36 has to be expressed at a very precise level in order to maintain a normal insulin secretion. Recently, Leite et al. have suggested that, during the maturation process of neonatal islet, the gain of capacity of the islet to secrete insulin was associated with an increase in Cx36 expression levels. Therefore, variations of the endogenous Cx36 expression levels could have major repercussions on β-cell function. Considering the fundamental implication of glucose on gene regulation in β-cells, we investigated the effect of glucose on the Cx36 levels in insulin-producing cells. Early morphological studies have demonstrated that glucose induces a remodeling of the gap junctions aggregates in pancreatic islets (In't Veld et al., 1986; Meda et al., 1980). Here, we have demonstrated for the first time that glucose induces a time- and dose-dependent decrease of Cx36 mRNA and protein content in several insulin-secreting cell lines and adult pancreatic rat islets.

In β-cells, glucose metabolism generates fluctuations in the ATP/ADP ratio, leading to oscillations of membrane potential and cytoplasmic calcium concentrations, which then trigger insulin release (Deeney et al., 2000). Thus, β-cell stimulation is associated with regular Ca^{2+} oscillations (Lenzen et al., 2000). Surprisingly, glucose-induced Ca^{2+} oscillations are synchronized among the thousands of β-cells that form each pancreatic islet (Jonkers and Henquin, 2001), probably because of a diffusible regulator (Meda, 1996). These oscillations become more regular and prominent in the presence of β-cell

contacts (Squires et al., 2000), suggesting that the intercellular communication is involved in the synchronization of β-cells. Calabrese et al. have shown that a marked decrease in the Cx36 expression levels was associated with an altered synchronization of the glucose-induced Ca^{2+} oscillations, indicating that Cx36 gap junctions control the ion-current flow from one cell to its coupled neighbors (Calabrese et al., 2003). This observation prompted us to study the effects of different compounds on Cx36 expression levels, particularly KCl and the inhibitor of Ca^{2+} entry nifedipine, in order to determine whether β-cell depolarization and Ca^{2+} entry are implicated in glucose-induced Cx36 regulation. We found that glucose's effect on Cx36 expression is independent of β-cell depolarization but required PKA activation. We further demonstrated the involvement of a novel cAMP responsive element (CRE) located in the *Cx36* promoter.

Materials and Methods

Cell-line and rat-islet isolation

The rat insulinoma cell lines INS-1, INS-1E (kindly provided by P. Maechler, University Medical Center, Geneva, Switzerland) and βTC3 were maintained in complete RPMI 1640 medium as previously described (Abderrahmani et al., 2001; Asfari et al., 1992; Le Gurun et al., 2003; Martin et al., 2003; Merglen et al., 2004). MIN6-B1 cells (kindly provided by P. Halban, University Medical Center, Geneva, Switzerland) were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (FCS), 25 mM glucose, 71 mM 2-mercaptoethanol, 2 mM glutamine, 100 U ml^{-1} penicillin, 100 mg l^{-1} streptomycin, 50 mg l^{-1} gentamycin (Lilla et al., 2003). Cells were kept at 37°C in a humidified incubator under 5% CO_2. Our institutional review committee for animal experiments approved all the procedures for rat care, surgery and euthanasia. Adult rats weighing 250-300 g were anaesthetized by inhalation of 5% halothane (Arovet, Zollikon, Switzerland), sacrificed and immediately used for pancreas sampling. Islets of Langerhans were isolated from the pancreas of male Wistar rats by collagenase digestion. After filtration on a 100 μM cell strainer (BD Biosciences, Basel, Switzerland), the islets were washed in a Hank's balanced saline solution (HBSS) and cultured in RPMI 1640 containing 11.1 mmol l^{-1} glucose supplemented with 10% FCS, 10 mmol l^{-1} HEPES, 2 mmol l^{-1} L-glutamine, 1 mmol l^{-1} sodium pyruvate, 50 μmol l^{-1} β-mercaptoethanol, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Glucose, leucine, D-galactose, 2-deoxy-D-glucose, 3-*O*-methyl-D-glucose, L-glucose, D-glucosamine, forskolin, nifedipine and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (Fluka Chemie, Buchs, Switzerland), and PD98059 and H89 from were purchased from Calbiochem (VWR International, Lucerne, Switzerland).

RNA isolation, northern-blot analysis and PCR

Cells were homogenized in Tripure isolation reagent (Roche Diagnostics, Rotkreuz, Switzerland) and total RNA was extracted using the kit procedure. 12 μg total RNA was size fractionated on 1% agarose gels containing 8% formaldehyde and 1× MOPS buffer (Fluka Chemie). RNAs were transferred overnight on Gene Screen membranes (PerkinElmer Life Science Europe, Zavemtem, Belgium) by capillary transfer in the presence of 10× saline sodium citrate SSC). Membranes were cross-linked using ultraviolet light and vacuum baked for 2 hours at 80°C. Transcript levels were determined by hybridization with random primed (Roche Diagnostics) cDNA probes labeled with α^{32}P-dCTP (Amersham Bioscience Europe, Otelfingen, Switzerland). Probes were prepared using cDNA clones for rat *Cx36* (Serre-Beinier et al., 2000), rat MIF (Waeber et al., 1997), and β-actin (Haefliger et al., 2002). Hybridizations were performed overnight at 68°C in Miraclehyb (Stratagene Europe, Amsterdam, Netherlands). Blots were washed twice in 1×SSC/1% SDS and twice in 0.1× SSC/1% SDS. RNA abundance was determined using the β-radiation counter Instant-Imager (PerkinElmer Life Science Europe).

For reverse-transcription PCR (RT-PCR), cytoplasmic RNA was extracted using the Rneasy kit (Qiagen, Garstligweg, Switzerland). Transcripts (1 μg) were reverse transcribed using the ImProm-II Reverse Transcription System (Catalys, Wallisellen, Switzerland). Quantitative PCR was performed, as already published, using the QuantiTect SYBR Green PCR Kit (Qiagen) in a Lightcycler (Roche Diagnostics), according to the manufacturer's instructions (Deglise et al., 2005; Tawadros et al., 2005). cDNAs were amplified using the following primers: rat *Cx36* (192 bp), 5′-ATACAGGTGTGA-ATGAGGGAGGATG-3′ (sense) and 5′-TGGAGGGTGTTACAGA-TGAAAGAGG-3′ (antisense); rat ribosomal protein L-27 (120 bp), 5′-GATCCAAGATCAAGTCCTTTGTG-3′ (sense) and 5′-CTGGG-TCTCTGAACACATCCT-3′ (antisense).

Measurement of intracellular cAMP levels

INS-1 cells were incubated for 24 hours with 2 mM or 20 mM glucose in the presence of nifedipine (10 μM) or forskolin (20 μM). The medium was removed and the cells were lysed with ice-cold lysis reagent as indicated in the cAMP RIA system kit (Amersham Bioscience Europe). The cAMP assay was carried out according to manufacturer's instructions.

Immunofluorescence

For immunofluorescence, cells were grown on glass coverslips, fixed for 5 minutes in –20°C acetone, air-dried, rinsed in PBS, permeabilized for 1 hour in PBS containing 1.5% bovine serum albumin (BSA), 0.1% Triton X-100. Coverslips were incubated overnight at 4°C in the presence of a polyclonal rabbit antiserum against residues 289–303 of rat Cx36 (Le Gurun et al., 2003), diluted 1:20. After a 1 hour exposure to fluorescein-conjugated antibodies against rabbit immunoglobulins (Igs) diluted 1:250, cells were counterstained with Evans blue, coverslipped and photographed using a fluorescence microscope (Leica Leitz DMRB, Nidau, Switzerland). Immunolabeling of Cx36 resulted in a punctate membrane labeling in INS-1 cells. Quantification of the fluorescent dots were performed under the microscope on many images by counting the immunofluorescent dots in a square containing approximately 100 cells.

Western blotting

INS-1 cells were solubilized by sonication in SDS buffer (62.5 mM Tris-EDTA, pH 6.8, 5% SDS). Protein content was measured using a detergent-compatible DC protein assay kit (Bio-Rad Laboratories, Reinach BL, Switzerland). Samples were equally loaded (100 μg) on a 12.5% polyacrylamide gel followed by electrophoresis and transferred onto PVDF membrane (Immobilon-P; Millipore, Volketswil, Switzerland). Membranes were incubated for 1 hour in PBS containing 5% milk and 0.1% Tween20 (blocking buffer). Saturated membranes were incubated overnight at 4°C with: rabbit polyclonal antibodies against Cx36 (Le Gurun et al., 2003; Martin et al., 2003), diluted 1:200; monoclonal anti-α-tubulin antibodies (Fluka Chemie, diluted 1:2000); monoclonal anti-β-actin antibodies (Fluka Chemie, diluted 1:2000); rabbit polyclonal anti-P-ERK antibodies (Cell Signaling, diluted 1:1000); or rabbit polyclonal anti-ERK1 antibodies (Santa Cruz Biotechnology, diluted 1:500). After incubation at room temperature for 1 hour with a convenient secondary antibody conjugated to horseradish peroxidase (Fluka Chemie, diluted 1:20,000), membranes were revealed by enhanced

chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Bioscience Europe). Densitometric analyses of immunolabeled proteins (western blots) were performed using the ImageQuant Software (Molecular Dynamics, Amersham Bioscience Europe).

Human *Cx36* promoter isolation and reporter-plasmid construction

A human genomic library in the pBeloBAC11 vector (Genome Systems, St Louis, MO) was screened using a specific *CX36* probe amplified by PCR with the following human *Cx36* primers [forward, 5′-AACGCCGCTACTCTACAGTCTTCC-3′ (intron); reverse, 5′-GATGCCTTCCTGCCTTCTGAGCTT-3′ (exon 2)]. The identity of a single positive clone was first confirmed by Southern hybridization with the 269-bp rat *Cx36* DNA probe (Belluardo et al., 1999). 3945 bp of the 5′ flanking sequence were sequenced (numbering from the translational start site). Five different fragments of the human *CX36* promoter were generated by PCR amplification using the same antisense primer for all the constructs: 5′-GCAGATCTCGCTG-TGCATCCGGAGGCAGCAGACAA-3′. The sense primers were: 5′-GCGGTACCGGCTTTCTACTGAGGG-3′ for the 3945 bp fragment; 5′-GCGGTACCGGATCTGCTGGTTGAGG-3′ for the 1384 bp fragment; 5′-GCGGTACCCTCCACACCTGCATTTA-3′ for the 1079 bp fragment; 5′-GCGGTACCTGCCACCCCGCCCCGCCC-3′ for the 316 bp fragment; and 5′-GCGGTACCCGCGCTTGGA-TTTCTTTCC-3′ for the 200 bp fragment. The fragments were subcloned into the promoterless expression vector pGL3 basic (Catalys) to obtain the plasmids: pGL3-3945, pGL3-1384, pGL3-1079, pGL3-316 and pGL3-200. Another plasmid was constructed, derived from the pGL3-1079 and containing a mutated *Cx36* CRE. The mutation was achieved using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene Europe) with the following primer: 5′-GACCTGGCGTAATGCAGGCGGAGACT-3′ (underlined amino acids indicate mutated residues. Plasmid constructs were verified by DNA sequencing. The sequence of the human *Cx36* promoter fragment (bases –3945/–2046; numbering from the translational start site) was submitted to GenBank™ as an update of the previously identified sequence corresponding to a fragment of 2045 bp of the human *Cx36* promoter [GenBank accession number AY341000 (Martin et al., 2003)].

Transient transfection and luciferase assays

INS-1 cells were transiently transfected using the liposome-mediated TRANSFAST reagent (Catalys) at a DNA:lipid ratio of 1:1 in RPMI 1640 according to manufacturer's instructions. After transfection, cells were incubated for 36 hours at 2 mM or 20 mM glucose before harvesting with 100 μl passive lysis buffer (Catalys). Using the Dual-Luciferase reporter assay system (Catalys), a 50 μl sample of the lysates was assayed for luciferase activities in a Turner TD-20/20 Luminometer. Promoter activity was normalized by co-transfection with pRLSV40renilla.

Electrophoretic mobility shift assay

Preparation of nuclear extracts from INS-1 cells and gel-shift analysis were performed as described previously (Martin et al., 2003). For electrophoretic-mobility-shift assay (EMSA), the following double-stranded DNA probes were prepared using oligonucleotides corresponding to the human and rodent *Cx36* CRE sequences: sense, 5′-GACCTGGCGTGACGCAGGCGGAGACT-3′; and antisense, 5′-AGTCTCCGCCTGCGTCACGCCAGGTC-3′. For non-specific competition experiments, the following mutant CRE sequences were used: sense, 5′-GACCTGGCGTAATGCAGGCGGAGACT-3′; and antisense, 5′-AGTCTCCGCCTGCATTACGCCAGGTC-3′ (underlined amino acids indicate mutated residues. The double-stranded DNA probes were labeled with (α^{32}P)-dCTP using the Klenow fragment of DNA polymerase I (Roche Diagnostic) and purified on microspin S300 HR columns (Amersham Bioscience Europe). 17 fmol ^{32}P-labeled double-stranded DNA probes were incubated with 2 μg poly(dI-dC) (Amersham Bioscience Europe) in binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mg ml^{-1} BSA, 3% glycerol). Equal amounts of nuclear protein (5 μg) were added and the mixture was incubated for 30 minutes at room temperature. For the supershift analysis, nuclear extract were previously pre-incubated for 10 minutes at room temperature with 1 μg antibody against: CRE-modulator protein 1 (CREM-1) (sc-440, Santa Cruz Biotechnology, LabForce, Nunningen, Switzerland); phosphorylated CRE-binding protein (phospho-CREB) (06-519, Upstate Biotechnology, Lucerne, Switzerland); activating transcription factor 3 (ATF-3) (sc-188, Santa Cruz Biotechnology); CCAAT/enhancer-binding protein β (C/EBPβ) (sc-746, Santa Cruz Biotechnology); or RE-1-silencing transcription factor (REST) (Martin et al., 2003). In the competition study, unlabeled specific double-stranded DNA competitors (100-fold excess) were added into the reaction mixtures before incubation. Finally, the samples were resolved on a non-denaturing polyacrylamide gel (4% acrylamide 19:1, 0.5× Tris-borate/EDTA), dried and subjected to autoradiography.

Statistical analysis

Data were expressed as means ± s.e.m. Differences between means were assessed using Student's *t* test. Statistical significance was defined at a value of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

Results

High glucose concentration represses the expression of *Cx36* mRNA and protein content in insulin-secreting cells

Freshly isolated pancreatic rat islets and several insulin-secreting cell lines were incubated for 24 hours at 2 mM or 20 mM D-glucose. The expression levels of *Cx36* mRNA were evaluated by quantitative RT-PCR (Fig. 1A). In rat islets, MIN6-B1 and βTC3, *Cx36* mRNA levels were reduced by 40% whereas in INS-1 and INS-1E cell lines, a 60% decrease in *Cx36* mRNA was observed. The effect of glucose on *Cx36* mRNA and protein expression was further evaluated in INS-1 cell lines. Glucose induced a dose- and time-dependent decrease in the *Cx36* mRNA and protein levels in INS-1 cells (Fig. 1B,C). This effect was already detectable after 8 hours and was maximal after 24 hours of incubation at 20 mM glucose. Northern-blot analysis confirmed that, between 2 mM and 20 mM glucose, *Cx36* transcript levels decreased by 60% in INS-1 cells (Fig. 1B). Similar results were obtained in the INS-1E cell line (data not shown). Macrophage-migration-inhibitory factor (MIF) was used as a positive control, because its transcript is positively regulated in a time- and a dose-dependent manner by glucose in β-cells (Waeber et al., 1997). Western-blot analysis further revealed a 70% decrease in the Cx36 content of INS-1 cells cultured with 20 mM glucose compared with those cultured with 2 mM glucose (Fig. 1C). The normalization of the Cx36 levels by tubulin or β-actin gave analogous results. Similar observations were performed in βTC3 and MIN6-B1 (data not shown).

To test the reversibility of this regulation, INS-1 cells preincubated for 24 hours at 2 mM, 10 mM and 20 mM glucose were further incubated at 10 mM glucose for 24 hours (Fig.

2A). After 8 hours at 10 mM glucose, Cx36 levels were already back to control levels, indicating that glucose-induced regulation of *Cx36* expression is a dynamic event.

Mechanisms of glucose regulation of Cx36

In order to investigate whether glucose-induced *Cx36* mRNA decrease is a transcriptional event, INS-1 cells preincubated for 24 hours in medium supplemented with 2 mM, 10 mM or 20 mM D-glucose, were exposed to the RNA-synthesis inhibitor actinomycin D for the indicated times (Fig. 2B). The stability of *Cx36* transcript was not affected by glucose, suggesting that glucose modulates *Cx36* mRNA at the transcriptional level.

To gain insight into the mode of action of glucose on *Cx36* expression, we tested the effects of several glucose analogs, nutrient stimuli and pharmacological agents. L-Glucose, an analog of glucose that cannot enter the cell, had no effect on Cx36 expression; neither had 3-*O*-methyl-D-glucose, a glucose analog that rapidly enters the β-cell via the glucose transporter GLUT2 but is not metabolized (Fig. 3). Leucine (which enters the Krebs cycle at the mitochondria level) and glucosamine [a specific substrate for the hexosamine synthesis pathway (Yoshikawa et al., 2002)] were also unable to reduce Cx36 levels. By contrast, 2-deoxy-D-glucose, which is phosphorylated by glucokinase (K_m 55 mM vs 10 mM for glucose) but poorly metabolized in the glycolytic or pentose-phosphate pathway (Girard et al., 1997), induced a 50% decrease in Cx36 protein levels (70% for D-glucose). Taken together, these results provide evidence that glycolytic steps beyond the initial phosphorylation of glucose are not required to repress Cx36 expression.

Glucose metabolism raises the ATP/ADP ratio, which induces closure of the ATP-sensitive K^+ channels, leading to membrane depolarization and subsequent opening of voltage-dependent Ca^{2+} channels (VDCCs). In an attempt to determine whether β-cell depolarization and Ca^{2+} entry were involved in glucose-induced Cx36 downregulation, INS-1 cells were incubated with 2 mM glucose in the presence of KCl (20 mM) or with 20 mM glucose in the presence of the VDCC blocker nifedipine (10 μM). Neither agent had any influence on Cx36 expression (Fig. 4).

The possible involvement of various signal-transduction pathways was then investigated. Long-term treatment (>18 hours) with PMA, which is thought to deplete and inactivate protein kinase C (PKC) (Seissler et al., 2000), failed to block the effect of glucose on Cx36 expression. The inhibition of MEK1/2 by 50 μM PD98059 did not alter the effect of glucose on Cx36 expression (Fig. 4). The ability of PD98059 to block the phosphorylated form of ERK1/2 after 24 hours was confirmed by western-blot analysis using specific antibodies directed against phosphorylated ERK1/2 and total ERK (data not shown) (Arnette et al., 2003; Briaud et al., 2003). Therefore, glucose inhibition of Cx36 expression probably occurred independently of PKC activation and subsequent activation of MEK1/2. By contrast, forskolin (20 μM), a strong stimulator of adenylate cyclase, induced a 50% decrease in Cx36 expression compared with control (Fig. 5A). In addition, H89 (10 μM), an inhibitor of PKA, completely blocked the effects of both glucose and forskolin on Cx36 levels, indicating that PKA activation is required for glucose-induced *CX36* downregulation.

These results were confirmed by immunofluorescence experiments. Using specific antibodies against Cx36, we were able to immunostain Cx36 aggregates as a characteristic punctate pattern at cell-cell contact sites.

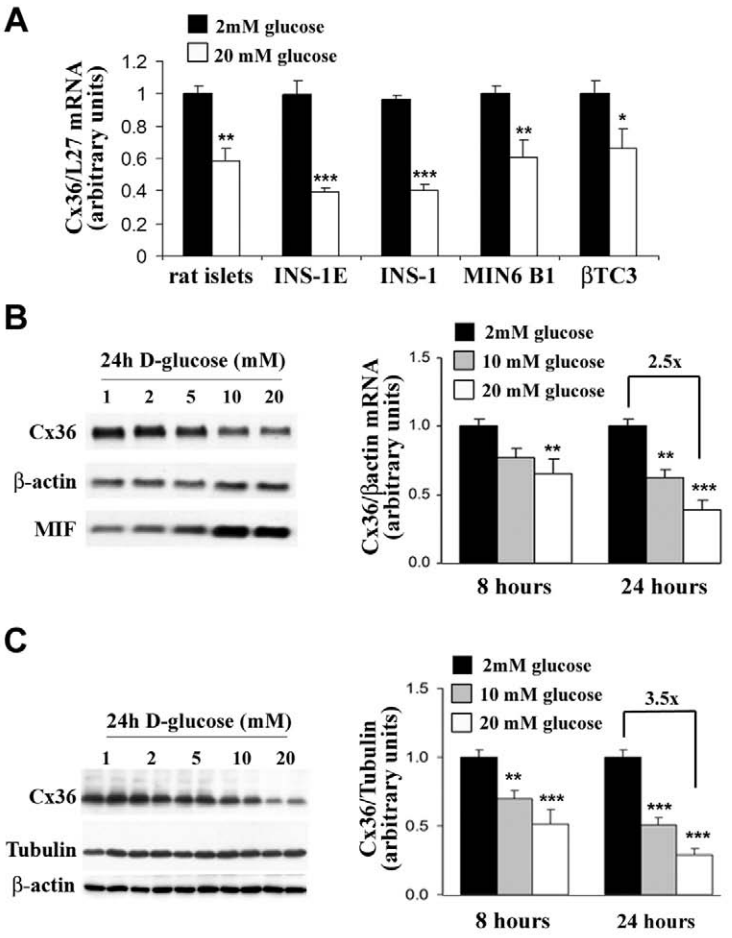

Fig. 1. Levels of *Cx36* mRNA and protein are decreased by glucose in insulin-secreting cells. (A) Quantitative RT-PCR analysis showed a decrease of *Cx36* mRNA in insulin-secreting cell lines and pancreatic rat islets after a 24 hour incubation at high (20 mM) versus low (2 mM) glucose concentration. Rat islets, βTC3 cells and MIN6-B1 cells display a 40% decrease in *Cx36* transcript whereas the INS-1 and INS-1E cell lines present a 60% decrease in *Cx36* mRNA level. Data are means ± s.e.m. of four independent experiments. Relative *Cx36* transcripts levels were normalized to the ribosomal protein L27. (B) Northern blots showed a dose-dependent decrease of *Cx36* mRNA in INS-1 cells after 24 hours. By contrast, the level of the macrophage inhibitory factor (MIF) encoding mRNA, used as a positive control, is increased by glucose. Quantitative assessment of *Cx36* mRNA confirmed that glucose induces a 60% reduction of *Cx36* transcript expression. Data are means ± s.e.m. of five independent experiments. (C) Western-blot analysis normalized with tubulin and β-actin showed a dose-dependent decrease of Cx36 in INS-1 cells after 24 hours. Densitometric analysis indicated that Cx36 protein levels were decreased by 70% in presence of a high glucose concentration. Data are means ± s.e.m. of six independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

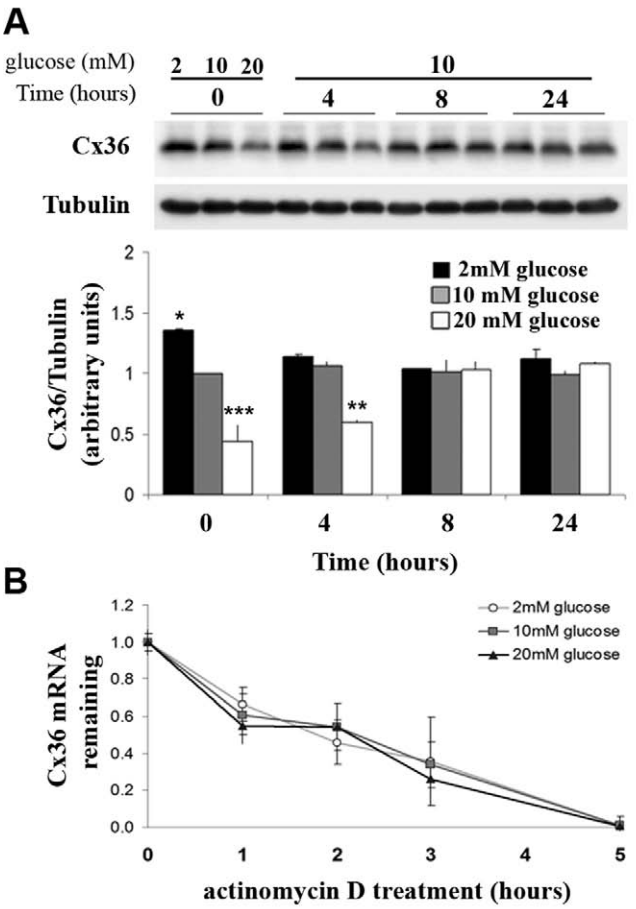

Fig. 2. Cx36 downregulation by glucose is reversible and occurs at the transcriptional level. (A) INS-1 cells preincubated for 24 hours at 2 mM, 10 mM and 20 mM glucose were further incubated at 10 mM glucose for 24 hours. Densitometric analysis of three independent northern blots revealed that *Cx36* expression levels returned to control values after 8 hours. Mean (± s.e.m.) of three independent experiments. ***, $P<0.001$; **, $P<0.01$. (B) After a 24 hour preincubation at 2 mM, 10 mM or 20 mM glucose, INS-1 cells were exposed to 5 μg ml^{-1} actinomycin D for 1 hour, 2 hours, 3 hours or 5 hours. Quantitative assessment of *Cx36* mRNA levels (northern blot) showed that glucose had no effect on the stability of the *Cx36* transcript.

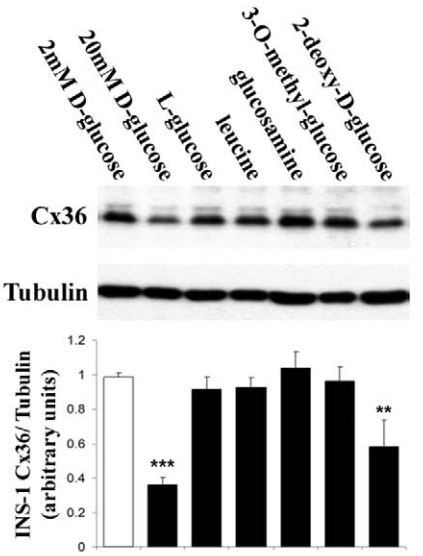

Fig. 3. Effects of various nutrients on Cx36 levels. Cells were incubated for 24 hours in the presence of glucose (2 mM or 20 mM), L-glucose (20 mM), 3-*O*-methyl-D-glucose (20 mM), 2-deoxy-D-glucose (20 mM), glucosamine (20 mM) or leucine (10 mM). Quantitative assessment of western blot demonstrated that 2-deoxy-D-glucose induced a 50% decrease in Cx36 levels. Data are means (± s.e.m.) from six independent experiments made in duplicate. **, $P<0.01$; ***, $P<0.001$.

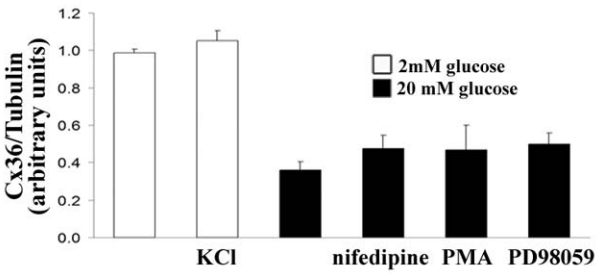

Fig. 4. β-Cell depolarization and MAPK pathway are not involved in Cx36 regulation. INS-1 cells were incubated for 24 hours at 2 mM or 20 mM glucose with or without 20 mM KCl, 10 μM nifedipine, 100 nM PMA and 50 μM PD98059, as indicated. Quantitative assessment (± s.e.m.) of six independent experiments revealed that none of these compounds influence Cx36 regulation by glucose. ***, $P<0.001$.

We observed that there were similar modulations to those observed in western-blot experiments (Fig. 5B, left). Quantitative assessment of the Cx36 immunofluorescent dots was achieved in many cells and experiments (Fig. 5B, right), and revealed a 70% decrease in Cx36 aggregates localized to the membrane of INS-1 cells cultured in presence of 20 mM glucose, in comparison with 2 mM glucose. Moreover, forskolin also had this effect in the presence of a low glucose concentration. By contrast, when cells were cultured in a medium containing a high glucose concentration, H89 treatment restored the number of Cx36 aggregates to the level observed with 2 mM glucose.

Measurement of the cAMP content of INS-1 revealed a 30% increase between cAMP levels in cells cultured for 24 hours at 2 mM or 20 mM glucose (Fig. 6). This effect was not inhibited by nifedipine and was therefore not mediated by Ca^{2+} influx. This result demonstrates the ability of glucose to stimulate the cAMP-PKA pathway independently of Ca^{2+} entry. As a positive control, forskolin induced a twofold increase in cAMP content regardless of the glucose concentration.

Effect of glucose on *Cx36* promoter activity

Using specific human *Cx36* primers, we PCR amplified and sequenced a 3945 bp fragment of the human *Cx36* promoter, and subcloned it in front of a reporter gene encoding luciferase (Fig. 7A). To evaluate the minimal region responsible for glucose responsiveness, five constructs consisting of full-length and 5′-deleted fragments of the isolated human *Cx36* promoter were transiently transfected in INS-1 cells incubated

with 2 mM or 20 mM glucose (Fig. 7A). These constructs drove high transcriptional activity compared with the pGL3 basic vector (basal activity). Apart from pGL3-316 and the pGL3-200, shifting the glucose concentration from 2 mM to 20 mM caused a 30-40% decrease in luciferase activity. These data suggest that putative glucose-responsive elements are located within the –1079 to –316 fragment of the human *Cx36* promoter. We have previously demonstrated (Martin et al., 2003) that this region contains a functional repressive motif called neuron-restrictive silencing element (NRSE) that binds the repressor REST, which is not expressed in unstimulated β-cells. Northern and western-blot analysis of REST expression revealed that glucose did not induce the expression of REST in INS-1 cells (data not shown), indicating that glucose-induced Cx36 downregulation is not caused by the repressor REST.

Bioinformatic analysis (http://www.genomatix.de/) of this region failed to identify any glucose-responsive element (GlRE) or carbohydrate-responsive element (ChoRE) (Girard et al., 1997). However, it revealed the presence between bases –566 and –556 of a putative CRE, which is the known target motif for the activated cAMP-PKA pathway. Sequence alignment (Fig. 7B) of functional CREs (Dumonteil and Philippe, 1996) with human and rodent *Cx36* promoter fragments revealed that this motif is highly conserved and corresponds to a partial consensus CRE. To investigate the involvement of this motif in glucose-induced *Cx36* downregulation, we generated a pGL3-1079 construct containing a mutated CRE (pGL3-1079m) (Fig. 7B). The promoter of this plasmid did not respond to glucose, indicating that the *Cx36* CRE is functional and required for glucose-induced *Cx36* downregulation. Using a *Cx36* CRE probe and nuclear extracts from INS-1 cells, EMSAs revealed the presence of two specific protein/DNA-binding complexes C1 and C2 (Fig. 8, lanes 2,5) when compared with the free probe (lane 1). EMSAs using the consensus CRE (Fig. 7B) displayed a similar pattern of interactions (data not shown). Competition assays performed using 100× excess of cold *Cx36* CRE (Fig. 8, lanes 3,6) totally extinguished the C1 complex and decreased the C2 complex. Competition using a mutated CRE (Fig. 7B) had no effect on the binding profile (Fig. 8, lanes 4,7). Nuclear extracts prepared from cells incubated in a medium containing a high glucose concentration showed an enhanced formation of the two protein-DNA complexes (Fig. 8, lane 5). Moreover, in the presence of a low glucose concentration, cAMP content elevation by forskolin resulted in increased appearance of these complexes (Fig. 8, lane 8), whereas these were decreased in presence of the PKA inhibitor H89 and a high glucose concentration (Fig. 8, lane 9).

In order to characterize the transcription factors involved in the control of *Cx36* expression by glucose, we performed EMSAs using antibodies

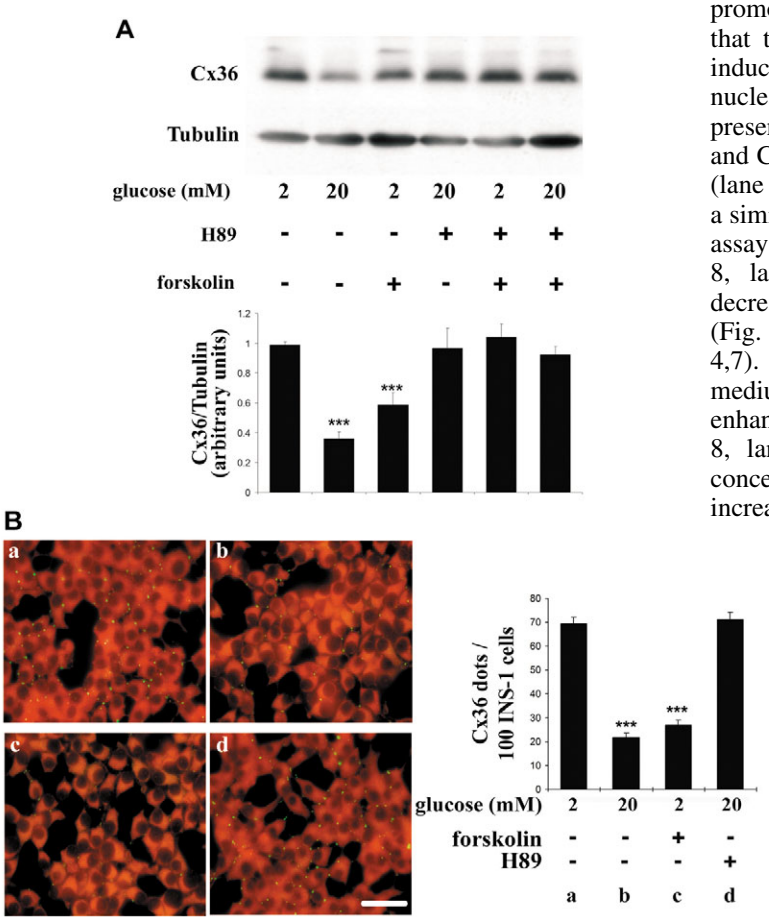

Fig. 5. The cAMP-PKA pathway mediates the effect of glucose on Cx36 levels. INS-1 cells were incubated for 24 hours at 2 mM glucose with or without 20 μM forskolin or at 20 mM glucose with or without 10 μM H89. (A) Western-blot analysis showed that forskolin had a similar effect to glucose on Cx36 expression, whereas H89 fully blunted the effects of glucose and forskolin. Data are means ± s.e.m. of densitometric analyses of six independent experiments made in duplicate. **, $P<0.01$, ***, $P<0.001$. (left) Immunolabeling of Cx36 (Cx36 recognized by fluorescein-conjugated secondary antibodies) resulted in a punctate membrane labeling in INS-1 cells (white tab represents 50 μm). (a) 2 mM glucose; (b) 20 mM glucose; (c) 2 mM glucose, 20 μM forskolin; (d) 20 mM glucose, 10 μM H89. Pictures are representative of four independent experiments. (right) Quantitations of the fluorescent dots were performed under the microscope on many images by counting the immunofluorescent dots in a square containing ~100 cells. Data are means ± s.e.m. of four independent experiments. ***, $P<0.001$.

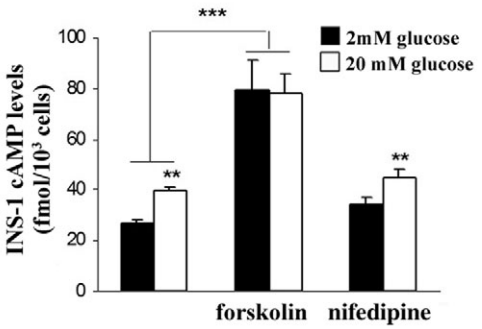

Fig. 6. Glucose increases cAMP levels in INS-1 cells. INS-1 cells were incubated for 24 hours at either 2 mM or 20 mM glucose with or without 10 μM nifedipine or 20 μM forskolin, as indicated. Measurements of the intracellular cAMP levels showed that glucose increased cAMP levels independently of Ca^{2+} entry. Data are means ± s.e.m. of three independent experiments made in duplicate. **, $P<0.01$; ***, $P<0.001$.

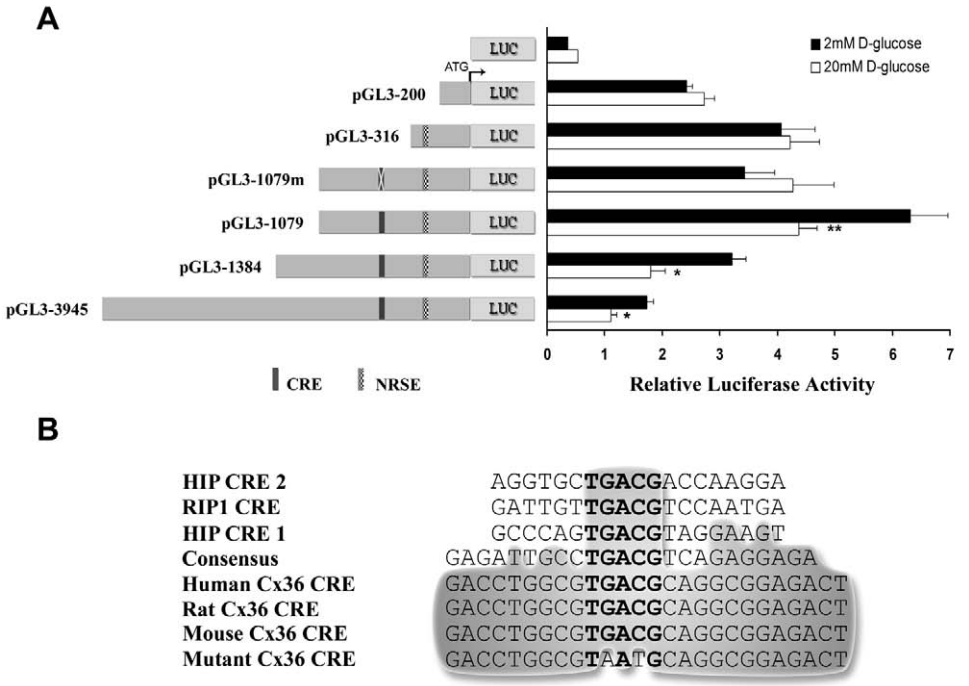

Fig. 7. Glucose decreases the transcriptional activity of the human *Cx36* promoter through a CRE. (A) Progressive deletions of the human *Cx36* promoter were transfected into INS-1 cells. After transfection, cells were incubated at 2 mM or 20 mM glucose. The –1079 to –316 region of the *Cx36* promoter drives glucose responsiveness. The mutation of a CRE (pGL3-1079m) located in this region abolished the inhibitory effect of glucose on the luciferase activity. Results are means ± s.e.m. of four experiments performed in triplicate. Luciferase activities were normalized using the pRLSV40renilla construct. *, $P<0.05$. NRSE, neuron-restrictive silencing element. (B) Alignments of CREs: the TGACG core region (bold) of the consensus CRE is 100% conserved between the three species. HIP CRE1, human insulin promoter CRE 1; HIP CRE 2, human insulin promoter CRE 2; RIP1 CRE, rat insulin promoter 1 CRE.

directed against proteins related to CRE. A specific antibody against CREM-1 supershifted the C1 complex (Fig. 8, lane 10) and the supershift was clearly enhanced in presence of 20 mM glucose (Fig. 8, lane 15). Moreover, an antibody directed against the activated (phosphorylated) form of CREB also induced a supershift of the C1 complex (Fig. 8, lane 13), which is more pronounced at 20 mM glucose (Fig. 8, lane 18). The anti-ATF3 (Fig. 8, lanes 11,16), anti-C/EBPβ (Fig. 8, lanes 12,17) and anti-REST (Fig. 8, lanes 14,19) (Martin et al., 2003) antibodies had no effect on protein-DNA complexes.

Discussion

Glucose upregulates the expression of many genes in β-cells after 24 hours (Schuit et al., 2002), such as GLUT2 (Waeber et al., 1996), the major β-cell autoantigen IA-2 (Seissler et al., 2000) and the acetyl-CoA carboxylase ACC (Brun et al., 1993). Although many genes are repressed by glucose after 24 hours (Schuit et al., 2002), only a few have been well studied, such as the peroxisomal proliferator-activated receptor α (PPARα) (Roduit et al., 2000) or the SUR/Kir6.2 channel (Moritz et al., 2001). The present study identified *Cx36* as a new gene whose expression is transcriptionally decreased in insulin-secreting cells by glucose after 24 hours. Recently, it has been demonstrated that Cx36 expression levels are

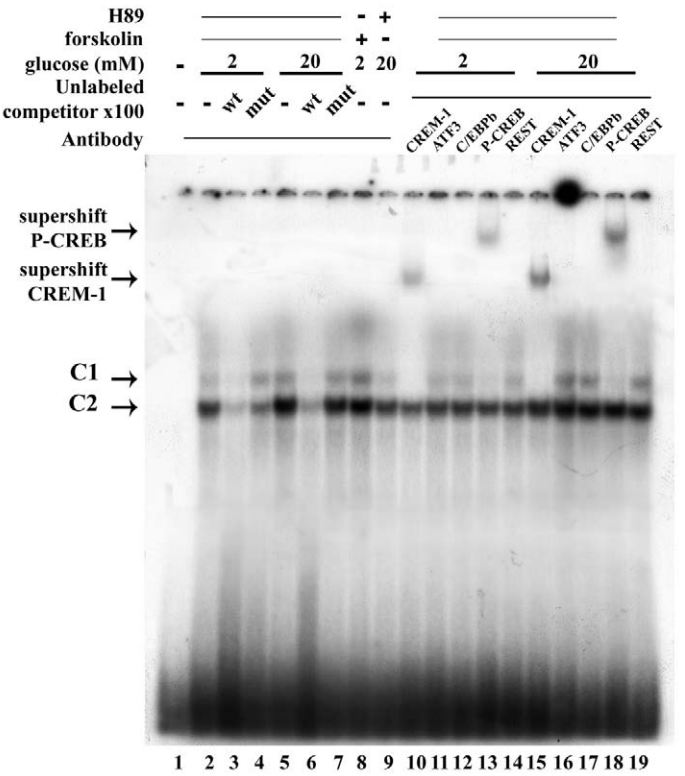

Fig. 8. Glucose and PKA activation enhance the binding activity of CREM1 to the human *Cx36* CRE motif in INS-1 cells. Electrophoretic mobility shift assays (EMSAs) were performed using the *Cx36* CRE and nuclear extracts (NE) from INS-1 cells cultured at either 2 mM or 20 mM glucose with or without forskolin or H89. For the competition assay, a 100-fold excess of the unlabelled oligonucleotides were used (cold competitor ×100). mut, mutated *Cx36* CRE; wt, wild-type *Cx36* CRE. Compared with the free probe, two slow-migrating complexes (C1 and C2) were detected. The levels of binding are increased in presence of high levels of glucose or forskolin and reduced by H89 treatment. For the supershift analysis, nuclear extract from INS-1 cells cultured at either 2 mM or 20 mM glucose were exposed to specific antibodies directed against CRE modulator protein 1 (CREM-1), activating transcription factor 3 (ATF3), CCAAT/enhancer-binding protein β (C/EBPβ), phosphorylated CRE-binding protein (P-CREB), RE-1-silencing transcription factor (REST, also known as NRSF). CREM-1 and P-CREB supershifted the C1 complexes. Data are representative of four independent experiments.

increased during the maturation process of neonatal islets and that this effect is enhanced by glucose (Leite et al., 2005). Here, we demonstrated that glucose decreases Cx36 expression in a dose- and time-dependent manner in several insulin-secreting cell lines and in adult rat islets, suggesting that glucose plays a major role in the control of Cx36 expression levels in β-cells.

Considering the various effects of different glucose analogs that are phosphorylated or not by glucokinase and the lack of effect of other secretagogues, our results indicate that glucose-induced *CX36* downregulation shares many characteristics with those previously observed for the glucose modulation of the genes encoding liver-type pyruvate kinase (Marie et al., 1983), ACC (Brun et al., 1993) and PPARα (Roduit et al., 2000). These similarities suggest that an analogous metabolic intermediate, possibly glucose-6-phosphate, mediates the action of glucose on the expression of its target genes (Scott et al., 1998).

Glucose elicits its better-known molecular actions on insulin-secreting cells via cellular uptake and mitochondrial metabolism, increased ATP/ADP ratio, closure of K^+/ATP channels and calcium influx via VDCCs (Maechler and Wollheim, 2001). The fact that none of these events were implicated in glucose-induced *Cx36* downregulation confirmed the hypothesis that glucose metabolism beyond phosphorylation by glucokinase is not mandatory to inhibit expression of *Cx36* and that the classical glucose-induced mechanism of insulin secretion is not involved in this regulation.

We further analysed two different transduction pathways, the PKC and the cAMP-PKA pathways, that have been shown to be implicated in glucose-induced gene regulation in β-cells (Arnette et al., 2003; Lawrence et al., 2002). Downregulation of PKC by PMA (Seissler et al., 2000) or inhibition by PD98059 of the MEK1/2 kinases, which can be activated by PKC (Gutkind, 1998), both failed to block the effect of glucose. These results demonstrate that the PKC signaling pathway is not involved in glucose-induced *Cx36* repression. By contrast, forskolin (a strong activator of adenylate cyclase) induced a decrease in Cx36 levels. Although forskolin (in the presence of low or high glucose concentration) stimulated a larger synthesis of cAMP than glucose (Fig. 6), it was unable to induce a further decrease in Cx36 expression levels (Fig. 5). Several recent studies indicate that elevation of the internal concentration of cAMP ($[cAMP]_i$) occurs in discrete microdomains and that each PKA substrate would have its own anchored pool of PKA and its own local gradient of cAMP (Tasken and Aandahl, 2004). Thus, the large amount of cAMP produced in response to forskolin treatment might not be fully available to the specific cAMP-PKA pathway involved in Cx36 downregulation. Previous work has indicated that glucose induces $[cAMP]_i$ elevation in INS-1 cells through an upstream increase of the cytosolic Ca^{2+} concentration (Briaud et al., 2003). Here, we demonstrated that long-term (24 hour) exposure to 20 mM glucose increased the cAMP content of INS-1 cells independently of Ca^{2+} entry. However, we cannot rule out the possibility that glucose could increase $[Ca^{2+}]_i$ independently of Ca^{2+} entry by mobilizing the intracellular Ca^{2+} pool. Nevertheless, our results indicate that cAMP could mediate the impact of glucose on Cx36 expression. cAMP exerts most of its effects by activating PKA and the role of the PKA in glucose-dependent signaling pathways is well documented (Vaulont et al., 2000). In the presence of H89, an inhibitor of PKA, the effects of glucose or forskolin on Cx36 expression levels were abolished. Moreover, forskolin did not synergize with glucose to decrease Cx36 levels. Taken together, these data suggest that, in INS-1 cells, glucose induces the accumulation of cAMP, leading to the release of the active catalytic subunit of PKA which, in turn, phosphorylates a range of substrates.

Transient transfection analysis indicated that glucose-induced *Cx36* downregulation requires the presence of a CRE 550 bp upstream of the translational start site of the *Cx36* gene. Sequence alignment (Fig. 6B) of the *CX36* CRE with functional insulin CREs (Dumonteil and Philippe, 1996) highlighted the fact that, even if the *Cx36* CRE is not a perfect consensus CRE sequence (TGACGTCA), it contains the highly conserved core TGACG that is found in many functional CREs (Manna et al., 2002; Sassone-Corsi, 1995). In this study, human *Cx36* promoter constructs were transiently transfected in rat insulin-secreting cells. However, we have shown in the past that rodent and human *Cx36* promoter sequences were highly homologous in the region studied (Martin et al., 2003). Thus, our results might imply that a similar mechanism of regulation could occur in human and rodents.

CREs are the main DNA targets of a large family of PKA-activated basic leucine-zipper (bZIP) CRE-binding factors including CREB, CREMs and ATFs. These factors are characterized by multiple alternatively spliced forms and some lack transactivation domains and hence function as transcription suppressors (Habener et al., 1995; Hai and Hartman, 2001). Previous studies have indicated that the transcription of genes encoding insulin and somatostatin is modulated by PKA through CRE-binding proteins (Chepurny et al., 2002; Montminy et al., 1996). Interestingly, it has been shown that, in rat islets, glucose induces the overexpression of CREM-17x and ICER, two repressive isoforms of CREM (Chepurny et al., 2002; Zhou et al., 2003). Recently, it has also been demonstrated that, in MIN6 cells exposed to 25 mM glucose for 24 hours, total CREB protein levels were decreased by 50% (Costes et al., 2004), reinforcing the idea that long-term exposure to elevated glucose concentrations leads to the accumulation of repressive isoforms of the CRE-binding protein family.

Using EMSAs, we observed that the level of binding to the *Cx36* CRE was increased by glucose or forskolin. Supershift analysis showed that the repressor CREM-1 binds to the *Cx36* CRE, whereas ATF3 and C/EBPβ, two factors suspected to bind to CREs (Servillo et al., 2002), did not. Moreover, this binding was increased in presence of a high glucose concentration, suggesting that glucose enhances the formation of a repressive complex on the *Cx36* CRE. We also demonstrated that glucose increases the binding of the PKA-activated form of CREM (phosphorylated on Ser117) or CREB (phosphorylated on Ser133) (Servillo et al., 2002). Taken together, our data suggest that glucose activates, through the release of active PKA via cAMP accumulation, the activation of a CRE-binding factor, probably a repressive isoform of CREM, which might be part of a repressive complex binding to the *Cx36* CRE. Surprisingly, the C2 complex did not interact with the CRE-binding proteins we tested. It has been demonstrated that active PKA regulates additional proteins (like the CREB-binding protein CBP) that are required for

recruitment of the transcriptional apparatus to cAMP-responsive genes (Brindle et al., 1995). Thus, one could hypothesis that the C2 complex is formed by partners of the CRE binding proteins involved in a repressive complex contributing to glucose-induced *CX36* repression. Further studies remain to be carried out to identify the exact isoform(s) involved in this regulation and whether this regulation is merely due to the activation of a repressor or whether it also involves modifications in the expression levels of these factors.

These data provide the first evidences of a regulation of *Cx36* by glucose in β-cells and demonstrate a mechanism whereby pathophysiological conditions of glucose stimulation (20 mM glucose for 24 hours) modulate endogenous Cx36 levels. Glucose-induced transcriptional downregulation of *Cx36* was dependent on an upstream release of the active form of PKA through the elevation of $[cAMP]_i$. In view of the complexity of the transcription machinery binding to CREs, it is most likely that PKA phosphorylates and/or activates several effectors, including the repressive isoform CREM-1, that lead to the formation of a complex that binds on the *Cx36* CRE, inhibiting *Cx36* gene expression.

Considering the fact that adequate levels of Cx36 are crucial for proper β-cell function (Caton et al., 2003; Le Gurun et al., 2003), modulation of endogenous Cx36 levels could result in altered insulin secretion. Thus, *Cx36* might be a potential candidate for participating in the impaired glucose-induced insulin secretion observed during the glucose desensitization and/or early glucotoxicity phenomena.

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