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Exendin-4 regulates GLUT2 expression via the CaMKK/CaMKIV pathway in a pancreatic β-cell line

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Abstract

The GLUT2 glucose transporter plays an important role in glucose-induced insulin secretion in pancreatic β-cells by catalyzing the uptake of glucose into the cell. In this study, we investigated whether exendin-4, a long-acting agonist of glucagon-like peptide–1, mediates stimulatory effects on GLUT2 gene expression through the Ca^{2+}/calmodulin (CaM)-dependent protein kinase IV (CaMKIV) cascade. GLUT2 expression was examined by real-time polymerase chain reaction, Western blot analysis, and a reporter gene assay in rat insulin-secreting INS-1 cells incubated with exendin-4. An increased expression level of GLUT2 protein was noted in response to increasing concentrations of exendin-4, with maximal induction at 10 nmol/L. Real-time polymerase chain reaction analysis similarly revealed a significant increase in the amount of GLUT2 messenger RNA by 10 nmol/L exendin-4. Exendin-4 also stimulated GLUT2 promoter activity in response to increasing exendin-4 concentrations, but failed to do so in the presence of STO-609, a CaMKK inhibitor. We also investigated the effect of the constitutively active form of CaMKK (CaMKKc) on GLUT2 promoter activity. The result is consistent with the observations that CaMKKc/CaMKIV enhanced or up-regulated GLUT2 promoter activity in INS-1 cells. Furthermore, exendin-4 induction of GLUT2 protein expression was significantly suppressed in the cells knocking down the CaMKIV. In summary, activation of the CaMKK/CaMKIV cascade might be required for exendin-4–induced GLUT2 gene transcription, indicating that exendin-4 plays an important role in insulin secretion in pancreatic β-cells.

1. Introduction

The insulin secretagogue hormone glucagon-like peptide–1 (GLP-1) and its long-acting agonist exendin-4 are new treatment agents for diabetes [1]. Glucagon-like peptide–1 is a peptide hormone formed by alternative enzymatic cleavage of proglucagon, the prohormonal precursor of GLP-1 [1,2]. The enteroendocrine cells of the gut secrete GLP-1 in response to ingestion of food. Glucagon-like peptide–1 stimulates glucose-dependent insulin secretion and lowers blood glucose levels in persons with type 2 diabetes mellitus (DM). Initial studies established that GLP-1 is a potent insulin secretagogue [2]. Subsequently, GLP-1's multiple antidiabetogenic functions were discovered, including stimulation of the proliferation of insulin-producing pancreatic β-cells and inhibition of their apoptosis [1,3]. Exendin-4 can also protect β-cells from apoptosis induced by elevated concentrations of glucose and lipids [4].

GLUT2 is present in the plasma membrane of hepatocytes, intestine, kidney, and pancreatic β-cells [5]. The glucose-induced insulin secretion is first initiated by the uptake of glucose. Adenosine triphosphate (ATP) is then

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generated during glucose processing through glycolysis and mitochondrial metabolism. The increased ATP to adenosine diphosphate ratio promotes ATP-dependent K^+ channel closure, membrane depolarization, and the opening of voltage-dependent Ca^{2+} channels, thereby increasing cytosolic Ca^{2+} concentrations and finally the exocytosis of insulin granules [6]. The GLUT2 glucose transporter plays an important role in glucose-induced insulin secretion in pancreatic β-cells by catalyzing the uptake of glucose into the cell [7]. GLUT2 is a facilitative glucose transporter, and its expression is strongly reduced in glucose-unresponsive islets in various animal models of diabetes [7,8]. In the present study, we analyzed the intracellular Ca^{2+}-mediated signaling (CaMKK/CaMKIV) involved in exendin-4–induced GLUT2 expression in pancreatic β-cells.

2. Materials and methods

2.1. Cell culture

The INS-1 cells were derived from a rat insulinoma cell line developed and propagated at the Division of Clinical Biochemistry, University Medical Centre, Geneva, Switzerland (courtesy of Dr CB Wollheim). These cells were cultured in RPMI-1640 medium (GIBCO BRL, Tokyo, Japan) containing 11.2 mmol/L glucose (unless otherwise stated) and supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical, Tokyo, Japan), 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. We isolated pancreatic islets from adult Wistar rats as described previously [9]. Isolated islets were then maintained in RPMI medium containing 11.2 mmol/L glucose, 10% fetal bovine serum, 200 U/mL penicillin, and 200 μg/mL streptomycin in humidified 5% CO_2, 95% air at 37°C. The protocol used in this experiment was reviewed and approved by the Kagawa University Institutional Animal Care and Use Committee.

2.2. Real-time reverse transcriptase–polymerase chain reaction

Polymerase chain reactions (PCRs) were performed in a final volume of 20 μL in LightCycler glass capillaries (Roche, Mannheim, Germany). The reaction mixture consisted of 2 μL LightCycler-FastStart DNA Master SYBR Green I (Roche), 2.4 μL 25 mmol/L $MgCl_2$ stock solution, 11.6 μL sterile PCR-grade H_2O, 2 μL of the complementary DNA template for each gene of interest, and 1 μL of 10 μmol/L of each primer. The sequences of the forward and reverse rat GLUT2 primers were 5′-TTAGCAACT-GGGTCTGCAAT-3′ and 5′-GGTGTAGTCCTACACT-CATG-3′, respectively. The cycling program consisted of initial denaturation for 10 minutes at 95°C followed by 55 cycles of 95°C for 5 seconds, 62°C for 5 seconds, and 72°C for 15 seconds, with a 20°C/s slope. Each set of PCRs included a water sample as a negative control and 5 dilutions of a standard. Known amounts of DNA were diluted to provide the standards, and a regression curve of crossing points vs concentration was generated using the LightCycler. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping standard.

2.3. Western blot analysis

INS-1 cells were processed as described previously [10]. The proteins were separated using a 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane for immunoblotting. The membranes were incubated in 0.1% Tween-20 in phosphate-buffered saline (PBS-T) containing anti-GLUT2 antibody (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). An antibody for GAPDH (diluted 1:1000; TREVIGEN, Gaithersburg, MD) was used as the internal standard for the cytosolic extract. The membranes were then washed with PBS-T and incubated for 1 hour at room temperature in PBS-T containing a second antibody linked to horseradish peroxidase. The signal was visualized using an enhanced chemiluminescence detection kit (ECL; GE Healthcare, Buckinghamshire, United Kingdom).

2.4. Transfection of INS-1 cells and luciferase reporter gene assay

To confirm the transcriptional regulation of the GLUT2 promoter by exendin-4, we used a plasmid construct containing the rat pancreatic GLUT2 promoter, obtained by PCR amplification, cloned in front of the luciferase reporter gene as previously described [11]. Purified reporter plasmid was cotransfected with a CaMKK/CaMKIV-expressing plasmid or an empty vector and a β-galactosidase expression plasmid for determining transfection efficiency into the INS-1 cells (at 80% confluence)) using Lipofectamine (Life Technologies, Gaithersburg, MD). The complementary DNAs of both the Ca^{2+}/CaM-independent mutant of CaM-KIV (CaM-KIVc, 305 HMDT to DEDD) and the constitutively active CaM-KK mutant (CaM-KKc, residues 1-434) were generated as described previously [12,13]. Transfected cells were maintained in control media for 24 hours as previously described [14]. These cells were subsequently harvested, and the β-galactosidase activity was measured in an aliquot of cytoplasmic preparation. Twenty-microliter aliquots of the same preparation were used for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

2.5. Transfection of small interfering RNA

The small interfering RNAs (siRNAs) designed to target CaMKIV or scramble were purchased from Santa Cruz. Transfection of CaMKIV siRNA was performed

using siPORT Amine (Ambion, Austin, TX) as described previously [2].

2.6. Statistical analysis

Statistical comparisons were performed using one-way analysis of variance and Student *t* test, with a *P* level < .05 considered as significant.

3. Results

3.1. Exendin-4 increases the expression of GLUT2 in INS-1 cells

The effect of exendin-4 on GLUT2 expression in INS-1 cells was examined by exposing the cells to varying concentrations (0-10 nmol/L) of exendin-4 for 24 hours. GLUT2 expression was examined by Western blot analysis with anti-GLUT2 antibody. The results showed an induction of the GLUT2 protein in response to increasing concentrations of exendin-4, with maximal induction observed at 10 nmol/L (Fig. 1A). To confirm this observation, we used real-time PCR analysis to assess the level of GLUT2 messenger RNA (mRNA) transcription in the cells. Consistent with the induction of the amount of GLUT2 protein by exendin-4 (Fig. 1A), a significant increase of GLUT2 mRNA transcription was observed following treatment with 10 nmol/L exendin-4 (Fig. 1B). In contrast, GAPDH expression levels remained unaltered by this treatment. We have confirmed the effect of exendin-4 using rat pancreatic islets. Fig. 1C showed that exendin-4 also stimulated the expression of GLUT2 in rat pancreatic islets.

3.2. Effects of exendin-4 on GLUT2 promoter activity

On the basis of the aforementioned results indicating that exendin-4 induces increases in both the protein and mRNA levels of GLUT2 in INS-1 cells, we conjectured that the transcriptional activity of the GLUT2 promoter is regulated by exendin-4 in these cells. To examine this

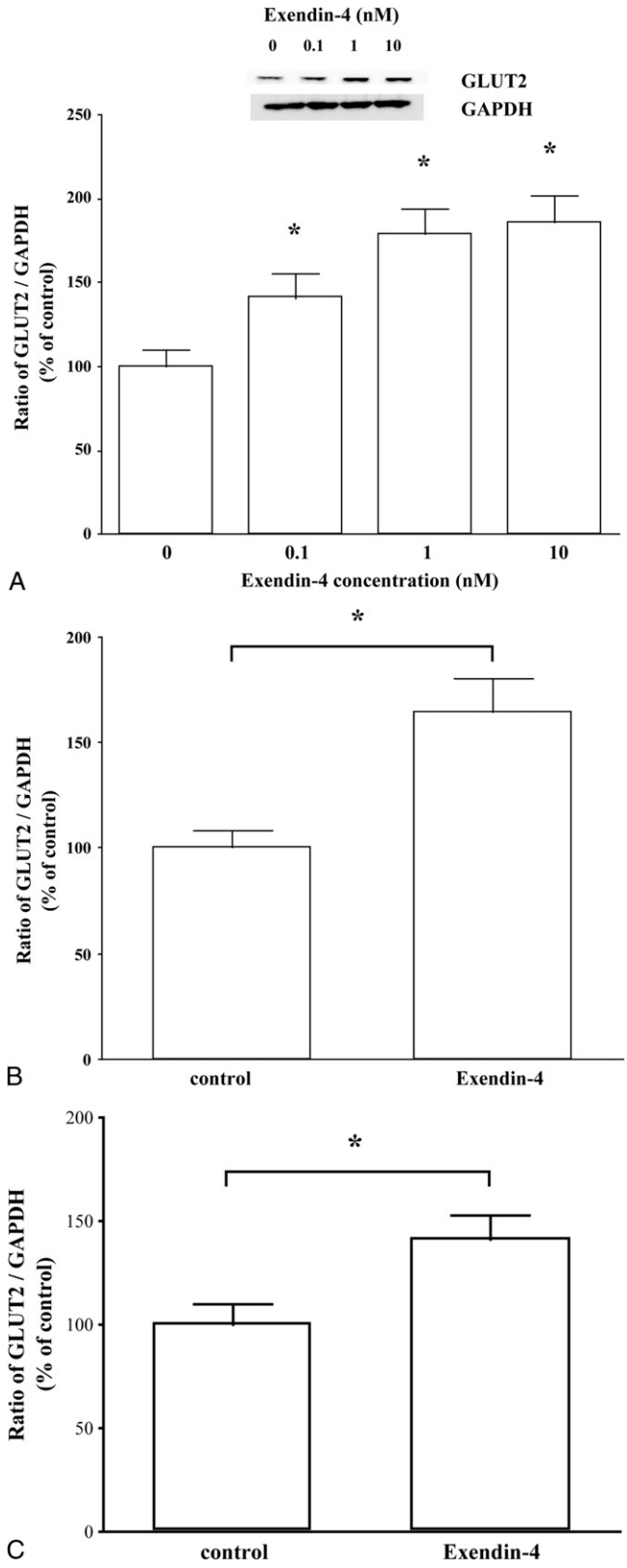

Fig. 1. Effect of exendin-4 on GLUT2 expression in rat pancreatic islets and INS-1 cells. A, Total cell lysate was purified from rat pancreatic islets treated with indicated concentrations of exendin-4 for 24 hours. Western blot analysis was performed to examine GLUT2 expression. Expression of GAPDH was studied as the control, and the results are shown in the bottom lanes. The plot shows the ratio of GLUT2/GAPDH. Results are represented as mean ± SEM of 3 experiments for each treatment group. *Significant difference ($P < .01$). B, Total RNA was extracted from the INS-1 cells treated with 10 nmol/L of exendin-4 for 24 hours. Real-time PCR was performed to analyze the GLUT2 mRNA expression. The plot shows the ratio of GLUT2/GAPDH mRNA. Results are represented as mean ± SEM of 3 experiments for each treatment group. *Significant difference ($P < .01$). C, Total RNA was extracted from rat pancreatic islets treated with 10 nmol/L of exendin-4 for 24 hours. Real-time PCR was performed to analyze the GLUT2 mRNA expression. The plot shows the ratio of GLUT2/GAPDH mRNA. Results are represented as mean ± SEM of 3 experiments for each treatment group. *Significant difference ($P < .05$).

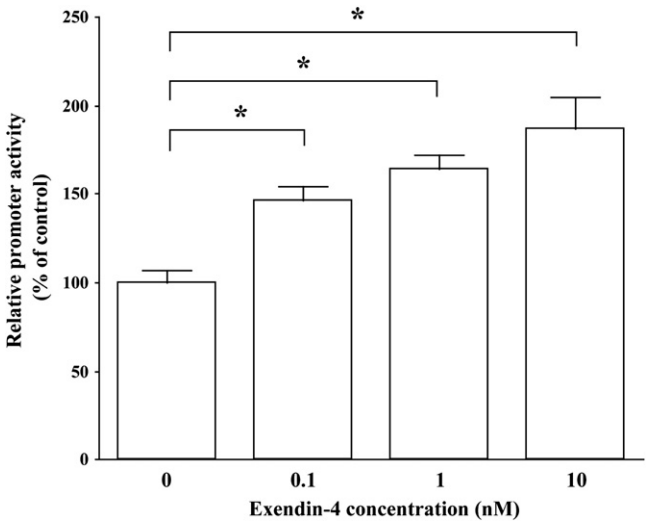

Fig. 2. Exendin-4 increases GLUT2 gene transcription. INS-1 cells were transfected with 1 μg pGLUT2-LUC and treated with the indicated concentrations of exendin-4 for 24 hours before cell harvesting. All assays were corrected for β-galactosidase activity, and the total amount of protein in each reaction was identical. The results were expressed as relative luciferase activity compared with that in the control cells arbitrarily set at 100. Each data point shows the mean ± SE of 4 separate transfections that were performed on separate days. *Significant difference ($P < .01$).

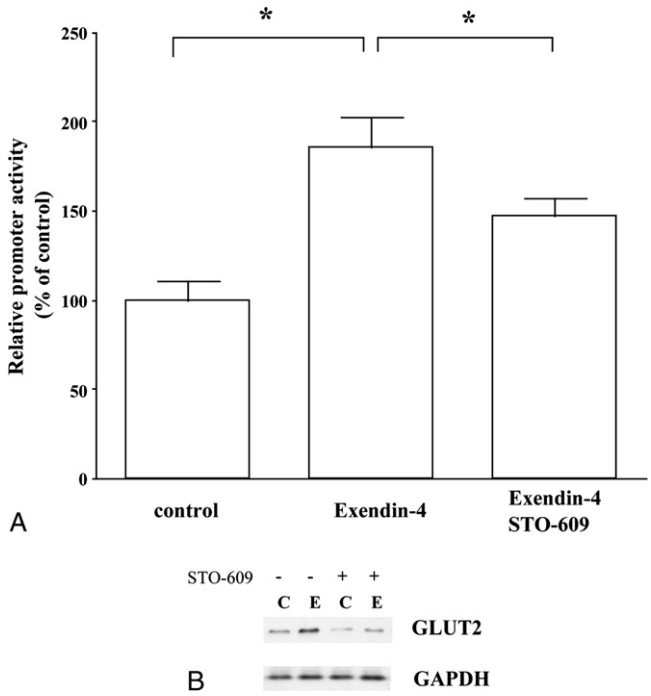

Fig. 3. Effects of the CaMK inhibitor STO-609 on GLUT2 transcriptional activity in INS-1 cells with 10 nmol/L exendin-4. A, INS-1 cells were transfected with 1 μg p-GLUT2-LUC and treated with 10 nmol/L exendin-4 for 24 hours before cell harvesting. The effects of the CaMK inhibitor (1 μmol/L STO-609) on GLUT2 transcriptional activity in INS-1 cells were examined with 10 nmol/L exendin-4. All assays were corrected for β-galactosidase activity, and the total amount of protein in each reaction was identical. The results were expressed as relative luciferase activity compared with that in the control cells arbitrarily set at 100. Each data point shows the mean ± SE of 4 separate transfections that were performed on separate days. *Significant difference ($P < .01$). B, GLUT2 expression after stimulation by exendin-4 in the presence and absence of the CaMKK inhibitor (STO-609). Western blot analysis of total cell protein extracted from INS-1 cells 24 hours after treatment with control media containing STO-609 (STO-609+) or DMSO (−) with (E) or without (C) 10 nmol/L exendin-4 is shown. Abundance of GAPDH served as a control and is shown on the bottom of each lane. An identical experiment independently performed gave similar results.

hypothesis, we measured GLUT2 promoter activity using the luciferase activity of pGLUT2-LUC in transfected INS-1 cells in the presence of the indicated amount of exendin-4 (Fig. 2A). As a consequence, exendin-4 was found to have a stimulatory effect on the activity of the GLUT2 promoter. The results showed an induction of GLUT2 promoter activity in response to increasing concentrations of exendin-4, with maximal induction being observed at 10 nmol/L (Fig. 2A).

3.3. CaMKK-mediated and exendin-4–induced expression of the GLUT2 gene

To further examine the mechanism by which exendin-4 stimulates GLUT2 gene expression, we examined the intracellular signaling mediated by exendin-4–induced stimulation of GLUT2 promoter activity. Previously, we reported that cells treated with exendin-4 exhibit an increased level of CaMKIV phosphorylation at Thr^{196} [15]. When INS-1 cells were exposed to 10 nmol/L exendin-4 in the presence of a CaMKK inhibitor (1 μmol/L STO-609), the stimulation of GLUT2 promoter activity by exendin-4 was reduced (Fig. 3). To confirm this result, we examined the effect of STO-609 on exendin-4–induced GLUT2 protein expression in intact cells. The expression level of GLUT2 stimulated by exendin-4 was accordingly attenuated by STO-609 treatment (Fig. 3B), indicating that the CaMKK-mediated signaling cascade may be involved in exendin-4–induced stimulation of GLUT2 promoter activity.

3.4. Effect of the CaMK cascade on GLUT2 gene transcription

These results indicate that the CaMKK/CaMKIV cascade is probably a signaling cascade that is involved in the activation of GLUT2 transcription by exendin-4. Therefore, we examined the potential role of the CaMKK/CaMKIV cascade by cotransfecting INS-1 cells with p-GLUT2-LUC plus expression vectors for CaMKK and/or CaMKIV to determine whether these exogenously expressed kinase affected GLUT2 gene transcription. The results showed that constitutively active CaMKK (CaMKKc) alone stimulated a 3-fold increase in GLUT2 promoter activity in unstimulated INS-1 cells (Fig. 4). Furthermore, the cotransfection of wild-type CaMKIV along with constitutively active CaMKK (CaMKKc)

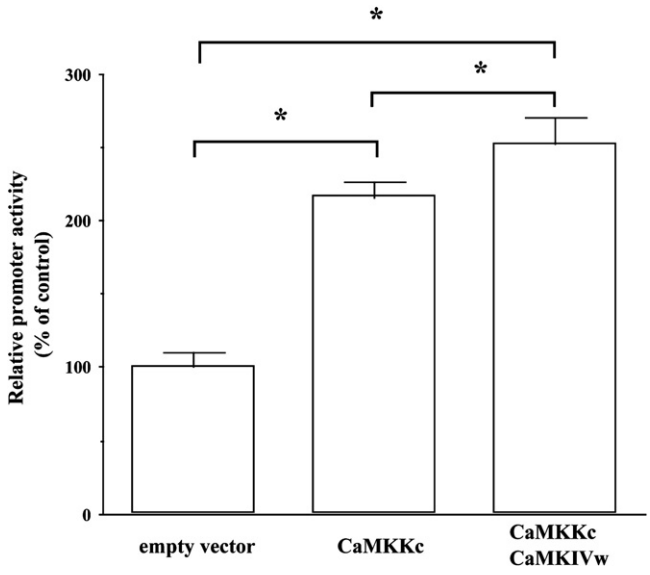

Fig. 4. Effect of the CaMKK/CaMKIV cascade on GLUT2 promoter activity. The cells were transfected with p-GLUT2-LUC and empty vector, CaMKKc, and CaMKKc/CaM-KIVw expression vectors. The results are expressed as relative luciferase activity compared with control cells arbitrarily set at 100. Each data point shows the mean ± SE (n = 3) of separate transfections. *Significant difference ($P < .05$).

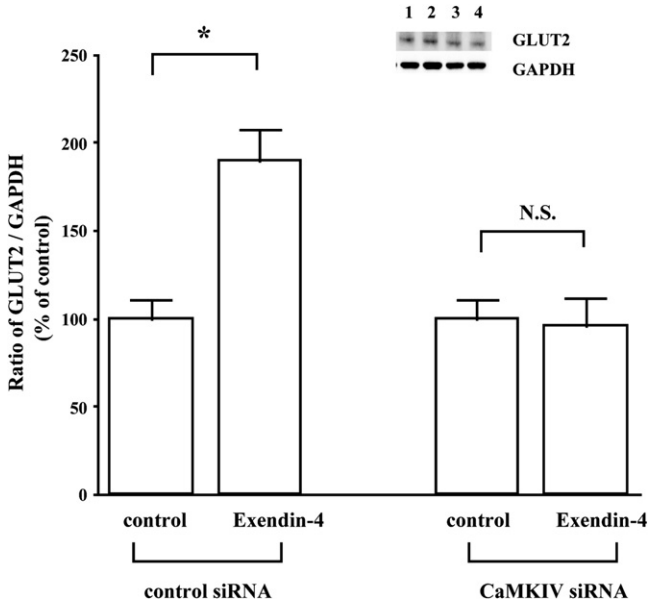

Fig. 5. Effects of CaMKIV knockdown on GLUT2 expression in INS-1 cells. SiRNA of CaMKIV (si CaMKIV) or scrambled siRNA (si Control) was transfected into INS-1 cells and then treated with exendin-4 (Ex-4). At 24 hours after transfection, the abundance of GLUT2 protein level was measured using Western blot analysis (upper panel). The ratio of GLUT2 to GAPDH is shown as the percentage of control. Each data point shows the mean ± SE (n = 3) of separate experiments. *Significant difference ($P < .05$). NS indicates no significant difference. Lanes 1 and 2, si Control; lanes 3 and 4, si CaMKIV; lanes 1 and 3, vehicle; lanes 2 and 4, 10 nmol/L exendin-4.

caused a 5-fold increase in transcriptional activation of the GLUT2 gene. This is likely due to the fact that the CaMKK/CaMKIV cascade is inactive in the absence of cell stimulation.

3.5. Role of CaMKIV in exendin-4–induced GLUT2 gene expression

Earlier studies identified the components of the CaMKK cascade in exendin-4–induced GLUT2 promoter activity. The lack of information on whether this signal transduction cascade mediates the action of exendin-4 in INS-1 cells led us to examine whether CaMKIV plays a role in exendin-4–induced transcription of the GLUT2 gene (Fig. 4). To further characterize the role of CaMKIV in the exendin-4–mediated signaling that enhances GLUT2 expression, we used siRNA to block CaMKIV expression. INS-1 cells were exposed to CaMKIV specific or scramble siRNA and then treated with exendin-4. As shown in Fig. 5, GLUT2 protein expression was increased in cells exposed to scrambled siRNA following stimulation with 10 nmol/L exendin-4. In contrast, exendin-4 induction of GLUT2 protein expression was significantly suppressed in cells treated with CaMKIV siRNA. The abovementioned findings support the idea that CaMKIV plays a role in exendin-4–induced GLUT2 expression.

4. Discussion

In this study, we found that the GLP-1 analogue exendin-4 stimulated GLUT2 expression in the INS-1 pancreatic β-cell line. The insulin secretagogue hormone GLP-1 and its structurally related peptide analogue, namely, exendin-4, are potent stimulators of the pancreatic β-cell GLP-1 receptor [1-3]. When administered to type 2 DM subjects, exendin-4 exerts multiple antidiabetogenic effects: it stimulates insulin secretion, lowers fasting blood glucose levels, and attenuates the elevation in blood glucose levels after ingestion of a meal. Such beneficial effects indicate its usefulness as a new treatment agent for diabetes [1].

GLUT2 contributes to the sensing of glucose [16] not only by fueling the metabolic signaling cascade but also by triggering a specific protein signaling pathway. Indeed, GLUT2 cannot always be replaced by another GLUT isoform, suggesting that the GLUT2 protein has particular qualities [17]. When β-cells are engineered with GLUT isoforms to provide a similar glucose flux, only GLUT2 allows normal insulin production in response to glucose [18]. Furthermore, there is a close correlation between the level of GLUT2 and glucose-sensitive gene expression in hepatomas [19] and in engineered β-cells [20]. In addition, only GLUT2-transported sugars are efficient stimulators of the transcription of glucose-sensitive genes [21]. This is directly supported by studies with GLUT2-null mice, in which the absence of GLUT2 impairs the glucose

stimulation of sensitive gene expression, for example, the insulin gene in pancreatic β-cells and L-pyruvate kinase in liver and intestine [22-24]. GLUT2 is generally considered to be a minor actor in the glucose-sensing apparatus involved in the glucose-induced secretion of insulin by pancreatic β-cells, glucokinase (GK) being the major player [25]. A mutation in GK can lead to maturity-onset DM in youth [26,27]. A recent study showed that haploinsufficiency of β-cell–specific GK (*GK*[+/−]) caused impaired insulin secretion in response to glucose stimulation [28]. Previously, we showed that exendin-4 also has a stimulatory effect on GK expression at the transcriptional level [15]. The activation of the CaMKK/CaMKIV cascade by exendin-4 stimulated *GK* gene transcription. These results together with those obtained in the present study suggest that exendin-4 may improve the function of pancreatic β-cells by the activation of GK and GLUT2 via the CaMKK/CaMKIV pathway. The activation of GK and GLUT2 by GLP-1 may offer a potential strategy for treating the decreased insulin secretion and decreased β-cell mass in type 2 DM patients.

Previous studies have demonstrated that GLP-1 activates multiple signaling pathways in the β-cells. These pathways involve protein kinase A, CaMK, mitogen-activated protein kinases (MAPK, ERK1/2), PI-3K, protein kinase B (*Akt*), and atypical protein kinase C-ζ [29]. The endogenous β-cell GLP-1 receptor is coupled to adenylyl cyclase, cell depolarization, activation of voltage-dependent Ca^{2+} channels, and induced extracellular Ca^{2+} influx [30]. We have identified the role of the CaMKK/CaMKIV cascade in GLUT2 expression in response to exendin-4. Numerous studies have demonstrated that the CaMKK/CaMKIV cascade is present and functional in various cell types, including pancreatic β-cells [31-33]. We previously reported that both pancreatic β-cells and the insulin-secreting cell line INS-1 have this CaMKK/CaMKIV cascade and that this signal cascade plays an important role in glucose–up-regulated transcriptional activation of the insulin gene [34]. In this study, exendin-4 induced the phosphorylation of CaMKIV at Thr^{196} by CaMKK and then stimulated the expression of GLUT2 catalyzing the uptake of glucose into the cell, raising the possibility that the activated CaMKIV might mediate the stimulatory effect of glucose-dependent insulin secretion by exendin-4. CaMKIV, which has significant nuclear localization, phosphorylates transcription factors such as cyclic adenosine monophosphate–responsive element binding protein and serum response factor [35]. GLUT2 is a glucose-sensitive gene found in liver cells [36], together with the genes encoding L-type pyruvate kinase, S14, and fatty acid synthase [37]. Carbohydrate response element-binding protein, a recently identified transcription factor, mediates glucose-induced transcription [38]. Intriguingly, the GLUT2 promoter does not appear to contain a carbohydrate response element-binding protein–binding sequence (carbohydrate response element); rather, it binds sterol regulatory element-binding protein–1C on a sterol-responsive element [39]. Further investigation is required to clarify the mechanisms by which the CaMKK/CaMKIV cascade mediates transcriptional regulation of the GLUT2 gene.

In summary, we examined the role of the CaMKK/CaMKIV cascade in exendin-4–induced GLUT2 gene expression in the insulin-secreting pancreatic β-cell line INS-1. The results indicate that activation of the CaMKK/CaMKIV cascade by exendin-4 stimulates GLUT2 gene transcription, suggesting that exendin-4 may improve the function of pancreatic β-cells by the activation of GLUT2.

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