The impact of Ca^{2+}/calmodulin-dependent protein kinase II on insulin gene expression in MIN6 cells

Short title: CaMKII regulates insulin promoter activity

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Abstract

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is expressed in insulin-secreting β cells. However, the effects of CaMKII on insulin synthesis are unknown. Although Ser133 phosphorylation of cyclic AMP-responsive element-binding (CREB) typically increases CREB transcriptional activity, CaMKII phosphorylates CREB at Ser142 and at Ser133 to exert a dominant inhibitory effect. Our objective was to characterize the role of CaMKII in insulin gene expression. In MIN6 cells, insulin gene promoter activity was significantly down-regulated by wild-type (WT) CaMKIIδ2, but was significantly upregulated after small interfering RNA (siRNA) knockdown of CaMKIIδ expression. These results were independent of glucose concentrations and membrane depolarization. Insulin mRNA levels were also decreased by WT CaMKIIδ2 and increased by CaMKIIδ siRNA. Downregulation of insulin gene promoter activity by WT CaMKIIδ2 was partly mediated via cyclic AMP-responsive element 2. WT CaMKIIδ2 significantly increased CREB phosphorylation at Ser142 and significantly decreased binding to CREB binding protein (CBP), whereas kinase dead CaMKIIδ2 did not. Our results indicate that CaMKIIδ2 downregulates insulin gene expression by Ser142 phosphorylation of CREB and reducing binding of CREB to CBP.

Keywords: CaMKIIδ2, CREB, CREB binding protein, CREB Ser142, Phosphorylation, Insulin gene promoter

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multi-functional serine/threonine protein kinase. It belongs to a family of 81 proteins in humans that play important role in cellular signaling via Ca^{2+} signal transmission [1]. In mammals, CaMKII is expressed by four closely related genes, α, β, γ, and δ, each of which produces mRNA that can be alternatively spliced to generate ~30 CaMKII variants. The major activators of CaMKII are Ca^{2+} and calmodulin [2]. Increases in intracellular Ca^{2+} cause the formation of a complex between Ca^{2+} and calmodulin. This complex then binds to the regulatory domain of CaMKII, inducing autophosphorylation of Thr287 in CaMKIIβ, γ and δ, or Thr286 in CaMKIIα, which activates CaMKII protein kinase activity. Activated CaMKII phosphorylates numerous target proteins and is involved in various cellular functions including trafficking, docking, fusion of secretory granules, regulation of gene expression, modulation of ion channel function, and intracellular signal transduction [3–6].

Several CaMKII isoforms, including CaMKII β, δ, and γ, have been identified in rat pancreatic islets [7, 8] and insulinoma cell lines [3, 9], although CaMKIIα mRNA expression was low in islet β cells [10]. Intracellular Ca^{2+} plays a critical role in glucose-induced insulin secretion in β cells [11] as it induces Ca^{2+}/calmodulin complex formation, followed by auto-phosphorylation and activation of CaMKII [12, 13]. CaMKIIδ2 is involved in glucose-induced insulin secretion, possibly by phosphorylating synapsin I [14], a critical step in insulin granule exocytosis in MIN6 cells.

Insulin synthesis in β cells is important for maintaining glucose homeostasis.

Transcriptional control of the insulin gene is modulated by *cis*-acting regulatory sequences located from the transcriptional start site to –400 bp upstream. Cyclic AMP (cAMP) responsive elements (CREs) bind to a broad array of closely related members of the basic leucine zipper domain cAMP-responsive element (CRE) binding protein (CREB)/activating transcription factor (ATF) family [15]. Wu et al. [4] previously reported dominant inhibitory effects of CaMKII on CREB. CaMKIV and protein kinase A (PKA) stimulate CRE-dependent gene expression by phosphorylating CREB at Ser133. Under these conditions, CREB exists as a dimer on CREs in its target genes and binds to CREB binding protein (CBP), which activates gene transcription. CaMKII phosphorylates CREB at Ser133 and Ser142. Phosphorylation at Ser142 prevents CREB dimerization on DNA and binding with CBP, thus exerting a dominant inhibitory effect [4]. Because CaMKII is a major CaMK isoform in β cells [15], we hypothesized that CaMKII is activated by hyperglycemia-induced Ca^{2+} influx, followed by inhibition of CREB and insulin gene transcription. Therefore, in this study, we investigated the effects of CaMKII on CREB phosphorylation and insulin gene promoter activity.

2. Materials and methods

2.1. Cells and reagents

MIN6 cells were a kind gift from Dr. Susumu Seino (Division of Cellular and Molecular Medicine, Kobe University, Graduate School of Medicine, Kobe, Japan) and were cultured under standard conditions in DMEM supplemented with 25 mM glucose and 15% heat-inactivated FBS. The anti-phospho-Ser142-CREB antibody was provided by Dr. Michael E. Greenberg (Harvard Medical School, Boston, MA). Mouse monoclonal and rabbit polyclonal anti-phospho-Ser133-CREB antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-CREB and mouse monoclonal anti-CBP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-synapsin I and rabbit polyclonal anti-phosho-Ser603-synapsin I antibodies were purchased from Millipore (Merck Millipore, MA). The rabbit polyclonal anti-CaMKIIδ antibody was purchased from Trans Genic Inc. (Kumamoto, Japan).

2.2. Expression and reporter plasmid construction

pCAGGSneo expression vectors carrying WT and kinase dead (KD) forms of CaMKIIδ2 were provided by Dr. Yusuke Takeuchi (Tohoku University; Supplemental Figure 1) [16]. Promoter activity of the human insulin gene was analyzed using pGL3, a low background promoterless luciferase reporter vector [17]. The phINS356LUC plasmid contained the –356 to +14 portion of the human insulin gene promoter. CRE2

and CRE1/2 site mutations were generated using a GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) with the respective primers (Supplemental Table 1). Plasmids mutated at CRE1 and CRE1/2 were designated phINS356m2LUC and phINS356m1/2LUC, respectively.

2.3. Transient transfection and luciferase reporter assays

MIN6 cells (5×10^5 cells/well) were seeded in 35 mm or six-well plates for plasmid transfection using HilyMax reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Cells were incubated for 3 h with 1 ml of serum-free transfection cocktail containing 1–2 μg WT or KD CaMKIIδ2 expression and luciferase vectors. Whole cell protein extracts were prepared 48 h later. Then, cells were cultured DMEM supplemented with 15% FBS plus either 25 mM glucose, 3 mM glucose or 30 mM KCl, for 12 h before the luciferase assay. Firefly and Renilla luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

2.4. RNA interference

Small interfering RNA (siRNA) against mouse CaMKIIδ, ON-TARGETplus SMARTpool siRNA(J-040821-05-0005), and negative control ON-TARGETplus siCONTROL Non-targeting pool(D-001810, siNC) were purchased from Dharmacon (Denver, CO). Approximately 1×10^6 MIN6 cells were microporated with 60 nmol of siRNA according to the manufacturer's instructions (LMS Co., Ltd, Tokyo, Japan) and

tested 24 h later.

2.5 .Immunoprecipitation.

MIN6 cells were transfected with pCAGGSneo plasmid alone, WT or KD CaMKIIδ2 expression plasmids. Immunoprecipitation was performed as previously described [16]. The eluate was then subjected to western blot analysis.

2.6. Immunoblotting

The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). Western blotting was performed as previously described [18].

2.7. Real-Time qRT-PCR

qRT-PCR was performed as previously reported [18] using primers for the human insulin gene (Supplemental Table 2). Insulin mRNA expression levels were quantified after normalization for β-actin mRNA levels. A melting curve analysis was performed to assess PCR product specificities.

2.8. Statistical analysis

Data are expressed as means ± standard error of the mean from at least three

experiments. Data were statistically analyzed using unpaired two-tailed *t* tests for two groups, while differences among more than two groups were assessed by analysis of variance and Fisher's protected least significant difference test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of WT or KD CaMKIIδ2 overexpression and CaMKIIδ siRNA in MIN6 cells

MIN6 cells were transiently transfected with pCAGGSneo carrying either WT or KD CaMKIIδ2. In KD CaMKIIδ2, Lys43 was substituted with Ala, which inhibited the ATP binding and kinase activities of CaMKIIδ2 [19] (Supplemental Figure 1). WT and KD CaMKIIδ2 overexpression and siRNA-induced knockdown of CaMKIIδ expression in MIN6 cells were confirmed by western blot analysis and qRT-PCR. WT and KD CaMKIIδ2 overexpression increased protein expression by ~2-fold compared with the expression of endogenous CaMKIIδ2 (Fig. 1A). CaMKIIδ siRNA significantly reduced CaMKII expression at the protein (Fig. 1A) and mRNA (Fig. 1B) levels. To evaluate CaMKII kinase activity, we performed western blot analysis using anti-phospho-Ser603-synapsin I antibodies. This test revealed that phosphorylation of synapsin I was significantly increased by WT CaMKIIδ2 and decreased by CaMKIIδ siRNA (Fig. 1C).

3.2. Effects of CaMKIIδ2 on insulin gene promoter activity in MIN6 cells

We performed luciferase assays using MIN6 cells cultured in DMEM supplemented with 3 mM glucose, 25 mM glucose, or 30 mM KCL (Fig. 2A). Insulin promoter activity was significantly increased in cells cultured in 25 mM glucose and significantly decreased in those cultured in 30 mM KCl compared with that in cells

cultured in 3 mM glucose. We then examined the effects of WT CaMKIIδ2, KD CaMKIIδ2, and CaMKIIδ siRNA on insulin gene promoter activity. Insulin promoter activity was significantly increased by suppressing CaMKII expression with CaMKIIδ siRNA (Fig. 2B). WT CaMKIIδ2 overexpression significantly decreased insulin gene promoter activity to 10.2 ± 1.5% of the control, whereas KD CaMKIIδ2 overexpression did not affect promoter activity (Fig. 2C). Next, we evaluated the effects of glucose or KCl-induced membrane depolarization on insulin gene promoter activity regulated by CaMKII. The effects of WT CaMKIIδ2 and KD CaMKIIδ2 on insulin gene promoter activity were generally unchanged in 25 and 3 mM glucose and 30 mM KCl (10.2 ± 3.0%, 17.1 ± 10.4%, and 19.5 ± 2.2%, respectively, for WT; 59.8 ± 23.8%, 75.6 ± 41.7%, and 84.5 ± 21.9%, respectively, for KD; Fig. 2C, D,E). These results suggest that insulin gene promoter activity was downregulated by CaMKIIδ2 independent of glucose concentration and membrane depolarization.

3.3. Effects of CaMKIIδ2 overexpression or knock-down on insulin mRNA levels in MIN6 cells

To determine whether the changes in insulin gene promoter activity also affected insulin mRNA levels, we measured insulin mRNA levels in MIN6 cells. Insulin mRNA was significantly decreased by 73.3 ± 16.0% ($p<0.05$) by WT CaMKIIδ2, but was significantly increased by 221.4 ± 26.7% ($p<0.01$) by CaMKIIδ siRNA (Fig. 3A). These observations further confirm that insulin gene promoter activity is downregulated by activation of CaMKIIδ2.

3.4. Identification of cis*-acting regulatory sequences in the insulin gene promoter*

To assess whether CREs in the human insulin gene promoter were involved in the downregulation of insulin gene promoter activity by WT CaMKIIδ2 overexpression, we performed luciferase assays using the insulin gene promoter bearing mutations in CREB binding sites, CRE1 and 2 (CRE1/2), and CRE2 (Supplemental Figure 2). As shown in Fig. 3B, a CRE2 mutation partially but significantly rescued the inhibitory effect of WT CaMKIIδ2 overexpression on insulin gene promoter activity (from 10.2 ± 1.5% to 76.8 ± 10.3%), whereas mutations of CRE1/2 did not have additional effects (from 10.2 ± 1.5% to 52.8 ± 0.1%). These data suggest that CaMKIIδ2-induced downregulation of human insulin gene promoter activity is mediated by CRE2 but not by CRE1.

3.5. Effects of CaMKIIδ2 on Ser142 phosphorylation of CREB and binding to CBP in MIN6 cells

Because downregulation of insulin gene promoter activity by CaMKIIδ2 was partially rescued by a CRE2 mutation, we hypothesized that Ser142 of CREB was phosphorylated by CaMKIIδ2, which might decrease binding of CREB to CBP and suppression of CREB-mediated transcription. To test this hypothesis, we performed western blot analysis using an anti-phospho-Ser142-CREB antibody. While CREB phosphorylation at Ser142 and Ser133 was significantly increased by WT CaMKIIδ2

(Ser142: 168.4 ± 16.3%; Ser133: 172.5 ± 12.6%), CaMKIIδ siRNA markedly decreased phosphorylation of CREB at Ser142 and Ser133 (Ser142: 21.4 ± 8.7%; Ser133: 47.3 ± 8.1%; Fig. 4A). To confirm that CaMKIIδ2 regulates insulin gene transcription by preventing CREB binding to CBP, we performed immunoprecipitation followed by western blot analysis using anti-CBP and anti-CREB antibodies (Fig. 4B). CREB binding to CBP was significantly decreased by WT CaMKIIδ2 (48.3 ± 5.9%), but not by KD CaMKIIδ2 (107.9 ± 62.7%). Taken together, these data indicate that CaMKIIδ2 inhibits CREB binding to CBP by phosphorylating CREB at Ser142, which downregulates insulin gene promoter activity.

4. Discussion

In this study, we demonstrated that CaMKIIδ2 inhibited insulin gene promoter activity in MIN6 cells. Several studies have described the role of CaMKIIδ2 in Ca^{2+}-dependent insulin secretion [3, 9, 10, 13, 20-22]. However, the role of CaMKIIδ2 in insulin gene transcription has not been thoroughly investigated. In MIN6 cells, we demonstrated that insulin gene promoter activity and insulin mRNA expression were decreased by WT CaMKIIδ2 and increased by CaMKIIδ siRNA.

Insulin gene transcription is regulated by increases in extracellular glucose and Ca^{2+} levels, nutrient availability, and hormone signaling [23]. In MIN6 cells, we found that insulin promoter activity was activated by 25 mM glucose and suppressed by 30 mM KCl compared with that in 3 mM glucose. We hypothesized that KCl may enhance the inhibitory effects of WT CaMKIIδ2 overexpression on insulin gene promoter activity because β cell stimulation with insulin secretagogues, such as glucose, tolbutamide, and high K^+, enhanced CaMKII activity [3, 22]. However, high glucose and K^+ did not affect CaMKIIδ2-mediated downregulation of insulin gene promoter activity (Fig. 2C–E), suggesting that the inhibitory effects of CaMKIIδ2 are independent of glucose and membrane depolarization.

CaMKII inhibits the transcriptional activity of various genes with CREs in the promoter to a greater degree than the stimulatory effects of PKA [21] and CaMKIV [24-26]. Only primates possess CRE1~4 in the insulin gene, but the CRE2 sequence corresponds to a single CRE in other mammals, including rodents [27]. Our results suggest that CRE2, but not CRE1, is the primary target of CaMKIIδ2-mediated

downregulation of insulin gene promoter. The most likely explanation for this observation is that CRE1 overlaps with an A3 site (Supplemental Figure 2). The A3 site is a binding site for pancreatic and duodenal homeobox gene-1 (PDX-1), which is mainly expressed in β cells and is required for pancreatic development and glucose-induced insulin gene transcription [28]. Overlapping of the A3 site with CRE1 might result in occupation of CRE1 by PDX-1, thereby preventing other transcription factors from binding to this region.

A mechanism for CaMKII-mediated down-regulation of CRE-dependent genes expression has been proposed. CREB phosphorylation at Ser142 inhibits CREB dimerization, and reduces binding of CREB to CBP [4, 29]. CaMKII-induced phosphorylation of CREB at Ser133 and Ser142 does not stimulate CREB-dependent gene transcription [24, 25]. This mechanism is consistent with our current results.

We found that CRE1/2 and CRE2 mutations partially rescued the downregulation of insulin gene promoter activity caused by CaMKIIδ2. The insulin gene promoter is organized in a complex arrangement of discrete *cis*-acting sequence motifs, and its activity is controlled by various mechanisms involving several *trans*-activating factors, including CREB, PDX-1, and NeuroD. PDX-1 promotes insulin gene transcription in a glucose-dependent manner [30]. CaMKII mediates phosphorylation of NeuroD at Ser336 in primary neurons [31]. Therefore, the partial rescue of insulin gene promoter activity mediated by CRE1/2 and CRE2 mutations might be due to interactions between CaMKIIδ and other transcriptional factors, including PDX-1 and/or NeuroD.

Osterhoff et al. [32] demonstrated that insulin gene transcription was downregulated by CaMKIIδ2 inhibition and upregulated by CaMKIIδ2 overexpression.

They proposed that the mechanism involved CaMKIIδ2 in the activation of PDX-1, and that CaMKIIδ2 did not directly phosphorylate the transcription factors. However, our study showed that insulin promoter activity was increased by CaMKIIδ siRNA and decreased by phosphorylation of CREB at Ser142 as a result of WT CaMKIIδ2 overexpression. The cause of this discrepancy is unknown, but may be due to differences in experimental methods including the use of antisense-nucleotides.

Although WT CaMKIIδ2 overexpression sufficiently affected insulin gene promoter activity, KD CaMKIIδ2 did not affect insulin gene promoter activity. We used a KD form of CaMKIIδ2 that prevents ATP binding [19]. Wu et al. reported that CaMKII modifies CREB at Ser142, which inhibited CREB dimerization and binding to CBP, and that CREB phosphorylated at Ser142 has a dominant inhibitory effect [4]. KD CaMKIIδ2 expression might be insufficient to inhibit Ser142 phosphorylation of CREB caused by endogenous CaMKII.

Some patients with type 2 diabetes show increases in blood glucose levels after long-term sulfonylurea therapy [33]. This phenomenon, known as secondary sulfonylurea failure, is thought to be a result of progressive β cell functional loss over the course of type 2 diabetes [34]. In β cells, prolonged hyperglycemia and long-term use of sulfonylurea may induce chronic Ca^{2+} influx followed by prolonged CaMKII activation. Therefore, we propose that downregulation of insulin gene transcription by CaMKII overexpression may be involved in decreased insulin synthesis caused by sustained hyperglycemia and/or long-term use of sulfonylurea. Further studies are needed to examine the effects of CaMKII on glucose toxicity and secondary sulfonylurea failure.

In conclusion, our study showed that CaMKIIδ2 regulates the insulin gene promoter. CaMKIIδ2 enhanced CREB phosphorylation at Ser133 and Ser142, which might inhibit CREB binding to CBP, and downregulation of insulin gene promoter activity. Therefore, CaMKIIδ2 may play an important role in insulin gene expression and the pathogenesis of diabetes.

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Figure legends

Fig. 1. Expression of WT and KD CaMKIIδ2. (A) Effects of WT and KD CaMKIIδ2 overexpression and siRNA knock down of CaMKIIδ in MIN6 cells. After transfection with the WT and KD CaMKIIδ2 vectors or CaMKIIδ siRNA, the lysates of MIN6 cells were subjected to western blot analysis using an anti-CaMKIIδ2 antibody. Upper panel: representative western blots. Lower panel: densitometric analysis of the western blot. (Mean ± SE, n=8); *$P < 0.01$ versus basal levels, (B) mRNA expression of CaMKII in MIN6 cells transfected with CaMKIIδ siRNA. Values are means ± SE (n=8). **$P <$ 0.001 versus control siRNA (C) CaMKII kinase activity evaluated by phosphorylation of Synapsin I, a CaMKII-specific substrate. MIN6 without vector, white dotted bar; Control vector, white bar; WT CaMKIIδ2 vector, striped bar; KD CaMKIIδ2 vector, black dotted bar; control siRNA, gray bar; CaMKIIδ siRNA, black bar. Values are means ± SE (n=8). *p<0.05 and **p<0.001 versus control (analysis of variance and Fisher's protected least significant difference test).

Fig. 2. Effects of CaMKIIδ2 overexpression or CaMKIIδ knock down on human insulin gene promoter activity. (A) Insulin promoter activity in MIN6 cells transfected with control vector and incubated in 3 mM glucose (white bar), 25 mM glucose (white dotted bar), or 30 mM KCl (checked bar). Values are means ± SE (n=8). *p<0.05 between the indicated groups. (B) Effects of CaMKIIδ siRNA transfection on insulin promoter activity in MIN6 cells. Control siRNA, gray bar; CaMKIIδ siRNA, black bar. Values are means ± SE (n=8) *p<0.001 between the indicated groups. (C–E) Effects of WT or KD

CaMKIIδ2 overexpression on insulin gene promoter activity after incubation in 25 mM glucose (C), 3 mM glucose (D), or 30 mM KCl (E). Control vector, white bar; WT CaMKIIδ2 vector, striped bar; KD CaMKIIδ2 vector, black dotted bar. Values are means ± SE (n=12), *p<0.05 and **p<0.001 vs control vector.

Fig. 3. (A) Effects of WT or KD CaMKIIδ2 expression vectors or CaMKIIδ siRNA overexpression on insulin mRNA levels in MIN6 cells. MIN6 cells were transfected with the indicated vector or CaMKIIδ siRNA, and lysates subjected to quantitative RT-PCR. Control vector, white bar; WT CaMKIIδ2 vector, striped bar; KD CaMKIIδ2 vector, black dotted bar; control siRNA, gray bar; CaMKIIδ siRNA, black bar. Values are means ± SE (n=10). *p<0.01 and **p<0.001 vs control. (B) Effects of CRE mutations on insulin gene promoter activity. MIN6 cells were transfected with a pGL3 luciferase vector containing the CRE-mutated human insulin gene promoter and each vector. Control vector, white bar; WT CaMKIIδ2 vector, striped bar; KD CaMKIIδ2 vector, black dotted ba. Values are means ± SE (n=14). *p<0.01 between the indicated groups.

Fig. 4. Effects of CaMKIIδ2 overexpression or CaMKIIδ siRNA on phosphorylation of CREB at Ser142 and Ser133, and binding to CBP. (A) Upper panel: representative western blot analysis of MIN6 cells transfected with WT or KD CaMKIIδ2 and CaMKIIδ siRNA using anti-phospho-Ser142 and -Ser133-CREB antibodies. Lower panel: densitometric analysis of the western blot for Ser142 phosphorylation. (B) Upper panel: immunoprecipitation and western blot analysis. MIN6 cell lysates were

immunoprecipitated with an anti-CREB antibody followed by immunoblotting with anti-CBP and anti-CREB antibodies. Lower panel: densitometric analysis of the western blot for CBP. Data are normalized for the expression of β-actin in the same sample and are expressed as a percentage of the control value. Control vector, white bar; WT CaMKIIδ2 vector, striped bar; KD CaMKIIδ2 vector, black dotted bar; control siRNA, gray bar; CaMKIIδ siRNA, black bar. Values are means ± SE (n=8). *p<0.01 vs control.