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ARTICLE

Calcium/calmodulin-dependent kinase IV controls glucose-induced *Irs2* expression in mouse beta cells via activation of cAMP response element-binding protein

S. J. Persaud · B. Liu · H. Barbosa Sampaio · P. M. Jones · D. S. Muller

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Abstract
Aims/hypothesis Irs2, which is upregulated by glucose, is important for beta cell plasticity. Cyclic AMP response element-binding protein (CREB) stimulates beta cell *Irs2* expression and is a major calcium/calmodulin-dependent kinase $(CaMK)_{IV}$ target in neurons. We therefore hypothesised that $CaMK_{IV}$ mediates glucose-induced *Irs2* expression in beta cells via CREB activation.
Methods The functions of $CaMK_{IV}$ and CREB were investigated in MIN6 beta cells and mouse islets using the CaMK inhibitor KN62, the calcium chelator bapta-(AM) and the voltage-dependent calcium channel inhibitor nifedipine. Small interfering RNAs were used to silence endogenous $CaMK_{IV}$ production and expression vectors to overproduce constitutively active and dominant negative forms of $CaMK_{IV}$ and CREB. *Irs1* and *Irs2* expression were determined by quantitative PCR and Western blotting, and the role of CREB was also investigated by assessing its phosphorylation on serine 133.
Results Increasing the glucose concentration from 2.5 to 25 mmol/l stimulated CREB phosphorylation on serine 133 and specifically stimulated *Irs2* but not *Irs1* expression. Similarly, overproduction of a constitutively active form of $CaMK_{IV}$ promoted sustained CREB phosphorylation and a significant increase in *Irs2* but not *Irs1* expression. In contrast, these stimulatory effects of glucose were all suppressed by overproducing an inactive $CaMK_{IV}$ mutant. Inhibition of glucose-induced calcium influx with nifedipine or chelation of intracellular calcium with bapta-(AM), as well as silencing of $CaMK_{IV}$ or inhibition of its activity with KN62 resulted in similar observations. Finally, overproduction of a dominant negative form of CREB completely suppressed glucose and $CaMK_{IV}$ stimulation of *Irs2* expression.
Conclusions/interpretation Our results suggest that the $Ca^{2+}/CaMK_{IV}$/CREB cascade plays a critical role in the regulation of *Irs2* expression in beta cells.

Keywords Beta cell · $CaMK_{IV}$ · CREB · *Irs2*

Abbreviations

CaMK	Calcium/calmodulin-dependent kinase
$\Delta CaMK_{IV}$	Constitutively active form of $CaMK_{IV}$
CREB	cAMP response element-binding protein
$CREB_{DIEDML}$	Constitutively active form of CREB
$CREB_{m1}$	Dominant negative form of CREB
GLP-1	Glucagon-like peptide-1
$\Delta^{K75E}CaMK_{IV}$	Dominant negative form of $CaMK_{IV}$
PKA	Protein kinase A
siRNA	Small-interfering RNA

Introduction

Although it is generally accepted that type 2 diabetes mellitus is caused by a progressive decline in beta cell function [1] and beta cell mass [2, 3] following the development of insulin resistance [4, 5], several reports suggest that beta cell failure, rather than insulin resistance,

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is the primary defect, which occurs years before the onset of diabetes [1, 6]. This notion is supported by the general observation that type 2 diabetes mellitus does not develop in most obese individuals or in pregnant women, who can have severe insulin resistance [3, 7, 8], due to a compensatory process involving increased beta cell function and notably beta cell mass expansion [3, 9–11].

Short-term glucose administration promotes beta cell mass expansion [12–14], and recent observations implicate glucose-stimulated insulin secretion [15, 16] and *Irs2* expression [17, 18] as the main upstream mechanisms. A strong correlation between *IRS2* expression in beta cells with apoptosis, proliferation and type 2 diabetes suggests that *IRS2* could become a target gene for future therapeutic intervention. Thus, *Irs2* knockout mice exhibited a significant reduction in beta cell mass and developed the full phenotype of diabetes [19, 20], whereas targeted re-expression of *Irs2* in beta cells increased their survival and promoted their growth through stimulation of proliferation [21]. More recently, it was reported that exendin-4, a stable glucagon-like peptide-1 (GLP-1) receptor agonist known to stimulate *Irs2* expression and beta cell mass expansion, failed to do so in *Irs2* knockout mice, thus linking the cAMP signalling pathway with *Irs2* expression and activity [22].

The role of the cAMP/protein kinase A (PKA) pathway in glucose-regulated *Irs2* expression and beta cell mass expansion was recently investigated and experiments using PKA inhibitors indicated that glucose-stimulated *Irs2* expression was reduced by only 20% to 25% [17, 18]. These observations indicate that GLP-1 and glucose do not share the same signalling cascade to increase *Irs2* expression, and that activation of the cAMP/PKA pathway is not the major mechanism by which glucose stimulates *Irs2* expression.

Interestingly, calcium-dependent stimulation of dendritic growth in neurons is mediated by calcium/calmodulin-dependent kinase $(CaMK)_{IV}$-induced cAMP response element-binding protein (CREB) activation, independently of cAMP/PKA stimulation [23]. Thus, since *CamkIV* (also known as *Camk4*) is expressed in beta cells [24], is activated by increases in intracellular Ca^{2+} levels as occurs following glucose metabolism [25] and CREB is known to stimulate *Irs2* gene expression [26], it is possible that glucose regulates *Irs2* levels in beta cells through a Ca^{2+}/$CaMK_{IV}$/CREB signalling cascade.

In the current study, the role of $CaMK_{IV}$-induced CREB activation in mediating the glucose effects on *Irs2* expression in MIN6 beta cells and mouse islets was examined through downregulation and overexpression studies that used small-interfering RNAs (siRNAs) and constitutively active or dominant negative forms of $CaMK_{IV}$ and CREB.

Methods

Ethics Experiments involving animals were approved by the local ethics committee.

Cells, plasmids and reagents MIN6 cells were a gift from Y. Oka and J.-I. Miyazaki (Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Japan) and ICR mice were purchased from Harlan (Blackthorn, UK). Plasmids encoding the mouse constitutively active form of $CaMK_{IV}$ ($\Delta CaMK_{IV}$) and human kinase-dead form of $CaMK_{IV}$ ($\Delta^{K73E}CaMK_{IV}$) were kindly provided by A. Ghosh (Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, USA). pcDNA3.1 plasmids encoding the following forms of CREB were a gift from Professor D. D. Ginty (Department of Neuroscience, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, USA): wild-type; constitutively active forms of CREB (DIEDML); and dominant negative forms of CREB ($CREB_{m1}$). Metafectene Pro was from Biontex (Martinsried/Planegg, Germany). Glucose-free DMEM and FBS were purchased from Invitrogen (Paisley, UK). KN62 was from Calbiochem (Nottingham, UK). The mouse monoclonal anti-α-tubulin antibody, culture media, nifedipine, bapta-(AM), Histopaque-1077, collagenase (type XI), penicillin/streptomycin and L-glutamine were from Sigma Aldrich (Poole, UK). The rabbit polyclonal anti-IRS1 and anti-IRS2 antibodies were from Millipore (Watford, UK), the mouse monoclonal anti-$CaMK_{IV}$ antibody was from Clontech (Oxford, UK), and mouse monoclonal anti-CREB and anti-(^{133}phosphoserine) CREB antibodies were from New England Biolabs (Hitchin, UK). The siRNA duplexes were obtained from Dharmacon (Cramlington, UK). The mRNA purification kit (RNeasy) was from Qiagen (Crawley, UK). Enhanced chemiluminescent kits for Western blotting were from GE Healthcare (Little Chalfont, UK). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Pierce Biotechnology (Rockford, IL, USA).

Cell culture, treatment and transfection MIN6 beta cells were maintained in culture at 37°C in DMEM (25 mmol/l glucose) supplemented with 2 mmol/l glutamine, 10% (vol./vol.) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. To study the effect of glucose on *Irs1* and *Irs2* expression and on CREB phosphorylation, cells were maintained at 6 mmol/l glucose for 24 h followed by exposure to the experimental conditions described below ("Results"). Pre-treatment with the CaMK inhibitor KN62 (or DMSO vehicle, 45 min) and transient transfection with non-silencing RNA or siRNA duplexes designed to knock down $CaMK_{IV}$ levels were performed before final adjust-

ment of the glucose concentration. High efficiency (~60–80%) transient transfection of MIN6 cells was achieved by electroporation (Nucleofector II; Amaxa, Cologne, Germany).

Mouse islets isolation, culture and siRNA transfection Islets were isolated by collagenase digestion of mouse pancreas as described previously [27, 28] and maintained in culture for 24 to 48 h in RPMI 1640 medium (11 mmol/l glucose) supplemented with 10% (vol./vol.) FBS before use. To study the role of $CaMK_{IV}$ in primary tissue, mouse islets were transiently transfected with either a commercially available non-silencing RNA or with four siRNA duplexes designed to specifically reduce endogenous $CaMK_{IV}$ levels. The target sequences used were: 5′-gagauccucugggcgauuu-3′, 5′-ucaag gaaauauucgaaac-3′, 5′-ggugcuacauccauugugu-3′ and 5′-gggaugaagugucuuuaaa-3′. Transient transfection of mouse islets was performed using a two-step transfection protocol with Metafectene Pro as described [29].

Reverse transcription and quantitative polymerase chain reaction Total RNA was isolated from ~5×10^5 MIN6 cells or ~150 mouse islets using RNeasy (Qiagen) according to the manufacturer's instructions. Complementary DNAs were synthesised and quantitative PCR amplifications were performed as described previously [30]. Each sample value was normalised to beta-actin copy numbers. In all quantitative PCR experiments, the presence of possible contaminants was checked by control reactions in which amplification was performed in reaction mixtures without cDNA templates. Specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis of PCR products.

Western blot analysis MIN6 cell protein extracts (50–75 μg) were separated on 10% (wt/vol.) polyacrylamide gels and transferred to polyvinylidene fluoride membranes, which were incubated for 16 h with antibodies directed against IRS1 (1:166 dilution), IRS2 (1:1,000 dilution), α-tubulin (1:2,000 dilution), $CaMK_{IV}$ (1:750 dilution), CREB (1:750 dilution) or phospho-CREB(Ser133) (1:750 dilution). After three washes in Tris buffered saline (pH 7.4) containing 0.05% Tween 20, the polyvinylidene fluoride membranes were incubated for another hour with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgGs as appropriate (1:5,000 dilution). Binding of secondary antibodies was revealed by chemiluminescence.

Statistical analysis Numerical data are expressed as means ± SEM. Differences between two groups were analysed by unpaired Student's *t* test and considered significant at $p<0.05$. Differences between several groups

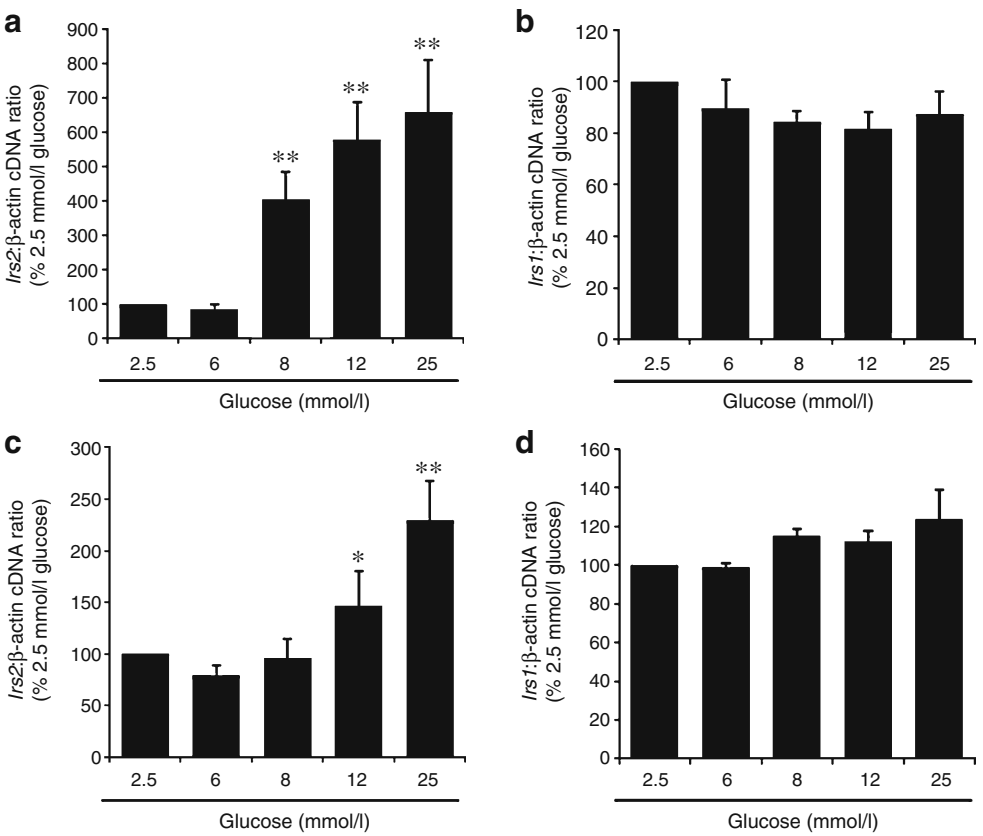

Fig. 1 Glucose regulates *Irs2*, but not *Irs1* mRNA expression in beta cells. **a**, **b** Isolated mouse islets or (**c**, **d**) MIN6 beta cells were maintained in culture for 24 h in the presence of 6 mmol/l glucose and then exposed to 2.5 to 25 mmol/l glucose for another 24 h. Quantitative RT-PCR was used to determine mRNA expression of (**a**, **c**) *Irs2* and (**b**, **d**) *Irs1* relative to beta-actin. Values are per cent of those at 2.5 mmol/l glucose, shown as means ± SEM of three to five independent experiments. *$p<0.05$ and **$p<0.01$ vs 2.5 mmol/l glucose

were analysed by one-way analysis of variance followed by Tukey's honestly significant differences test.

Results

Glucose stimulates Irs2, but not Irs1 mRNA expression in beta cells The effect of glucose on *Irs1* and *Irs2* mRNA expression was initially assessed using isolated mouse islets (Fig. 1a, b) or insulin-secreting MIN6 cells (Fig. 1c, d) that were maintained in culture for 24 h in the presence of 2.5 to 25 mmol/l glucose. As previously reported [17, 18], *Irs2*, but not *Irs1* mRNA expression was significantly stimulated in a concentration-dependent manner by glucose in mouse islets and MIN6 cells, with a maximal effect occurring at 25 mmol/l.

Glucose-induced IRS2 production is calcium-dependent As observed at the mRNA level, increasing the glucose concentration from 2.5 to 25 mmol/l also stimulated IRS2, but not IRS1 protein production in mouse islets (Fig. 2a, Electronic supplementary material [ESM] Fig. 1a) and MIN6 cells (Fig. 2b, ESM Fig. 1b). In addition, prevention of glucose-induced calcium influx and increase of intracellular calcium by addition of 10 μmol/l nifedipine or 10 μmol/l bapta-(AM) prior to glucose exposure completely suppressed this stimulatory effect on IRS2 production, but again without altering IRS1 protein levels. Taken together, these results are consistent with the notion that glucose specifically regulates IRS2 levels in a calcium-dependent manner.

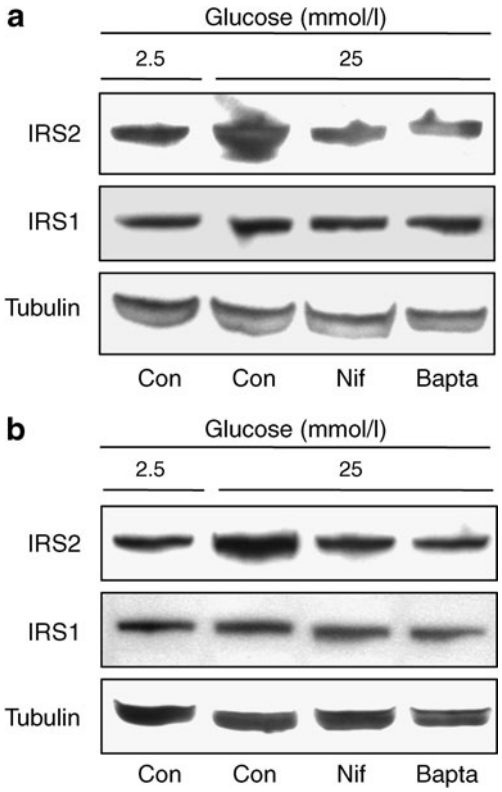

Fig. 2 Glucose-stimulated IRS2 upregulation is mediated by glucose-induced calcium influx. **a** Mouse islets and (**b**) MIN6 beta cells were maintained in culture for 24 h in the presence of 6 mmol/l glucose, pre-treated for 45 min with 10 μmol/l nifedipine (Nif), 10 μmol/l bapta-AM (Bapta) or DMSO vehicle (Con), and then exposed to 2.5 or 25 mmol/l glucose for another 24 h in the presence of the chemicals. Equivalent loading was confirmed by mouse monoclonal anti-α-tubulin antibody. Immunoblots are representative of three independent experiments

Glucose stimulation of Irs2 production is mediated by a CaMK pathway The data in Figs 1 and 2 indicate that glucose stimulates *Irs2* mRNA expression and IRS2 protein levels in a concentration-dependent manner in MIN6 beta cells and in mouse islets. We therefore used MIN6 beta cells to further examine the molecular mechanisms downstream of calcium influx, which mediate this effect. Thus, knowing that CREB has the ability to induce beta cell *Irs2*, but not *Irs1* expression [26], and that $CaMK_I$ and $CaMK_{IV}$ mediate the calcium effect on CREB activation in the GH3 growth hormone-secreting cell line [31], we tested the hypothesis that glucose-stimulated *Irs2* expression in beta cells is regulated by a CaMK pathway. This was achieved by assessing the effect of the non-selective CaMK inhibitor KN62 (30 μmol/l) on glucose-stimulated CREB phosphorylation at serine 133 and on IRS2 protein levels.

As shown in Fig. 3a and ESM Fig. 2a, a low level of CREB phosphorylation at serine 133 could be detected at 6 mmol/l glucose (t=0 min). Challenging the MIN6 cells with 12 mmol/l glucose resulted in a rapid, but transient increase of CREB phosphorylation, with a maximal effect observed after 5 min of exposure. In addition, exposure of MIN6 cells to 12 and 25 mmol/l glucose for 24 h resulted in enhanced IRS2 protein production (Fig. 3b, ESM Fig. 2b), consistent with data displayed in Fig. 2. However, the presence of KN62 suppressed the stimulatory effects of glucose on CREB phosphorylation (Fig. 3a, ESM Fig. 2a) and greatly reduced the glucose-dependent upregulation of IRS2 (Fig. 3b, ESM Fig. 2b). These observations are consistent with the notion that glucose-stimulated *Irs2* expression is mediated, at least in part, by a CaMK–CREB signalling cascade. In addition, the specific involvement of this signalling cascade in regulation of *Irs2* expression by glucose was demonstrated by our observations that neither increased glucose concentrations nor the use of KN62 altered *Irs1* expression levels (Figs 2 and 3c, ESM Fig. 2c).

Glucose-stimulated Irs2 expression is controlled by CaMK$_{IV}$ $CaMK_I$, $CaMK_{II}$ and $CaMK_{IV}$ all have the ability to stimulate CREB phosphorylation at serine 133, but a recent

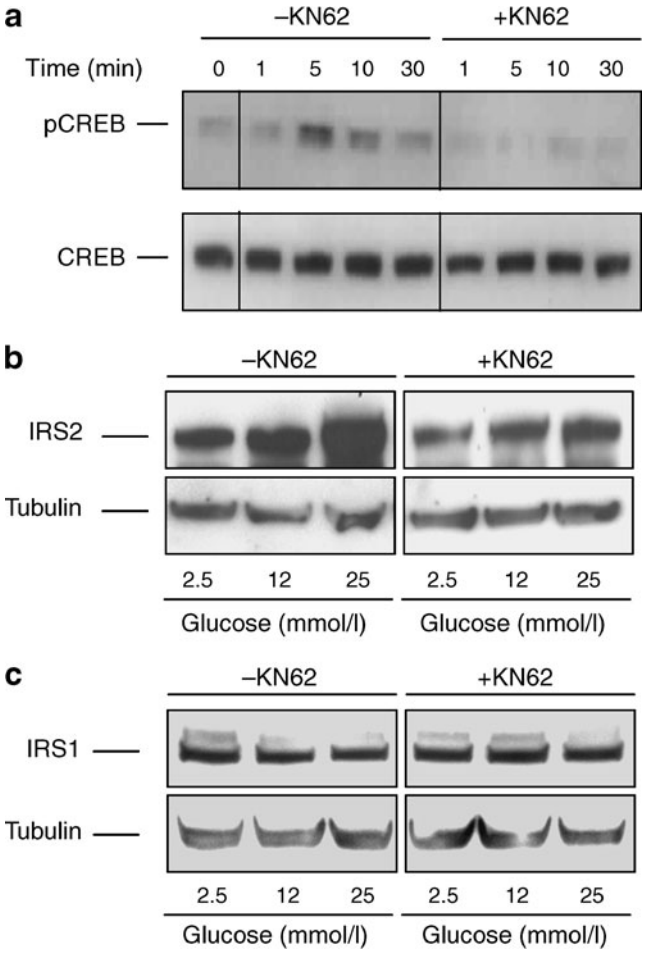

Fig. 3 Glucose stimulates IRS2 production via a CaMK. **a** Immunoblot showing the role of CaMKs in glucose-induced CREB activation as illustrated by detection of CREB phosphorylation (pCREB) at serine 133. MIN6 cells were pre-incubated with 6 mmol/l glucose for 24 h, treated with the CaMK inhibitor KN62 (30 μmol/l) or DMSO for 45 min, and then exposed to 12 mmol/l glucose for additional times as indicated (0 to 30 min). Levels of pCREB were monitored by Western blotting using total CREB as loading controls. **b** Immunoblot showing that glucose stimulates IRS2, but not (**c**) IRS1 protein production in a CaMK-dependent manner in MIN6 beta cells that were treated with 2.5, 12 and 25 mmol/l glucose for 24 h in the presence or absence of 30 μmol/l KN62. Equivalent loading was confirmed by mouse monoclonal anti-α-tubulin antibody. All immunoblots are representative of three independent experiments

study in astrocytes suggests that only $CaMK_{IV}$ has the capacity to mediate the calcium-dependent activation of this transcription factor [32]. Thus, to investigate whether the $CaMK_{IV}$ isoform also mediates glucose-stimulated *Irs2* expression in beta cells, MIN6 cells stably expressing $\Delta CaMK_{IV}$ or $\Delta^{K75E}CaMK_{IV}$ were generated and their expression profiles characterised by Western blotting. It can be seen from Fig. 4a that native MIN6 cells produced only the 60 kDa native $CaMK_{IV}$ (lane 1), whereas cells overproducing the dominant negative and constitutively active $CaMK_{IV}$ mutants also produced truncated 37 kDa (lane 2) and 35 kDa (lane 3) $CaMK_{IV}$ proteins. As expected, overproduction of $\Delta CaMK_{IV}$ was associated with sustained CREB phosphorylation (Fig. 4b, ESM Fig. 3), whereas overproduction of $\Delta^{K75E}CaMK_{IV}$ resulted in an almost complete loss of glucose-induced CREB phosphorylation (Fig. 4b, ESM Fig. 3). To further investi-

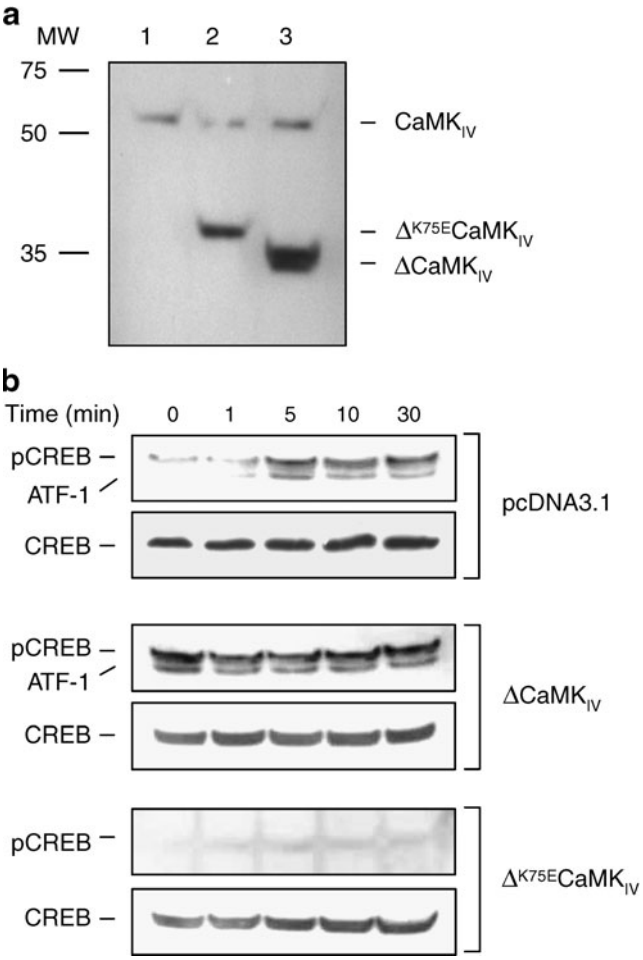

Fig. 4 Glucose-induced CREB phosphorylation at serine 133 is mediated by $CaMK_{IV}$. **a** Stable transfection of $\Delta CaMK_{IV}$ and $\Delta^{K75E}CaMK_{IV}$ in MIN6 beta cells. Sub-confluent MIN6 cells were co-transfected with pcDNA3.1/$\Delta CaMK_{IV}$ or pcDNA3.1/$\Delta^{K75E}CaMK_{IV}$ expression vectors or with pcDNA3.1 alone, and pcDNA3.1-positive colonies selected by maintenance in the presence of 800 μg/ml G418. Single colonies were selected, expanded in culture and tested for $\Delta CaMK_{IV}$ and $\Delta^{K75E}CaMK_{IV}$ by Western blotting using a monoclonal anti-$CaMK_{IV}$ antibody. The immunoblot shows the abundance of endogenous $CaMK_{IV}$ (~60 kDa), of constitutively active $\Delta CaMK_{IV}$ (~35 kDa) and of dominant negative $\Delta^{K75E}CaMK_{IV}$ (~37 kDa) in extracts of MIN6 cells stably expressing pcDNA3.1 (*lane 1*), pcDNA3.1/$\Delta^{K75E}CaMK_{IV}$ (*lane 2*) or pcDNA3.1/$\Delta CaMK_{IV}$ (*lane 3*). **b** CREB phosphorylation (pCREB) at serine 133 in response to $\Delta CaMK_{IV}$ or $\Delta^{K75E}CaMK_{IV}$ overabundance in MIN6 cells. MIN6 cells stably transfected with pcDNA3.1, pcDNA3.1/$\Delta CaMK_{IV}$ or pcDNA3.1/$\Delta^{K75E}CaMK_{IV}$ as labelled were maintained in culture in the presence of 6 mmol/l glucose for 24 h before being challenged with 12 mmol/l glucose for times as indicated (0 to 30 min). Total CREB production was used as loading control. Immunoblots shown are representative of three separate experiments

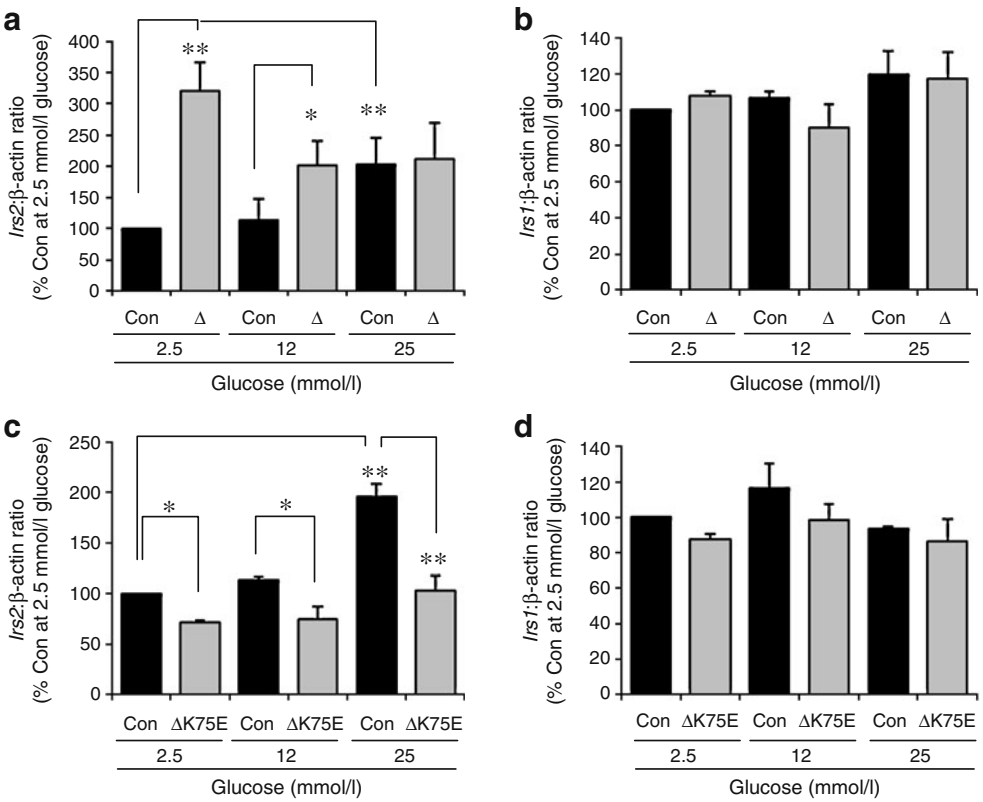

Fig. 5 $CaMK_{IV}$ mediates the stimulatory glucose effect on *Irs2* mRNA expression. **a**, **b** pcDNA3.1 (Con), pcDNA3.1/$\Delta CaMK_{IV}$ (Δ) and (**c**, **d**) pcDNA3.1/$\Delta^{K75E}CaMK_{IV}$ (ΔK75E) transfectant MIN6 beta cells were exposed to glucose as indicated for 24 h and quantitative RT-PCR was used to determine mRNA expression of (**a**, **c**) *Irs2* and (**b**, **d**) *Irs1* relative to beta-actin. Values are per cent of those at 2.5 mmol/l glucose (Con), shown as means ± SEM of three independent experiments. *$p<0.05$ and **$p<0.01$

gate whether these mutant-induced variations in CREB phosphorylation levels were also associated with a similar alteration in *Irs2* mRNA expression, we compared the effects of low (2.5 mmol/l), stimulatory (12 mmol/l) and supra-physiological (25 mmol/l) glucose concentrations. As shown in Fig. 5a, stable overproduction of $\Delta CaMK_{IV}$ significantly upregulated *Irs2* mRNA expression at 2.5 mmol/l and 12 mmol/l glucose, but did not modify it at 25 mmol/l glucose. In contrast, overproduction of $\Delta^{K75E}CaMK_{IV}$ reduced *Irs2* mRNA expression at all glucose concentrations (Fig. 5c). The specificity of these effects on *Irs2* was confirmed by observations that overproduction of these two $CaMK_{IV}$ mutants did not modify *Irs1* expression patterns at any glucose concentrations tested (Fig. 5b, d).

$CaMK_{IV}$ mediates glucose-induced Irs2 expression via CREB activation The data displayed in Fig. 6a show that similar results were obtained at the protein level, with glucose increasing IRS2 production in control cells. They also show that stable overproduction of $\Delta CaMK_{IV}$ resulted in a significant increase in IRS2 protein at 2.5 mmol/l glucose, with no further increase when glucose concentrations were increased to 12 and 25 mmol/l. In contrast, competitive inhibition of endogenous $CaMK_{IV}$ following stable expression of $\Delta^{K75E}CaMK_{IV}$ reduced IRS2 levels at 2.5 mmol/l glucose and also decreased glucose-induced IRS2 production.

To establish whether a direct link between glucose, $CaMK_{IV}$, CREB and IRS2 production exists in beta cells, $CREB_{m1}$ and the constitutively active form of CREB ($CREB_{DIEDML}$) were transiently overexpressed in MIN6 cells and the resulting effects on glucose- and $CaMK_{IV}$-induced stimulation of IRS2 protein levels were analysed by Western blotting. As shown in Fig. 6b, IRS2 production was stimulated in a glucose- and $CaMK_{IV}$-dependent manner, confirming the results obtained in our stable transfection studies (Fig. 6a). The data also show that excess levels of $CREB_{m1}$, as with excess $\Delta^{K75E}CaMK_{IV}$, suppressed the stimulatory effect of high glucose concentrations (12 and 25 mmol/l) on IRS2 production. As expected, Fig. 6c shows that overproduction of $CREB_{DIEDML}$ at a non-stimulatory glucose concentration of 6 mmol/l resulted in a marked increase in IRS2 protein in MIN6 cells. In addition, whereas simultaneous excess of $\Delta CaMK_{IV}$ and the wild-type form of CREB generated an additive effect on IRS2 production, excess levels of $CREB_{m1}$ completely abolished the stimulatory effect of $\Delta CaMK_{IV}$.

Reduction of $CaMK_{IV}$ levels decreases glucose-induced Irs2 expression To confirm that the data obtained in our transfection studies were not the consequence of non-specific effects of $\Delta CaMK_{IV}$ or $\Delta^{K75E}CaMK_{IV}$ overproduction, we used siRNA duplexes to knock down $CaMK_{IV}$ levels in native MIN6 beta cells. In these experiments, a marked reduction in $CaMK_{IV}$ protein production was

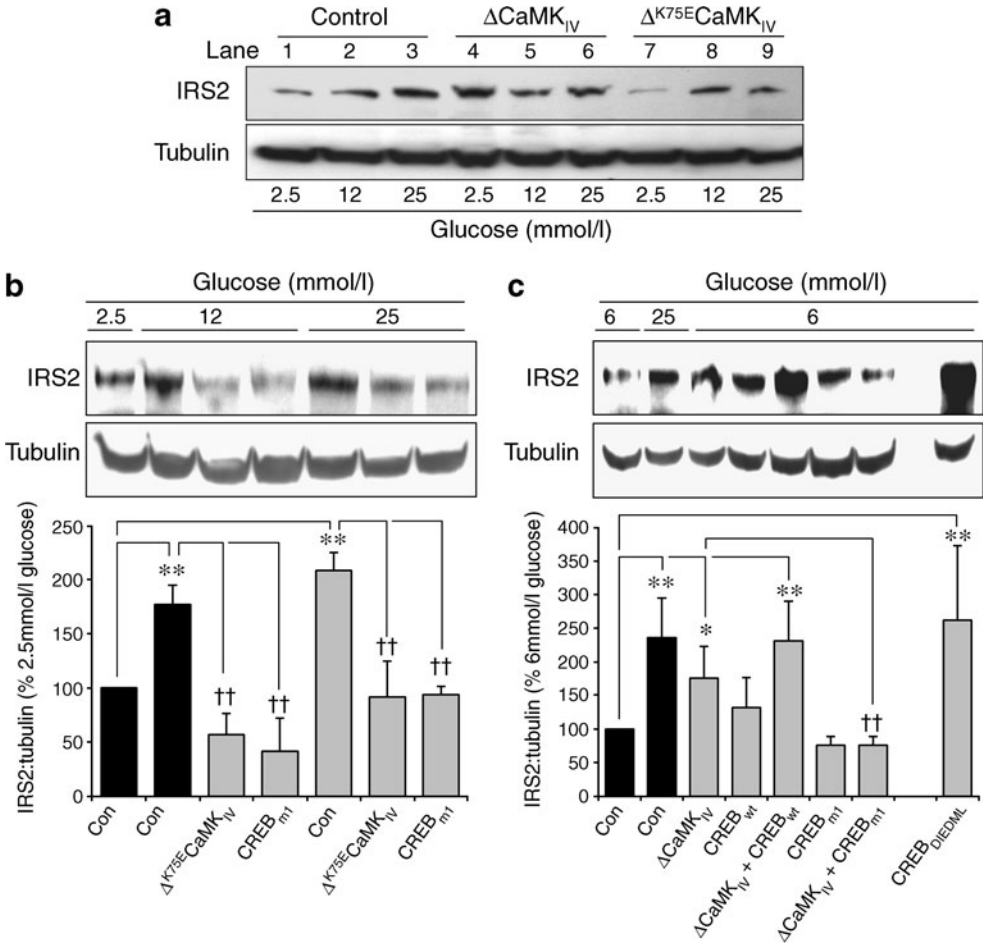

Fig. 6 The glucose/CaMK$_{IV}$ signalling cascade uses CREB activation to stimulate IRS2 production. **a** pcDNA3.1 (control), pcDNA3.1/ΔCaMK$_{IV}$ and pcDNA3.1/Δ^{K75E}CaMK$_{IV}$ transfectant MIN6 cells were maintained in culture with 6 mmol/l glucose for 24 h, then exposed to glucose as indicated for another 24 h before IRS2 protein levels were determined by Western blotting. Equivalent loading was confirmed by a mouse monoclonal anti-α-tubulin antibody. The immunoblot is representative of three independent experiments. **b**, **c** MIN6 cells were transiently transfected with pcDNA3.1 (Control, Con) or various expression vectors coding for ΔCaMK$_{IV}$, Δ^{K75E}-CaMK$_{IV}$, wild-type CREB, CREB$_{m1}$ or CREB$_{DIEDML}$. At 24 h after transfection, the cells were treated with glucose as indicated for an additional 24 h before determination of IRS2 protein levels by Western blotting. Equivalent loading was confirmed with mouse monoclonal anti-α-tubulin antibody. Each immunoblot is representative of three independent experiments. Values are per cent of those at 2.5 and 6 mmol/l glucose (**b**, **c**) respectively, shown as means ± SEM of optical density ratios from three independent experiments. *p<0.05 and **p<0.01; $^{\dagger\dagger}p$<0.01

obtained after 24 h exposure to siRNAs and sustained for at least 48 h (Fig. 7a). Interestingly, increasing the glucose concentration from 2.5 to 25 mmol/l for 24 h not only increased *Irs2* mRNA levels (Fig. 7b) as already observed (Figs 1 and 5), but also resulted in significant stimulation of *CamkIV* mRNA expression (Fig. 7c), suggesting a correlation between CaMK$_{IV}$ expression/activity and *Irs2* expression. Consistent with this inter-relationship, knock-down of CaMK$_{IV}$ levels with siRNA duplexes decreased *Irs2* mRNA expression at 25 mmol/l glucose to a level not significantly different from that observed at 2.5 mmol/l glucose (Fig. 7b) and also significantly reduced the stimulatory effect of 25 mmol/l glucose on *CamkIV* mRNA expression (Fig. 7c). The specificity of these effects was demonstrated by our observation that the use of these siRNAs did not alter *Irs1* mRNA expression (Fig. 7d). In addition, these siRNAs did not reduce *CamkIIa* (also known as *Camk2a*) mRNA expression 24 h after transfection of MIN6 beta cells (Fig. 7e), suggesting that CaMK$_{II}$ expression and function were also not altered in our experimental conditions.

To extend the observations we made in islets that glucose stimulates *Irs2* upregulation in a calcium-dependent manner (Figs 1 and 2) and to determine whether this occurs via activation of the CaMK$_{IV}$–CREB pathway as it does in MIN6 beta cells, several key experiments were performed using freshly isolated mouse islets. It can be seen from Fig. 8a–d that exposing islets to 25 mmol/

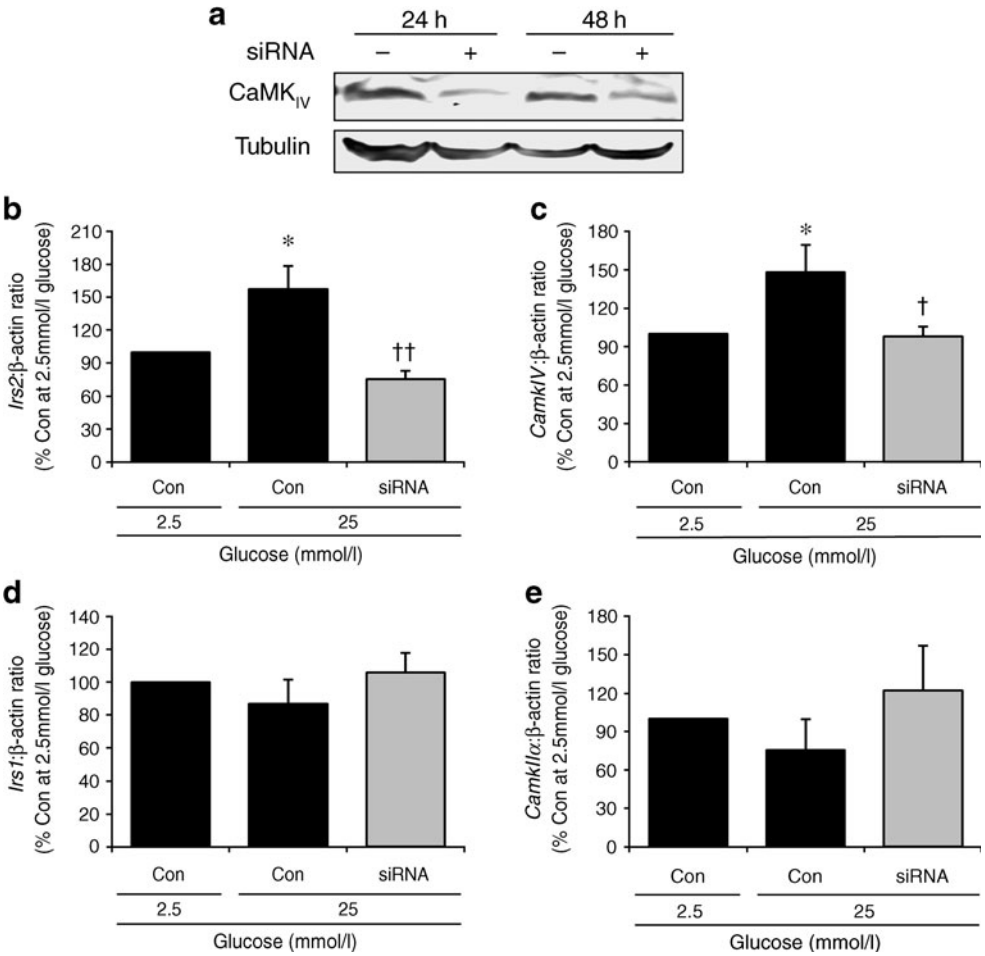

Fig. 7 siRNA-mediated reduction of $CaMK_{IV}$ levels suppresses glucose-induced *Irs2* mRNA expression in MIN6 beta cells. **a** Immunoblot showing the effect of four siRNA duplexes designed to reduce $CaMK_{IV}$ protein levels in MIN6 cells that were maintained in culture for 24 or 48 h in the presence of 25 mmol/l glucose. **b**–**e** MIN6 cells were transfected either with a non-interfering siRNA duplex (Con) or four duplexes designed to silence $CaMK_{IV}$ production, and exposed to glucose as indicated for 24 h. Quantitative RT-PCR was used to determine mRNA expression of *Irs2* (**b**), *CamkIV* (**c**), *Irs1* (**d**) and *CamkIIa* (**e**) relative to those of beta-actin. Values are per cent of those at 2.5 mmol/l glucose, shown as means ± SEM of three to four independent experiments. *$p<0.05$ relative to Con mRNA determined at 2.5 mmol/l glucose; $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ relative to Con mRNA determined at 25 mmol/l glucose

l glucose for 24 h resulted in a significant stimulation of *CamkIV* mRNA expression and that this effect was associated with a significant increase in *Irs2* mRNA expression. In contrast, 25 mmol/l glucose failed to upregulate *Irs2* mRNA expression in mouse islets that had been transfected with *CamkIV* siRNA duplexes, which reduced *CamkIV* mRNA expression (Fig. 8a), but not *CamkIIa* (Fig. 8d) to a level not significantly different from that observed in the presence of 2.5 mmol/l glucose. As shown in Fig. 8e and ESM Fig. 4a–c, increasing the glucose concentration from 2.5 to 25 mmol/l also stimulated $CaMK_{IV}$ and IRS2 production at the protein level in mouse islets, while downregulation of $CaMK_{IV}$ levels by siRNAs entirely suppressed these effects. Figure 8 and ESM Fig. 4 also demonstrate that *Irs1* mRNA expression and protein levels were not modified when the glucose concentration was increased from 2.5 to 25 mmol/l or when the islets were transfected with the *CamkIV* siRNA duplexes. Finally, the observation that CREB phosphorylation at serine 133 was significantly increased in mouse islets in response to 25 mmol/l glucose (Fig. 8d, ESM Fig. 4d) confirms our results in MIN6 cells (Figs 3 and 4) and further supports the hypothesis that glucose stimulates *Irs2* expression via activation of a $CaMK_{IV}$–CREB signalling cascade in beta cells.

Discussion

$CaMK_{IV}$ is a multifunctional enzyme whose function is best understood in neurons, where it inhibits apoptosis and stimulates growth in a calcium- and CREB-dependent manner [23, 33–37]. *CamkIV* is also expressed by pancreatic beta cells [24], but its roles in beta cells have not been fully defined. Earlier reports that glucose and GLP-1 receptor agonists regulate beta cell mass through CREB- and IRS2-dependent inhibition of apoptosis and stimulation of proliferation [17, 18, 21, 26, 38–41] led us to examine the role of the $CaMK_{IV}$–CREB cascade in the regulation of *Irs2* expression by beta cells.

Our results demonstrate that glucose stimulates *Irs2* expression in islets and MIN6 cells in a calcium-dependent manner and provide evidence for the first time that this is mediated by the $CaMK_{IV}$–CREB pathway. Indeed, glucose-dependent *Irs2* mRNA and protein upregulation were substantially reduced when either

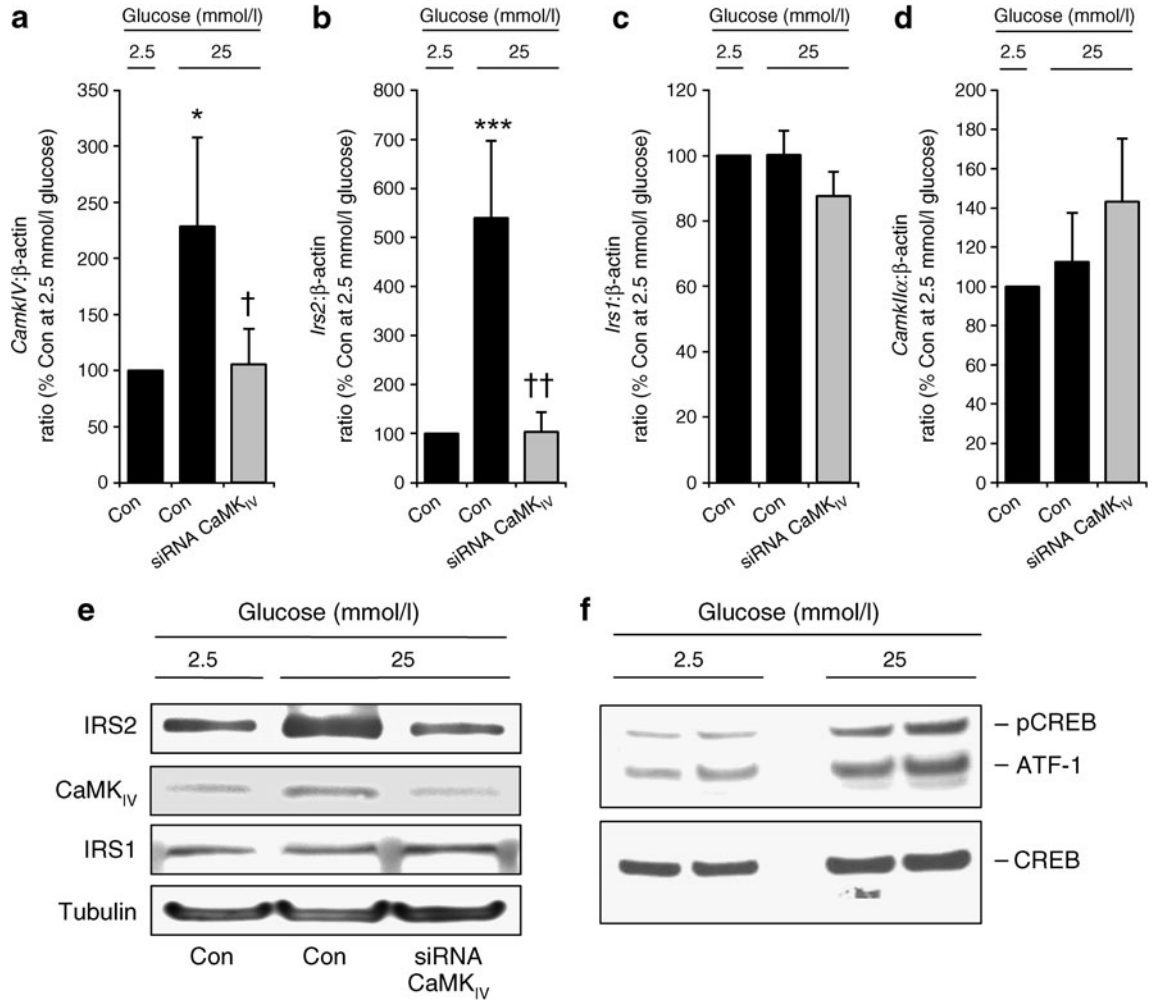

Fig. 8 siRNA-mediated reduction in $CaMK_{IV}$ levels suppresses glucose-induced *Irs2* mRNA expression and protein levels in mouse islets. **a**–**e** Mouse islets were transfected either with a non-interfering siRNA duplex (Con) or four duplexes designed to silence $CaMK_{IV}$ production, and maintained in culture for 24 h in the presence of 5.5 mmol/l glucose, then exposed to glucose as indicated for another 24 h. Quantitative RT-PCR was used to determine mRNA expression of (**a**) *CamkIV*, (**b**) *Irs2*, (**c**) *Irs1* and (**d**) *CamkIIa* relative to those of beta-actin. Values are a percentage of those at 2.5 mmol/l glucose, shown as means ± SEM of three independent experiments. *$p<0.05$ and **$p<0.01$ relative to mRNA determined at 2.5 mmol/l glucose; $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ relative to mRNA determined at 25 mmol/l glucose. **e** Mouse islets were treated as above and $CaMK_{IV}$, IRS2 and IRS1 protein levels were analysed by Western blotting. Equivalent loading was confirmed by mouse monoclonal anti-α-tubulin antibody. **f** Immunoblot showing the stimulatory effect of glucose on CREB phosphorylation (pCREB) at serine 133 in mouse islets. Mouse islets were maintained in culture for 24 h in the presence of 2.5 mmol/l glucose and then exposed to 25 mmol/l glucose for another 7 min. Immunoblots are representative of three independent experiments

$\Delta^{K75E}CaMK_{IV}$ or $CREB_{m1}$ were overabundant in MIN6 cells or following siRNA-induced reduction of endogenous $CaMK_{IV}$ content. In contrast, excess levels of the constitutively active forms of $CaMK_{IV}$ ($\Delta CaMK_{IV}$) or of CREB (CREBDIEDML) resulted in enhanced *Irs2* expression, while the stimulatory effect of $\Delta CaMK_{IV}$ was suppressed by coproduction of the dominant negative CREB mutant ($CREB_{m1}$). These novel observations in MIN6 cells are consistent with previous in vitro [23, 42] and in vivo [33] reports in other tissues, demonstrating that $CaMK_{IV}$ controls CREB transcriptional activity. The observation that only *Irs2*, but not *Irs1* expression levels were modified when glucose concentration was increased or the constitutively active form of $CaMK_{IV}$ was overproduced also confirms previous observations of selective upregulation of *Irs2* by glucose [17, 18], as well as demonstrating the specificity of this glucose–$CaMK_{IV}$ effect. We also observed that stable $\Delta CaMK_{IV}$ production by MIN6 cells produced significant elevations of *Irs2* mRNA at 2.5 and 12 mmol/l glucose compared with native MIN6 cells, but this did not occur at 25 mmol/l glucose. These observations suggest that production of the constitutively active $\Delta CaMK_{IV}$ in MIN6 cells bypassed the requirement for glucose-stimulated calcium entry and enabled the cells to maximally stimulate *Irs2* expression independently of a glucose stimulus.

However, whereas glucose-induced CREB phosphorylation at serine 133 was abolished by the calcium/calmodulin kinase inhibitor KN62 or in MIN6 cells stably transfected with $\Delta^{K75E}CaMK_{IV}$ (Figs. 3a and 4b), glucose-stimulated IRS2 protein production was only partially reduced (Figs 3b and 6a). This suggests that part of the stimulatory effect of glucose on IRS2 abundance may be independent of $CaMK_{IV}$ and that alternative mechanisms of action exist. One possible alternative signalling cascade might involve glucose-induced increases in intracellular cAMP levels [43], which have been shown to promote MIN6 cell CREB phosphorylation at serine 133 and CREB activation, but with a delayed time course compared with depolarising stimuli [26]. This hypothesis is supported by previous observations showing that H-89 and KT5720, two PKA inhibitors, reduced glucose-stimulated *Irs2* mRNA expression and protein levels by approximately 25% in rat islets [18].

MIN6 beta cells were used for many of the experiments presented in this study because they have several of the key functional characteristics of primary beta cells [44] and are readily amenable to stable transfection. However, MIN6 cells are a transformed beta cell line expressing the SV40 large T-antigen, which keeps them in a proliferative state, and they are also adapted to maintenance in media containing high glucose concentrations. Therefore, to ensure that the data obtained using MIN6 cells did not reflect signalling cascades present in cell lines but not in primary beta cells, key experiments were repeated using isolated mouse islets. We found that glucose stimulated a calcium-dependent upregulation of *Irs2* in islets, with stimulatory profiles similar to those seen in MIN6 cells; glucose also stimulated CREB phosphorylation. In addition, our experiments in mouse islets, in which glucose-stimulated *Irs2* upregulation was lost when $CaMK_{IV}$ production was transiently knocked down, confirmed the existence, in mouse islets, of the glucose/$CaMK_{IV}$/*Irs2* cascade that we had identified in MIN6 cells. Taken together, our data imply that $CaMK_{IV}$ plays a central role in the regulation of *Irs2* expression in islets. Previous studies using the HIT-T15 and INS-1 insulin-secreting cell lines suggest that $CaMK_{IV}$ also mediates glucose-induced insulin gene expression and insulin secretion [24, 25], and that it stimulates glucokinase expression [44]. In addition, *CamkIV* is a target gene for the canonical wingless-type MMTV integration site family (WNT)/β-catenin signalling pathway [45], whose signalling-associated transcription factor *TCF7L2* is a diabetes susceptibility gene [46, 47] known to regulate beta cell proliferation [48], insulin gene expression and insulin secretion [49]. Thus, a central role for *CamKIV* in islet function implicates it as a useful target gene for the development of future drug therapies to treat type 2 diabetes mellitus.

The results presented here establish a critical role for $CaMK_{IV}$ in *Irs2* expression. This, together with previous observations showing a reduction in beta cell mass due to increased beta cell apoptosis in *Irs2* knockout mice [19–21], suggests that $CaMK_{IV}$ might regulate beta cell survival and proliferation. Preliminary data, obtained in our laboratory and demonstrating that overexpression of the constitutively active form of $CaMK_{IV}$ in MIN6 cells stimulates proliferation and reduces caspase-3/7 activities, are consistent with this hypothesis (D. S. Muller, S. J. Persaud, B. Liu and P. M Jones, unpublished data). The precise role of *Irs2* in these $CaMK_{IV}$-mediated effects is now being investigated.

In conclusion, the current study demonstrates for the first time that $CaMK_{IV}$ has a central role in CREB-dependent mechanisms by which glucose regulates *Irs2* expression in beta cells. Moreover, since *Irs2* deficiency has been linked with the progressive development of type 2 diabetes mellitus, our results suggest that finding a mechanism to stimulate *CamKIV* expression and/or activity could have a significant clinical impact in the future for patients with type 2 diabetes mellitus.

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