View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Biochimica et Biophysica Acta 1783 (2008) 1929–1934

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

PKCepsilon mediates glucose-regulated insulin production in pancreatic beta-cells

Nasim Warwar, Avital Dov, Eva Abramovitch, Ren Wu, Marina Jmoudiak, Esther Haber, Erol Cerasi, Rafael Nesher *

Endocrinology and Metabolism Service, Department of Medicine, The Hebrew University – Hadassah Medical Center, Jerusalem, Israel

ARTICLE INFO

Article history:
Received 12 February 2008
Received in revised form 27 March 2008
Accepted 18 April 2008
Available online 29 April 2008

Keywords:
β′COP1
Anchoring proteins
Translocation inhibiting peptides
siRNA
Proinsulin biosynthesis

ABSTRACT

Endocrine cells produce large amounts of one or more peptides. The post-translational control of selective production of a single protein is often unknown. We used 3 unrelated approaches to diminish PKCε in rat islets to evaluate its role in preferential glucose-mediated insulin production. Transfection with siRNA (siR-PKCε) or expression of inactive PKCε (PKCε-KD) resulted in a significant reduction in insulin response to glucose (16.7 mmol/l). Glucose stimulation resulted in concentration of PKCε in the perinuclear region, an area known to be rich in ER–Golgi systems, associated with insulin-containing structures. ß'COP1 (RACK2) is the anchoring protein for PKCε. Glucose-stimulated proinsulin production was diminished by 50% in islets expressing PKCε-KD, and 60% in islets expressing RACK2 binding protein (εV1-2); total protein biosynthesis was not affected. In islets expressing εV1-2, a chase period following glucose stimulus resulted in a reduced proinsulin conversion to mature insulin. We propose that PKCε plays a specific role in mediating the glucose-signal into insulin production: binding to ß'COP1 localizes the activated enzyme to the RER where it modulates the shuttling of proinsulin to the TGN. Subsequently the enzyme may be involved in anterograde trafficking of the prohormone or in its processing within the TGN.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Highly specialized cells, such as hormone producing cells, produce one or two peptides at much higher rates than all other cellular proteins. Little is known on the mechanism of selectivity of this process. In pancreatic β-cells glucose metabolism affects most major signal pathways, including those of insulin production, storage and exocytosis [1,2]. Multiple families of protein kinases are known to function downstream to glucose metabolism; however the precise role of many is poorly defined [3]. Several subtypes of these kinases are also activated by receptor-generated signals and thus may function as the site of convergence of the metabolic signals with those generated by hormones and neurotransmitters. When activated, many protein kinases, including the protein kinase C family, translocate to the site of their effectors and bind an anchoring protein which keeps them in close proximity of their downstream target [4]. We identified six isoforms of PKC in rat β-cells, four of which, PKCα, PKCε, PKCζ and PKCθ, exhibited glucose-induced activation and translocation [5]. Abnormalities in level or translocation characteristics in response to glucose were observed for PKCα, PKCε, PKCζ, PKCδ, and PKCθ in β-cells of the GK rat, a rodent with spontaneous type 2-like diabetes [5]. Using anchoring-competing short peptides, we have also shown that inhibition of translocation of PKCα and PKCε diminishes insulin release from isolated rat islets, the former in a calcium-dependent mode and the latter in a calcium-independent manner [6].

Histochemical analyses indicated a strong association of PKCα and PKCε with insulin granules and with their movements during the biphasic dynamics of glucose-induced insulin secretion [5]. In other cells, PKCε has been reported to play an essential role in diverse signaling systems related to cell proliferation [7–10], gene expression [7,10], and vesicle trafficking and exocytosis [10]. β′COP-1, or RACK2, which is a member of the COP complex involved in the shuttling of newly formed proteins, has been identified as PKCε's anchoring protein [10,11] and is responsible for maintaining the activated form of the enzyme in close proximity of its effector protein(s) [4,12]. Because we observed that glucose stimulation led to concentration of PKCε in the perinuclear region in β-cells [5], in the present study we explored the role of PKCε in insulin production and secretion in rat islets of Langerhans.

2. Materials and methods

2.1. Animals

Male Wistar rats (Harlan Animal Farms, Jerusalem) weighing 180–200 g were used for islet perifusion and for biosynthesis studies, while 250–300 g males were used for pancreas perfusion. Animal usage was with full adherence to Institutional Ethical Committee guidelines. For perfusion studies, rats were anesthetized by IP injection of

Abbreviations: ER, endoplasmic reticulum; PKC, protein kinase C; RACK2, receptor for activated C kinase 2; COP1, coatomer protein 1; AUC, area under the curve; siRNA, small interfering RNA; DAG, diacylglycerol; PIP3, phosphatidylinositol 3,4,5-triphosphate; TGN, trans Golgi network; RP-HPLC, reverse-phase high pressure liquid chromatography

* Corresponding author. Endocrinology and Metabolism Service, The Hebrew University – Hadassah Medical Center, P.O. Box 12000, 91120 Jerusalem, Israel. Tel.: +972 2 677 6787; fax: +972 2 643 7940.

E-mail address: rafael.nesher@huji.ac.il (R. Nesher).

0167-4889/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2008.04.007

100 mg/kg thiopentone sodium, the pancreas quickly isolated and prepared for perfusion as previously described [13,14]. The pancreas was fixed with 4% formaldehyde in PBS by a brief perfusion, and kept at 4 °C in PBS until staining and imaging [5,15].

2.2. Islets

Rat islets were isolated by collagenase digestion (Collagenase P; Roche, Boehringer-Mannheim, Mannheim, Germany) as previously described [14], hand picked and washed 3 times in Hanks solution. Islets were cultured in bacteriological dish in RPMI 1640 medium, containing 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2.0 mmol/l L-Glutamine (all from Biological Industries, Beit Haemek, Israel).

2.3. Immunohistochemical staining and imaging

Pancreata were sectioned, stained and imaged using a Zeiss LSM 410 confocal laser scanning system attached to the Zeiss Axiovert 135 M inverted microscope as previously described [5]. Antibodies for PKCε histochemistry and for Western blot were obtained from Sigma-Aldrich Biochemicals (Rehovot, Israel).

2.4. siRNA

Double-strand 21 mer siRNA (siR-PKCε) was designed as described in detail by Elbashir et al. [16] (Fig. 1A) and ordered from Dharmacon (Dharmacon Inc. Chicago, IL). The DS Scrambled Negative was from IDT (Integrated DNA Technologies, Coralville, IA). DS siRNA-containing micelles were prepared by mixing 5 µl Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, California, USA) and 100 µl OptiMEM (Invitrogen) and transfected as described by the manufacturer in an antibiotic-free medium. The final concentration of siRNA was 100 nmol/l. Transfection medium was replaced after 6 h with the regular growth medium (RPMI), and the islets were maintained in culture for three additional days. Efficiency of penetration was monitored in INS-1E cells using 5'-Cye3 labeled inactive construct (Dharmacon) followed by FACS analysis 16 h after transfection, and was routinely >94%. In isolated islets, the efficiency was monitored by confocal imaging of the same construct using 5 µm apart *z*-plane sections (Supplementary Fig. 1). Efficiency of PKCε shutoff was estimated by Western blot and densitometric analysis using Tina 2.0 Image Analysis software (an open source library and toolkit), and ranged between 60% and 80% (Fig. 2A).

2.5. Expression of PKCε

The adenoviruses expressing kinase-dead PKCε (PKCε-KD) or authentic PKCε ([17] (Fig. 1B) were kindly provided by Drs. T. Kuroki, Showa University, Tokyo and T. Tennenbaum, Bar-Ilan University, Israel. The virus was propagated in HEK 293 cells grown in DMEM medium (Biological Industries). Freshly isolated islets were infected with 200 IFU/islet in RPMI medium (Biological Industries) and incubated overnight at 37 °C. Levels of PKCε expression were determined by Western blot using anti-PKCε antibody (Sigma, see Section 2.3 above) and islets treated with control adenovirus were used as reference for basal levels of PKCε. Reduction in novel PKC activity in islets infected with PKCε-KD was 70% (Fig. 2B), determined by SignaTECT PKC Activity Assay (Promega, Madison WI). For novel PKC activity assay, endogenous phospholipids were removed by DE52 column extractions and calcium was excluded using EGTA, thus eliminating the contribution of the Classical family of isoenzymes. The activity of the atypical family of isoenzymes was subtracted using duplicate extracts activated by phospholipids alone. Data shown in Fig. 2B are the net DAG-activated fractions, in the

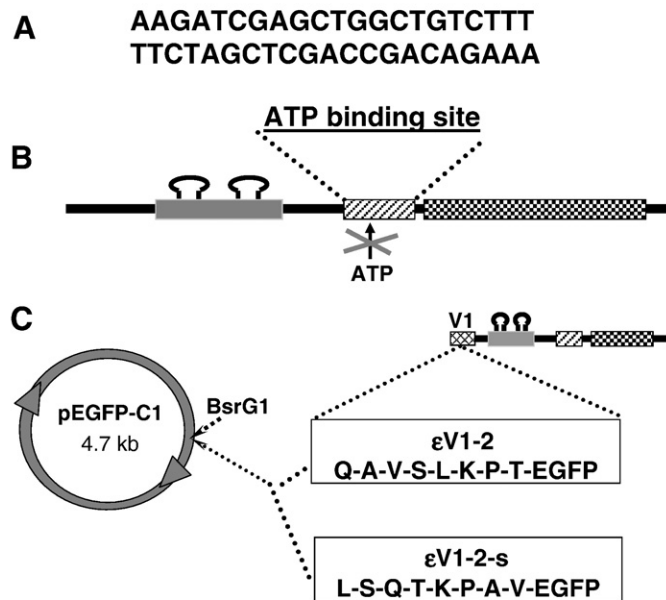

Fig. 1. A–C: The three unrelated procedures used to diminish PKCε activity in rat pancreatic β-cells. A: The RNAi, siR-PKCε; B: the expression of inactive PKCε, PKCε-KD; C: map of the adenovirus expressing a short peptide that competes with PKCε for RACK2, εV1-2, a translocation-inhibitory peptide generated from the V1 region of PKCε [11]. The scrambled sequence is also illustrated.

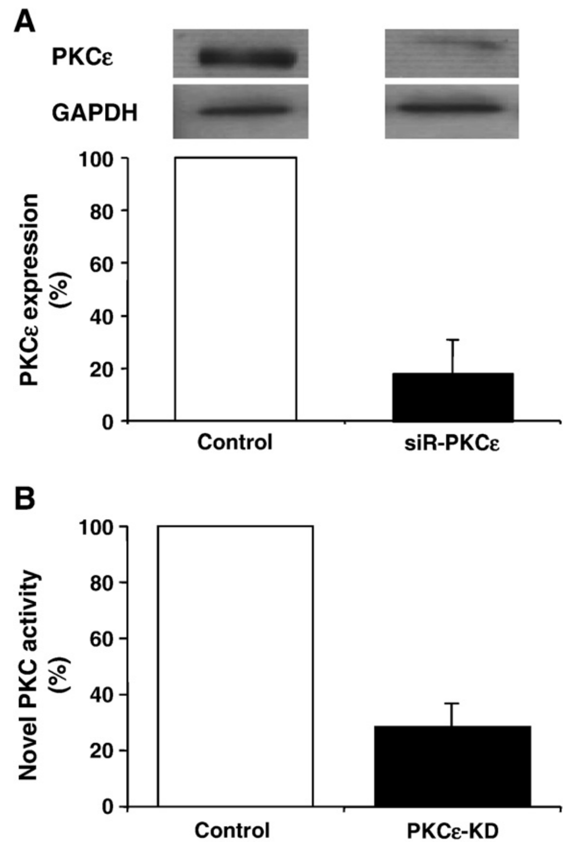

Fig. 2. Diminished PKCε levels or activity in isolated rat islets. A: Quantitative Western blot of PKCε levels, corrected for levels of GAPGH, in rat islets transfected with siR-PKCε. Control islets were transfected with an inactive sequence of 21 mer siRNA. B: Diminished novel PKC activity in isolated islets infected by adenovirus expressing mutant PKCε, PKCε-KD. Control islets were infected with the adenovirus devoid of added sequence. A and B are means ± SD of 3 determinations.

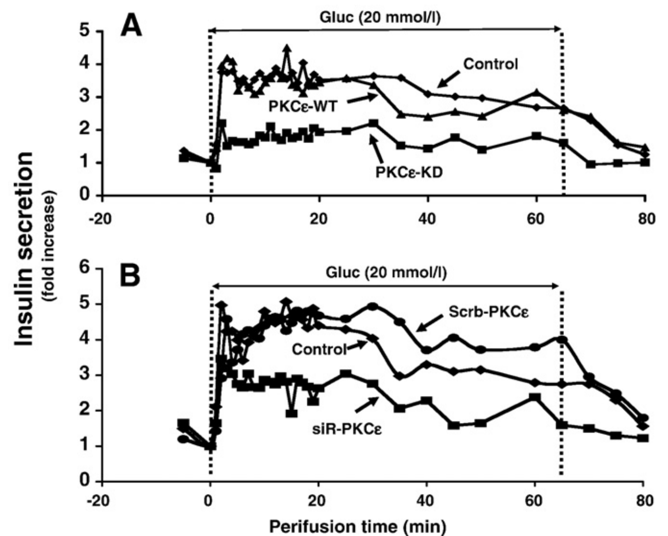

Fig. 3. The effect of diminished PKCε activity on the dynamics of insulin response to glucose in perifused rat islets. A: Control islets; islets expressing active PKCε, PKCε-WT; or inactive PKCε, PKCε-KD. B: Control islets, islets transfected with siR-PKCε, or with scrambled PKCε, scrb-PKCε. Each line represents means of 4–9 studies.

Table 1
Biphasic insulin response to glucose during diminished PKCε activity

	First phase	Second phase
	(ng/50 isl/6 min)	(ng/50 isl/h)
Cont. (scrmb)	4.2±1.0	44.5±10.3
PKCε-DN	1.4±0.6	8.9±1.1#¶
PKCε-WT	4.0±1.5	32.7±11.5
siR-PKCε	2.6±0.8	7.7±3.8*

Area under the insulin release curves in response to glucose in isolated perifused rat islets. Cont., islets transfected with inactive, scrambled siRNA; PKCε-DN, islets expressing inactive PKCε at 5 fold concentrations of control islets as determined by densitometric evaluation of Western blot analysis; PKCε-WT, islets expressing active PKCε at 5 fold concentrations; siR-PKCε, islets transfected with siRNA to diminish PKCε levels. Data are means±SEM of 4–9 observations; #, $p<0.001$ vs. control; ¶, $p<00.05$ vs. PKCε-WT; *, $p<0.001$ vs. control.

presence of phosphatidylserine, after subtraction of the activity determined with phosphatidylserine and without DAG.

2.6. Translocation-inhibitory peptides

Plasmids coding for PKCε translocation-inhibitory peptide (εV1-2) and for scrambled control (εV1-2s) were kindly provided by Dr. D. Mochly-Rosen, Stanford University, CA, and inserted into adenoviral vectors. Single-strand nucleotide sequences for peptides generated from the V1 region of PKCε and for the scrambled controls were inserted into pEGFP-C1 adenoviral vector as described in detail by He et al. [18] (Fig 1C).

2.7. Insulin synthesis

Batches of 25 islets were used in a final volume of 500 µl to study total protein and proinsulin biosynthesis. Islets were preincubated in 3.3 mmol/l glucose for 60 min, then further incubated for 1 h in Krebs Ringer Bicarbonate (KRB) solution containing 16.7 mmol/l glucose, then for an additional 15 min in the same buffer supplemented with 25 µCi ^{3}H-leucine (leucine, L-2, 3, 4, 5-^{3}H, American Radiolabeled Chemicals, Inc., St. Louis, MO). For proinsulin processing studies, following the labeling period, the medium was changed to KRB with 3.3 mmol/l glucose supplemented with 1.0 mmol/l non-labeled leucine. The studies were terminated by the addition of 1.0 ml glucose-free KRB solution, centrifugation, and addition of 500 µl GB/NP40 buffer (0.2 mol/l glycine buffer containing 0.1% RIA grade BSA and 0.5% NP-40, pH 8.8) to the precipitate. For determination of total insulin I+II plus proinsulin, 50 ml samples were pre-treated with 4.5 mg Protein A-Sepharose beads (Sigma-Aldrich) in 100 µl of GB/NP40 buffer by shaking at room temperature for 1 h. After centrifugation (1000 RPM, 1 min), the supernatant was transferred to fresh beads of Protein A-Sepharose, suspended in GB/NP40 buffer; anti-insulin antibody (1/25 dilution, Sigma-Aldrich) was added, and the mix was incubated using gentle shaking for 1 h at room temperature. The antiserum (Sigma I-8510) was evaluated for cross-reactivity with proinsulin by comparing the labeled insulin+proinsulin recovered by immunoprecipitation and by RP-HPLC in identical samples. Incubation was terminated by centrifugation at 1000 RPM for 1 min, two washes of the precipitate with GB/NP40 buffer, and the addition of 100 µl water. Scintillation fluid (4.5 ml, QuickSafe A, Zinsser Analytic, Frankfurt, Germany) was added and the sample was counted using TriCarb 1500 beta counter (Packard Instrument, Downers Grove, IL). Production of insulin and insulin intermediates was determined by the RP-HPLC separation protocol as described by Gadot et al. [14] modified after Halban et al. [19] and Zambre et al. [20]. For the determination of total newly synthesized protein, 0.5 ml of 0.1% BSA was added to 20 ml of sample, followed by 0.5 ml 20% trichloroacetic acid (TCA). Following 20 min of standing on ice, the sample was washed twice with 10% TCA by filtration through a Whatman GF/A filter, dried, and counted in 8 ml QuickSafe A as above.

2.8. Insulin assay

In dynamic islet perifusion studies, basal samples (2.5 mmol/l glucose) were collected every 5 min, then during glucose stimulation (16.7 mmol/l) every minute for the first 10 min, followed by 5 min collections for the rest of the study. Net area under the curve (AUC) insulin released during the initial 6 min of stimulation was calculated for 1st phase insulin response and for 2nd phase insulin release, the subsequent 60 min was used. Insulin was determined using Rat Insulin RIA kit (Linco Research, St. Charles, Missouri, USA). Mean CV between assays was 8.9%, within assay 2.2%.

2.9. Statistical analyses

Kruskal–Wallis nonparametric ANOVA followed by Tukey–Kramer test was used to compare between the experimental conditions with 95% confidence level. Data was analyzed using InStat statistical software (GraphPad Software, San Diego, CA).

3. Results

3.1. Inhibition of PKCε reduces insulin release

Using cultured rat islets or mouse β-cells, we [6] as well as Mendez et al. [21] have shown that PKCε plays a role in the insulin response to glucose. To further elucidate the role of PKCε in the dynamics of insulin response to glucose, we employed two unrelated approaches in isolated rat islets to diminish endogenous PKCε activity: expression of inactive PKCε (PKCε-KD) and depletion of PKCε by siRNA (siR-PKCε). Fig. 3 shows that the two approaches resulted in similar results. Expression of PKCε-KD at 5 fold basal levels, resulting in 70% inhibition of novel PKC activity (Fig. 2B), led to inhibition of the insulin response to glucose (Fig. 3A). Likewise, diminished PKCε levels using siR-PKCε (routinely between 60% and 80% reduction, Fig. 2A), led to a similar inhibition of the insulin

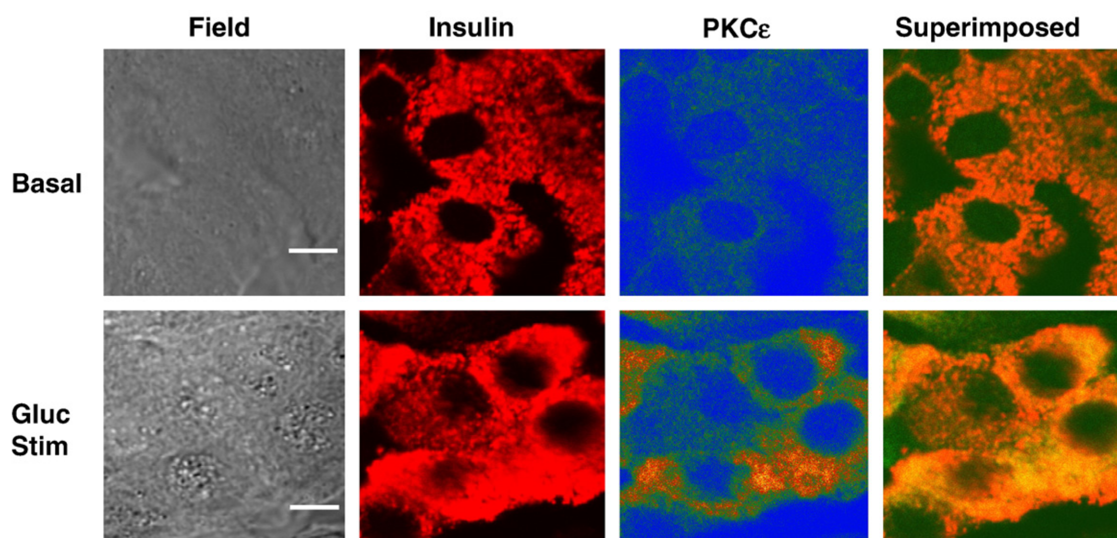

Fig. 4. Translocation of PKCε to the perinuclear region in pancreatic β-cells, 15 min after the onset of glucose stimulus. The isolated perfused rat pancreas was subjected to a 15-min glucose stimulation (16.7 mmol/l) or perfused with basal buffer (3.3 mmol/l glucose), then fixed with 4% formaldehyde in PBS by perfusion, and the sections were double stained for PKCε and insulin. Pancreatic sections were imaged by confocal microscopy. Signal intensity is presented as color scale: blue-background; green, red and bright orange, represent increasing signal intensity, respectively. A total of 48 recorded fields, obtained from 4 basal and 4 stimulated pancreata, were scanned and quantitatively analyzed. Analysis was done and compared with an earlier time point to determine statistical changes in the enzyme localization. At 15 min of stimulation with glucose, 67% of the scanned β-cell fields displayed perinuclear concentration of PKCε as compared with 5 min post-stimulation ($p<0.0001$). (Scale bar=5 µm).

response to glucose (Fig. 3B). Basal rates of insulin release were unaffected by infection with adenovirus expressing PKCε-KD or by transfection with siR-PKCε: in control, non-treated islets, basal insulin secretion was 0.26±0.06 ng/50 islets/min; in islets transfected with scrambled siRNA, 0.29±0.07 ng/50 islets/min; in islets transfected with siR-PKCε, 0.35±0.09 ng/50 islets/min; in islets infected with adenovirus expressing PKCε-KD, 0.30±0.09 ng/50 islets/min; in islets infected with adenovirus expressing PKCε-WT, 0.20±0.06 ng/50 islets/min (none significantly different). No change in insulin response was observed in islets infected with adenovirus over-expressing authentic PKCε (PKCε-WT) as compared with untreated islets (Fig. 3A), indicating that in non-treated islets, the enzyme was not rate-limiting in the exocytotic pathway. Transfection with an inactive (scrambled) construct of siRNA had no significant effect on rates of insulin response to glucose. Integrated evaluation (AUC) of the phasic response to glucose (Table 1) revealed a modest, but non-significant, decline of 1st phase insulin response (6 min) to glucose by treatment with either PKCε-KD or siR-PKCε. A significant decline in insulin response was seen in AUC during the 2nd phase (subsequent 1 h): the expression of PKCε-KD resulted in nearly 80% decline in insulin release and transfection with siR-PKCε resulting in 83% inhibition (Table 1).

3.2. Localization of activated PKCε

ß'COP1, a component of the COP1 trafficking complex was reported to be a specific anchoring protein for PKCε [11] This led us to consider the possibility that the inhibitory effect of PKCε on glucose-dependent insulin release could be related to a reduction in insulin production. The pool of newly synthesized hormone is known to be preferentially released upon glucose stimulation [22–25]. Fig. 4 shows that 15 min of stimulation of the isolated perfused rat pancreas with 16.7 mmol/l glucose resulted in concentration of the activated enzyme in granulated structures in the perinuclear region, known to be rich in ER and Golgi membranes. Within this short time frame, the majority of these perinuclear structures exhibited a signal super-imposable with insulin (or proinsulin), indicative of association with the insulin producing or storing structures. Forty-eight fields obtained from random sections of 4 control and 4 glucose-stimulated pancreata were imaged; 67% fields of pancreatic islets displayed glucose-induced β-cell perinuclear concentration of PKCε in association with insulin staining.

3.3. PKCε controls biosynthetic pathways

Given that PKCε localized to insulin-staining structures in the perinuclear regions, we next determined its role in the pathways of insulin production. Rat islets were infected with adenovirus expressing PKCε-KD, or εV1-2, stimulated with glucose for 75 min and pulse-labeled with ^{3}H-leucine for the final 15 min as described. Immediately at the end of the 75 min glucose stimulus, total islet proteins were extracted and subjected to immunoprecipitation with anti-insulin antibodies, or loaded on RP-HPLC for analysis of newly produced insulin-like products. At the end of 15 min labeling (0 time), the radioactivity recovered in the HPLC proinsulin peaks in non-infected control islets was 76,103±22,326 DPM/25 islets, N=8; in infected control islets expressing EGFP alone, 79,399±12,608 DPM/25 islets were measured, N=6; in infected control islets expressing εV1-2s, 108,840±16,234 DPM/25 islets were measured, N=5, (means±SEM, none significantly different). The insulin antibody was selected for its full cross-reactivity with proinsulin (see Materials and methods), therefore the products obtained by precipitation at the end of 15 min labeling would mostly be proinsulin. Islets expressing PKCε-KD produced 50% less proinsulin following the 15-min labeling, as compared to control islets as shown in Fig. 5A (p<0.01). The expression of inactive PKCε had no effect on total glucose-stimulated protein synthesis (Fig 5B) (p=NS). Similarly, at the end of the glucose stimulation, newly formed insulin-related products in islets infected with adenovirus expressing εV1-2 were more than 60% less than that observed in islets infected with adenovirus expressing the scrambled form of the peptide, εV1-2s (p<0.01) (Fig. 5C).

3.4. PKCε regulates proinsulin conversion to insulin

We next examined whether PKCε was also involved in the conversion of proinsulin to mature insulin I and II. Isolated rat islets were infected with adenovirus expressing the RACK2-competing peptide (εV1-2), and stimulated with 16.7 mmol/l glucose for 75 min; the final 15 min was pulsed with ^{3}H-leucine, then a 90-min chase period with 3.3 mmol/l glucose and 1.0 mmol/l leucine was applied (for details see Materials and methods). Islet proteins were analyzed by RP-HPLC as outlined [14,26]. The overall labeling in the control proinsulin peaks was 107,567±10,170 DPM/25 islets in the

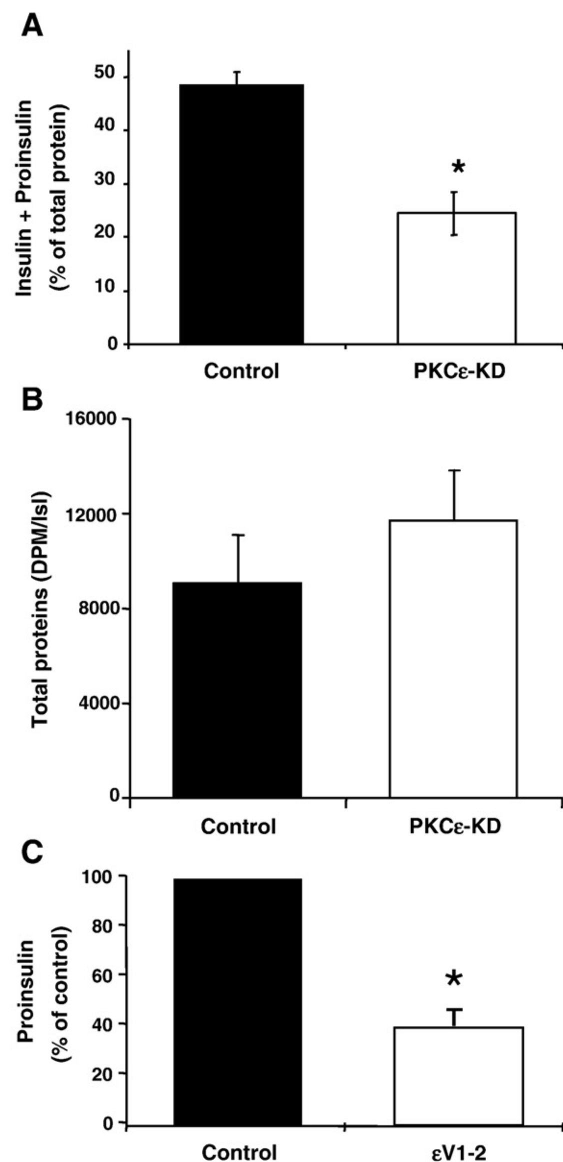

Fig. 5. Inhibition of PKCε results in diminished glucose-stimulated proinsulin production. Isolated rat islets expressing PKCε-KD (A), or εV1-2 (C), were stimulated with 16.7 mmol/l glucose for 75 min, and pulse-labeled with ^{3}H-leucine during the final 15 min. Islet extracts were subjected to immunoprecipitation with insulin/proinsulin antibody or analyzed by RP-HPLC and proinsulin production determined as fraction of total newly synthesized proteins (A), or of total insulin-related products (C). Shown are means±SE of 5–8 experiments; *=p<0.01. (B) No difference in total newly synthesized (TCA precipitate) protein was observed in islets expressing PKCε-KD.

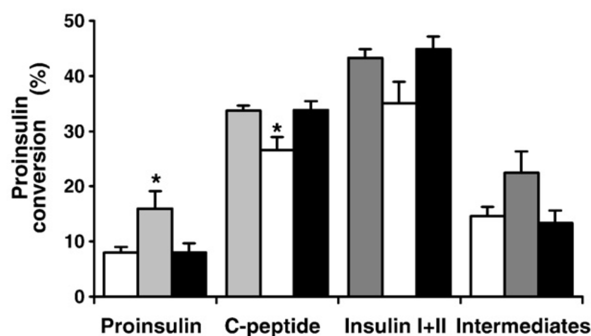

Fig. 6. PKCε affects early steps in the glucose-dependent processing of proinsulin. Isolated rat islets expressing εV1-2 or its scrambled control εV1-2s were stimulated with 16.7 mmol/l glucose for 75 min and pulse-labeled with ^{3}H-leucine during the final 15 min. Islet extracts were analyzed by RP-HPLC after 90 min chase with basal (3.3 mmol/l) glucose in the presence of excess leucine. Open bars represent control; grey bars, εV1-2; black bars, εV1-2s. Means±SE of 7–11 experiments; *= $p<0.05$.

non-treated islets; 99,062±11,330 DPM/25 islets in islets infected with adenovirus expressing EGFP alone and 127,125±19,187 DPM/25 islets in islets expressing the εV1-2s peptide (means±SEM, none significantly different). Fig. 6 presents the mean data of the individual peaks obtained in 7–11 studies, illustrating the relative amounts of proinsulin, insulin I+II, C-peptide and the insulin-related conversion intermediates detected at the end of the 90-min chase period. Diminished glucose-induced translocation of PKCε by εV1-2 led to a reduction in C-peptide and a small decrease in insulin fractions (insulin I+insulin II) compared to control islets or as compared with islets infected with adenovirus expressing the scrambled peptide, εV1-2s. These changes were significant for C-peptide ($p<0.05$) but not for insulin. Most significantly, the expression of εV1-2 resulted in the doubling of the fraction of proinsulin as compared to control (16%±3% vs. 8%±1%) or to islets expressing εV1-2s (16%±3% vs. 8%±2%) (both $p<0.05$). Finally, insulin-related conversion intermediates were also somewhat increased as compared to control islets or to islets expressing the scrambled peptide εV1-2s (22%±4% vs. 15%±2% control; 22%±4% vs. 13%±2% for εV1-2 vs. εV1-2s) (both NS). This analysis suggests that PKCε may affect two sites: the transport of proinsulin to the TGN, as well as its movement within the TGN, thus affecting its anterograde trafficking and the processing to mature insulin. The study also demonstrates that adenoviral infection and the expression of scrambled peptide had no effect on the synthesis of any of the insulin-related materials.

4. Discussion

Glucose metabolism controls the most essential functions in pancreatic β-cells through multiple complex pathways (reviewed in [1,2]). Several protein kinases play key functions in these cells, many also serve as cross points for signals originating from glucose metabolism and from activated receptors. Insulin synthesis as well as the synthesis of most other β-cell proteins is regulated by glucose metabolism; many components of this pathway are poorly defined. Glucose-enhanced overall protein synthesis has been linked with dephosphorylation of eIF2a and the formation of eIF2–GTP–Met-tRNA initiation complex [27], but the post-translational mechanism of glucose-selective production of insulin, a major component of the β-cell, is unknown. We have shown that glucose activated at least 6 isoforms of the PKC family, each with a unique kinetics and specific localization [5,6]. At the onset of the 1st phase (2 min), glucose-triggered co-localization of PKCε and insulin was observed near the β-cell membrane and subsequently, during the 2nd phase (>15 min) in the perinuclear region [5]. The latter persisted for more than 15 min following the cessation of the glucose stimulus. Stimulus-induced localization of PKCε to the ER–Golgi complex was reported in other cells (reviewed in [10]), and importantly, β′COP1, a vesicular trafficking protein, was the first specific anchoring protein (RACK2) identified for PKCε [11,28,29].

We next considered that the effect of inhibition of PKCε targeted the newly synthesized insulin, a pool of hormone preferentially released upon glucose stimulation [22–25]. Thus, the identification of ß'COP1 as the specific anchoring protein for PKCε prompted us to investigate the role of PKCε in vesicular trafficking in pancreatic β-cells. Indeed, employing three unrelated mechanisms to diminish β-cell activity of PKCε we were able to show that PKCε is involved in glucose-driven synthesis of insulin. The effect was quite early in the biosynthetic route: when evaluated at time 0, immediately after a short pulse with ^{3}H-leucine, production of proinsulin was strongly diminished under both conditions of diminished PKCε activity. Furthermore, PKCε was apparently required for further steps of the transport and processing of proinsulin, since 90 min after the glucose pulse, fractional C-peptide was significantly reduced, whereas the related insulin I and II and intermediate insulin split products were reduced but to a lesser extent (not significantly). Correspondingly, the fraction of proinsulin doubled in islets with diminished PKCε activity. Thus, PKCε could be the important linking kinase between glucose metabolism and insulin production, exerting its control at an early post-translational level. Its specificity was demonstrated from the lack of PKCε-dependent effects on newly synthesized total proteins.

Glucose-induced re-localization of PKCε to an area rich in ER and the trans Golgi network (TGN) was previously observed, and clearly illustrated here using the rat perfused pancreas preparation. Double staining also confirmed association with insulin-containing structures. The dramatic depletion in proinsulin production implies that PKCε plays an important part in recognition or anterograde trafficking of the newly produced proinsulin from the ER to the TGN, prior to further processing and packaging. In fact, the specific binding to β′COP would position PKCε action at this site. The reduction of proinsulin at an early time, before processing by the convertase PC1/3 reaches significant level, may indicate that the reduced transfer of proinsulin from the ER to the TGN leads to translational block and the elimination of the newly synthesized protein. We have never observed localization of PKCε within the nucleus, and therefore a transcriptional inhibitory action seems unlikely.

The specific role played by PKCε in glucose-dependent insulin production raises the question of which linking signal originates from glucose metabolism. DAG, PIP3 and some fatty acids were reported as physiological stimuli of PKCε in other systems [10]. Subcellular localization of PKCε may partially depend on which second messenger binds to its C1 domain: in some systems translocation to the plasma membrane and cytoskeleton was induced by DAG and tridecanoic acids, whereas localization to the TGN was induced by arachidonic and linoleic acids [30]. In pancreatic β-cells, glucose metabolism is known to promote, directly or indirectly, the production of all four messengers. The association of PKCε with β′COP occurs at the C2 domain, the domain that also binds phospholipids [10]. This needs to be reaffirmed in pancreatic β-cells. The target protein within the biosynthetic machinery for activated PKCε is yet unclear. Several non-vesicular proteins were reported to be targets for activated PKCε, none yet linked to early or late shuttling of newly translated peptides.

Schmitz-Peiffer et al. recently reported improved glucose tolerance, increased insulin production and reduced insulin disposal in transgenic mice with total body PKCε knockout [31]. Albeit inconsistent with our data utilizing islet-specific targeting methodology, it is however, impossible to preclude that extended, overall PKCε knockout may induce or activate alternative, undetermined signal pathways in the islet. The report that increased insulin production was typically significant in mice fed high fat diet would support the assumption that additional, PKCε-unrelated signals could be potentiated in the PKCε$^{-/-}$ mice.

In summary, the data presented here suggests that PKCε plays an important role in the transport of newly synthesized proinsulin from

the ER to the TGN. By affecting anterograde shuttling of the newly formed proinsulin, the enzyme may facilitate the processing of the prohormone to insulin. With interference in this function, diminished PKCε would overload the SER or ER with proinsulin, which would be promptly directed to a degradative pathway, resulting in diminished levels of proinsulin in that compartment. These effects of PKCε on the biosynthetic route of newly formed insulin may account for its indirect effect on glucose-triggered insulin secretion.

Acknowledgements

The authors wish to thank N. Kaiser, D. Gross and Y. Ariav for the technical advice. We are grateful to D. Mochly-Rosen for the εV1-2 plasmid and to T. Tenenbaum and T. Kuroki for the adenoviruses expressing PKCs. This work was supported in part by a grant from the Hadassah Medical Organization and a grant from Les Laboratoires Servier, France. N.W. was a fellow of the Israel Ministry of Science, Culture and Sport and of the Hebrew University Diabetes Center.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.04.007.

References

[1] F. Schuit, D. Flamez, A. De Vos, D. Pipeleers, Glucose-regulated gene expression maintaining the glucose-responsive state of beta-cells, Diabetes 51 (Suppl 3) (2002) S326–S332.
[2] J.T. Deeney, M. Prentki, B.E. Corkey, Metabolic control of beta-cell function, Semin. Cell Dev. Biol. 11 (2000) 267–275.
[3] P.M. Jones, S.J. Persaud, Protein kinases, protein phosphorylation, and the regulation of insulin secretion from pancreatic beta-cells, Endocr. Rev. 19 (1998) 429–461.
[4] D. Schechtman, D. Mochly-Rosen, Adaptor proteins in protein kinase C-mediated signal transduction, Oncogene 20 (2001) 6339–6347.
[5] N. Warwar, S. Efendic, C.G. Ostenson, E.P. Haber, E. Cerasi, R. Nesher, Dynamics of glucose-induced localization of PKC isoenzymes in pancreatic beta-cells: diabetes-related changes in the GK rat, Diabetes 55 (2006) 590–599.
[6] M. Yedovitzky, D. Mochly-Rosen, J.A. Johnson, M.O. Gray, D. Ron, E. Abramovitch, E. Cerasi, R. Nesher, Translocation inhibitors define specificity of protein kinase C isoenzymes in pancreatic beta-cells, J. Biol. Chem. 272 (1997) 1417–1420.
[7] V.U. Rao, H. Shiraishi, P.J. McDermott, PKC-epsilon regulation of extracellular signal-regulated kinase: a potential role in phenylephrine-induced cardiocyte growth, Am. J. Physiol. 286 (2004) H2195–H2203.
[8] D. Wu, T.L. Foreman, C.W. Gregory, M.A. McJilton, G.G. Wescott, O.H. Ford, R.F. Alvey, J.L. Mohler, D.M. Terrian, Protein kinase Cepsilon has the potential to advance the recurrence of human prostate cancer, Cancer Res. 62 (2002) 2423–2429.
[9] T.R. Xu, M.G. Rumsby, Phorbol ester-induced translocation of PKC epsilon to the nucleus in fibroblasts: identification of nuclear PKC epsilon-associating proteins, FEBS Lett. 570 (2004) 20–24.
[10] Y. Akita, Protein kinase C-epsilon (PKC-epsilon): its unique structure and function, J. Biochem. 132 (2002) 847–852.
[11] M. Csukai, C.H. Chen, M.A. De Matteis, D. Mochly-Rosen, The coatomer protein beta′-COP, a selective binding protein (RACK) for protein kinase Cepsilon, J. Biol. Chem. 272 (1997) 29200–29206.
[12] D. Schechtman, M.L. Craske, V. Kheifets, T. Meyer, J. Schechtman, D. Mochly-Rosen, A critical intramolecular interaction for protein kinase Cepsilon translocation, J. Biol. Chem. 279 (2004) 15831–15840.
[13] R. Nesher, L. Waldman, E. Cerasi, Time-dependent inhibition of insulin release: glucose–arginine interactions in the perfused rat pancreas, Diabetologia 26 (1984) 146–149.
[14] M. Gadot, G. Leibowitz, E. Shafrir, E. Cerasi, D.J. Gross, N. Kaiser, Hyperproinsulinemia and insulin deficiency in the diabetic *Psammomys obesus*, Endocrinology 135 (1994) 610–616.
[15] R. Nesher, N. Warwar, A. Khan, S. Efendic, E. Cerasi, N. Kaiser, Defective stimulus-secretion coupling in islets of *Psammomys obesus*, an animal model for type 2 diabetes, Diabetes 50 (2001) 308–314.
[16] S.M. Elbashir, J. Harborth, K. Weber, T. Tuschl, Analysis of gene function in somatic mammalian cells using small interfering RNAs, Methods 26 (2002) 199–213.
[17] H. Shinohara, N. Kayagaki, H. Yagita, N. Oyaizu, M. Ohba, T. Kuroki, Y. Ikawa, A protective role of PKCe against TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in glioma cells, Biochem. Biophys. Res. Commun. 284 (2001) 1162–1167.
[18] T. He, S. Zhou, L. da Costa, J. Yu, K. Kinzler, B. Vogelstein, A simplified system for generating recombinant adenoviruses, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2509–2514.
[19] P.A. Halban, C.J. Rhodes, S.E. Shoelson, High-performance liquid chromatography (HPLC): a rapid, flexible and sensitive method for separating islet proinsulin and insulin, Diabetologia 29 (1986) 893–896.
[20] Y. Zambre, Z. Ling, X. Hou, A. Foriers, B. Van Den Bogaert, C. Van Schravendijk, D. Pipeleers, Effect of glucose on production and release of proinsulin conversion products by cultured human islets, J. Clin. Endocrinol. Metab. 83 (1998) 1234–1238.
[21] C.F. Mendez, I.B. Leibiger, B. Leibiger, M. Hoy, J. Gromada, P.O. Berggren, A.M. Bertorello, Rapid association of protein kinase C-epsilon with insulin granules is essential for insulin exocytosis, J. Biol. Chem. 278 (2003) 44753–44757.
[22] G. Gold, M.L. Gishizky, G.M. Grodsky, Evidence that glucose "marks" beta cells resulting in preferential release of newly synthesized insulin, Science 218 (1982) 56–58 (New York, N.Y.
[23] P.A. Halban, Differential rates of release of newly synthesized and of stored insulin from pancreatic islets, Endocrinology 110 (1982) 1183–1188.
[24] H. Sando, J. Borg, D.F. Steiner, Studies on the secretion of newly synthesized proinsulin and insulin from isolated rat islets of Langerhans, J. Clin. Invest. 51 (1972) 1476–1485.
[25] C.F. Van Schravendijk, R. Kiekens, D.G. Pipeleers, Pancreatic beta cell heterogeneity in glucose-induced insulin secretion, J. Biol. Chem. 267 (1992) 21344–21348.
[26] M. Gadot, Y. Ariav, E. Cerasi, N. Kaiser, D.J. Gross, Hyperproinsulinemia in the diabetic *Psammomys obesus* is a result of increased secretory demand on the beta-cell, Endocrinology 136 (1995) 4218–4223.
[27] E. Gomez, M.L. Powell, I.C. Greenman, T.P. Herbert, Glucose-stimulated protein synthesis in pancreatic beta-cells parallels an increase in the availability of the translational ternary complex (eIF2-GTP.Met-tRNAi) and the dephosphorylation of eIF2 alpha, J. Biol. Chem. 279 (2004) 53937–53946.
[28] D. Mochly-Rosen, Localization of protein kinases by anchoring proteins: a theme in signal transduction, Science 268 (1995) 247–251.
[29] D. Mochly-Rosen, A.S. Gordon, Anchoring proteins for protein kinase C: a means for isozyme selectivity, Faseb J. 12 (1998) 35–42.
[30] Y. Shirai, K. Kashiwagi, K. Yagi, N. Sakai, N. Saito, Distinct effects of fatty acids on translocation of gamma- and epsilon-subspecies of protein kinase C, J. Cell Biol. 143 (1998) 511–521.
[31] C. Schmitz-Peiffer, D.R. Laybutt, J.G. Burchfield, E. Gurisik, S. Narasimhan, C.J. Mitchell, D.J. Pedersen, U. Braun, G.J. Cooney, M. Leitges, T.J. Biden, Inhibition of PKCepsilon improves glucose-stimulated insulin secretion and reduces insulin clearance, Cell Metab 6 (2007) 320–328.