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PKC ζ Enhances Insulin-like Growth Factor 1-Dependent Mitogenic Activity in the Rat Clonal β Cell Line RIN 1046-38

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Protein kinase C seems to be linked to the regulation of insulin secretion as well as mitogenic signaling in pancreatic β cells. To study the impact of different PKC isoforms on insulin secretion and mitogenic activity we stably overexpressed the PKC isoforms α, β2, ε, and ζ in the rat clonal β cell line RIN 1046-38. Under basal conditions PKC α, β2, ε, and ζ were identified mainly in the cytosol. Treatment with the phorbol ester TPA caused translocation of PKC α, β2, and ε to the plasma membrane. Glucose- and TPA-dependent increases in insulin release were comparable in all cell lines regardless of whether PKC was overexpressed or not. While PKC isoforms α, β2, and ε had no effect on the [^{3}H]thymidine incorporation rate, overexpression of PKC ζ specifically increased basal as well as IGF-1-dependent [^{3}H]thymidine incorporation. Incubation with the MAP-kinase inhibitor PD98056 abolished this effect. Furthermore, treatment with IGF-1 led to activation of the β cell-specific transcription factor PDX-1 in RIN 1046-38 cells overexpressing PKC ζ. Our data suggest that PKC ζ is involved in basal as well as IGF-1-dependent mitogenesis in RIN 1046-38 cells, while none of the PKC isoforms tested seem to be related to glucose-stimulated insulin release.

Key Words: **PKC ζ; insulin secretion; mitogenic activity; PDX-1; RIN 1046-38 cells.**

Insulin secretion in β cells is triggered by complex events and the coupling between secretagogue signals and insulin release is still not completely understood. Protein kinase C (PKC) seems to be linked to the regulation of insulin secretion as well as mitogenic signaling (1, 2). Several studies showed that basal as well as secretagogue-dependent insulin secretion is influenced by phorbol esters interpreted as evidence for an involvement of PKC in these processes (1). It is also known that glucose is able to stimulate PKC in β cells (3). While these data support a role of PKC in β cells in general, the exact mechanisms and cellular functions are not understood. In recent years, several PKC isoenzymes have been identified and characterized according to their molecular and biochemical properties (4). They represent a family of structurally and functionally related serine/threonine kinases derived from multiple genes as well as from alternative splicing of single mRNA transcripts. PKC isoforms differ in their regulatory domains and in their dependence on Ca^{2+}, as well as in their tissue distribution. Due to these characteristics, PKC isoforms can be subdivided into three major groups: classical, Ca^{2+}-dependent cPKC isoforms (α, β1, β2, γ), new, Ca^{2+}-independent nPKC isoforms (δ, ε, η, θ), and atypical aPKC isoforms (ζ, λ) (4).

It has been reported that PKC isoforms α, β, ε, δ, λ and ζ are expressed in pancreatic islets (reviewed in (1)). Similar expression patterns have been found in β cell lines (reviewed in (1)). So far, a small number of studies have been focused on the specific role of these isoforms in insulin-secreting cells. Evidence was presented that PKC ζ might be involved in carbachol dependent stimulation of insulin secretion (5). Other investigators have suggested that PKC α mediates glucose-dependent insulin release (3), however, this concept was not supported by other studies (2, 6–8). Additionally, a role of PKC for the mitogenic activity in β cells has been suggested (2). To study the impact of different PKC isoforms on insulin secretion and mitogenic activity we used RIN 1046-38 cell clones stably overexpressing one of the PKC isoforms α, β, ε, or ζ.

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Our data suggest a specific role for PKC ζ in mitogenic activity but not in glucose-dependent insulin secretion in RIN 1046-38 cells.

MATERIALS AND METHODS

Materials. G-418 sulfate, Lipofectamine transfection reagent, media and fetal calf serum for cell culture were from GIBCO (Grand Island, NY). Aprotinin, phenylmethylsulfonyl fluoride, Na_3VO_4 and Triton X-100 were from Sigma (Munich, Germany). PD-98059 was obtained from BIOMOL (Hamburg, Germany). Recombinant human IGF-1 was from R&D Systems (Wiesbaden, Germany). All other reagents were of the best grade commercially available. Antibodies used in this study are commercially available: the polyclonal isoform-specific PKC antibodies were obtained from GIBCO (Grand Island, NY). Secondary antibodies for immunofluorescence microscopy conjugated with fluorescence dye were from Molecular Probes (Leiden, The Netherlands). Klenow enzyme, poly[d(I-C)] was obtained from Roche Diagnostics and [α-^{32}P]dATP was purchased from Hartmann (Braunschweig, Germany).

Cell culture. RIN 1046-38 cells were grown in medium 199–Earle's salts (M199) containing 10% fetal calf serum (FCS), and were used at passages 18–28. Cells were split once a week, using 0.1% trypsin–EDTA solution (Life Technologies) and kept under an atmosphere of 93% air and 7% CO_2 at 37°C.

Transfection of PKC cDNAs. RIN 1046-38 cells (passage 18) were seeded at a density of 2×10^5 cells/ml, treated for 4 h with transfection medium containing 2 μg pRK5 (expression vector) or PKC isoform plasmid DNA (in pRK5) and suspended with 15 μl Lipofectamine in 1 ml serum-free M199 medium. Subsequently, 1 ml of M199 medium supplemented with 10% FCS was added to the transfection mixture. After 24 h, the transfection medium was replaced with M199 supplemented with 10% FCS, and the cells were incubated for another 72 h. Thereafter, the cells were kept in selection medium containing 0.4 mg/ml G-418 sulfate. Individual G-418-resistant clones were collected and tested for PKC expression.

Insulin secretion. RIN cells were seeded in 24-multiwell plates at a density of 10^5 cells/ml. After 48 h, the medium was removed, and the cells were washed twice for 30 min at 37°C with glucose-free buffer containing 114 mmol/l NaCl, 25.5 mmol/l $NaHCO_3$, 10 mmol/l Hepes, 2.5 mmol/l $CaCl_2$, 4.7 mmol/l KCl, 1.21 mmol/l KH_2PO_4, 1.16 mmol/l $MgSO_4$, and 0.1% BSA (pH 7.4). The cells were then incubated in the presence or absence of glucose (3 mmol/l, 30 min) or TPA (100 nmol/l, 10 min). Aliquots of the supernatant were collected and stored at −20°C for measurement of insulin release. To determine the intracellular insulin content, cells were lysed overnight at 4°C with a solution of 150 μl EtOH (100%), 3 μl HCl (10 N) and 47 μl H_2O. Insulin amount was assayed by a rat insulin RIA Kit (RI-13K) as described by the manufacturer (Linco Research, St. Louis, MO).

Determination of [^{3}H]thymidine incorporation in RIN 1046-38 cells. To measure [^{3}H]thymidine incorporation, cells were grown to 50% confluence in 6-well culture plates and subsequently starved for 48 h in M199 medium with 0.5% FCS. After stimulation with insulin and IGF-1 in the presence or absence of PD-98059 for 16 h, [^{3}H]thymidine (0.5 μCi/ml) was added for 4 h. The dishes were rinsed twice with ice-cold PBS and 10% trichloroacetic acid was added on ice. After 20 min, dishes were washed once with ice-cold 10% trichloroacetic acid, cells were lysed with 0.5 ml of 0.2 N NaOH/1% SDS, and neutralized with 0.5 ml of 0.2 N HCl. Radioactivity was determined by liquid scintillation counting.

Immunofluorescence microscopy. RIN 1046-38 cells were grown on uncoated glass coverslips, starved overnight in medium with 0.5% FCS and then stimulated with 100 nmol/l TPA for 10 min or 100 nmol/l IGF-1 for 10 min and fixed for 20 min in methanol (−20°C). After washing with phosphate-buffered saline (PBS) cells were incubated for 10 min with glycine (100 mmol/l)/$NaBH_4$ (0.1%) in PBS to block autofluorescence. Nonspecific antibody binding was blocked with PBG (phosphate-buffered saline with 0.045% fish gelatin) containing 5% goat serum and 1% bovine serum albumin. The primary antibodies (anti-PKC isoform specific) were applied for 1.5 h at room temperature. After four washes with PBG, primary antibody binding was detected with secondary antibodies conjugated with Alexa546 fluorescent dye. The coverslips were mounted in PermaFluor (Immunotech, Marseille, France). Localization of the proteins was examined using confocal laser microscopy (Leica, Germany).

Preparation of nuclear extracts and electrophoretic mobility shift assay. Nuclear proteins were prepared as described by Andrews (9) and the protein concentrations were determined according to Bradford using the Bio-Rad protein assay reagent (10). Double stranded oligonucleotides corresponding to rat insulin-II A3 element (CCCCTGGTTAAGACTCTAATGACCCGCTGG) were end-labeled with [α-^{32}P]dATP (3000 Ci/mmol/l) and Klenow enzyme and were incubated with 10 μg nuclear protein in 20 μl 7 mmol/l Hepes–KOH, pH 7.9, 100 mmol/l KCl, 3.6 mmol/l $MgCl_2$, 10% glycerol on ice for 20 min. 0.05 mg/ml poly[d(I-C)] was added as unspecific competitor. The samples were run on a 5% nondenaturing polyacrylamide gel in a buffer containing 25 mmol/l Tris–HCl, pH 8.0; 190 mmol/l glycine and 1 mmol/l EDTA. Gels were dried and analyzed by autoradiography.

Statistics. Statistical analysis was done by multivariate analysis of variance, one-way analysis of variance and Student's *t*-test respectively. Multiple comparison testing was compensated using Tukey's procedure and Bonferroni correction as appropriate. Data in figures are expressed as means ± SE. *P* values less than 0.05 were considered significant.

RESULTS

To study the role of specific PKC isoforms for insulin secretion and mitogenic activity in insulin secreting cells we have stably overexpressed the classical PKC isoforms α and β2, the novel PKC isoform ε and the atypical PKC ζ in RIN 1046-38 cells. These PKC isoenzymes have been found in different β cell lines as well as pancreatic islets. RIN 1046-38 cells were derived from rat insulinoma and retain glucose-dependent insulin secretion at subphysiological glucose levels (11). Overexpression of the transfected PKC isoforms in RIN 1046-38 was confirmed by immunoblotting (data not shown) as well as confocal laser microscopy. We found that PKC delta was the predominant endogenous PKC in RIN 1046-38 cells (Fig. 1a). Under basal conditions PKC α, β2, ε, δ and ζ were identified mainly in the cytosol (Figs. 1a and 1b). TPA treatment of cells caused translocation of PKC α, β2 and ϵ to the plasma membrane and of PKC δ predominantly to the nuclear membrane (Fig. 1c). Therefore, overexpression of PKC α, β2, ϵ and ζ, endogenous expression of PKC δ as well as responsiveness to TPA (α, β2, ϵ, δ) could be demonstrated by confocal laser microscopy. We used high concentrations of IGF-1 (100 nmol/l, 10 min), which are also sufficient to stimulate the insulin receptor since it is known that the TPA-insensitive atypical PKC ζ can be stimulated by insulin and IGF-1. However, stimulation with IGF-1 did not induce a translocation of PKC ζ to the plasma or nuclear membrane under the experimental conditions chosen.

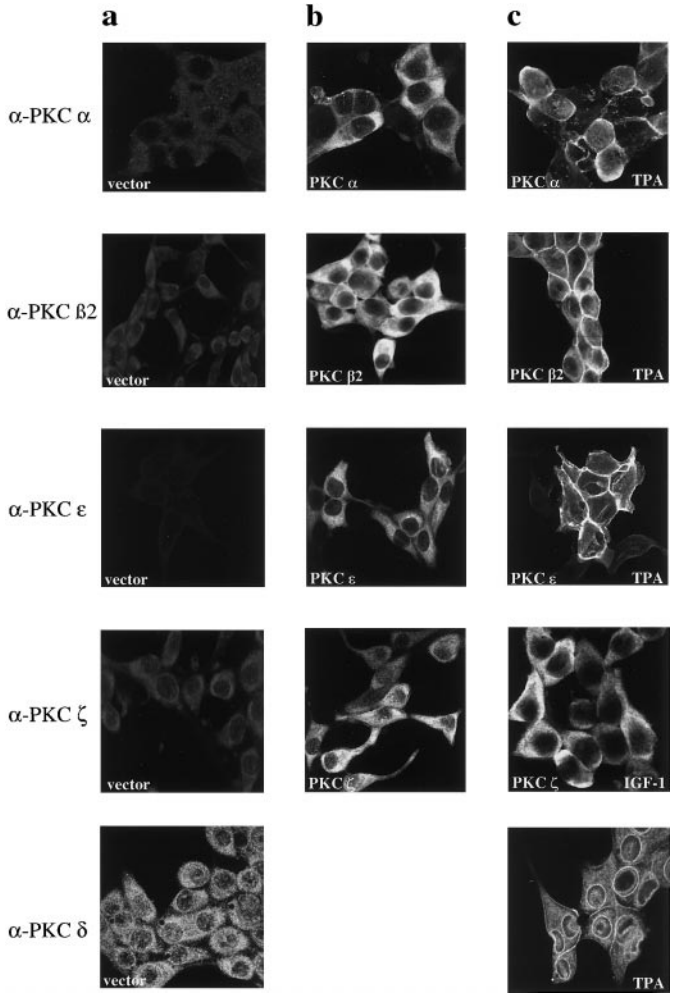

FIG. 1. Immunofluorescence staining of PKC α, β2, ϵ, δ, and ζ in RIN 1046-38 cells. RIN 1046-38 cells stably overexpressing vector (a) or PKC α, β2, ϵ, or ζ (b) were left untreated (b) or stimulated (c) with TPA (100 nmol/l, 10 min) or IGF-1 (100 nmol/l, 10 min) and then fixed with methanol. After blocking of autofluorescence and nonspecific antibody binding as described under Materials and Methods, immunostaining was performed by incubating the cells with antibodies directed specifically against each PKC isoform. Primary antibody binding was detected with secondary antibody conjugated with fluorescent dye.

To measure insulin secretion we used glucose (3 mmol/l, 30 min) as well as TPA (100 nmol/l, 10 min) in RIN 1046-38 cell clones overexpressing different PKCs. After stimulation with glucose a small increase of insulin secretion could be observed, i.e., 21 ± 4% ($P < 0.01$, $n = 6$) over basal level in vector transfected cells (Fig. 2). As we have studied RIN 1046-38 cells between passage 18 and 28 our results are in agreement with other investigators showing only a small increase of glucose dependent insulin secretion at passage 22 or above (11). Glucose-dependent increase of insulin release was comparable in all cell lines regardless of whether PKC was overexpressed or not (Fig. 2). This suggests that PKC α, β2, ε, and ζ do not play a major role for glucose-dependent insulin secretion in RIN 1046-38 cells. Stimulation of insulin secretion can be demonstrated with the phorbol ester TPA increasing insulin release by 67 ± 18% over basal ($P < 0.01$, $n = 6$, Fig. 2). However, there was no difference in TPA stimulated insulin secretion between vector transfected and PKC α, β2, and ε overexpressing cells (TPA insensitive PKC ζ was not tested).

Besides the potential role of PKC for insulin secretion it was suggested that PKC mediates mitogenic activity in β cells (2). Stimulation of β cell replication has been shown by glucose as well as several growth factors (12–16). We have studied mitogenic activity in RIN 1046-38 cells overexpressing PKC by measuring [^{3}H]thymidine incorporation. To reduce the variations due to different cell preparations all cpm values where normalized, taking the vector transfected cells in the basal state as 100%. The differences between the different PKC isoforms were tested for statistical significance using one-way ANOVA with Tukey's procedure to take multiple comparisons into account. While PKC isoforms α, β2 and ε had no effect on basal thymidine incorporation, overexpression of PKC ζ increased thymidine incorporation 2.39-fold in the basal state (Fig. 3, $P < 0.03$, $n = 5$). In addition, RIN 1046-38 cells overexpressing PKC ζ responded with a significant increase in thymidine incorporation after a 16-h incubation with 100 nmol/l insulin ($P < 0.03$, $n = 5$) or 10 nmol/l IGF-1 ($P < 0.04$, $n = 5$) while low insulin concentrations at 10 nmol/l had no effect. As a positive control we used 10% horse serum which leads to a high and significant increase in [^{3}H]thymidine incorporation in PKC ζ overexpressing RIN 1046-38 cells. In contrast, basal and stimulus dependent mitogenic activity was not different in cells overexpressing PKC α,

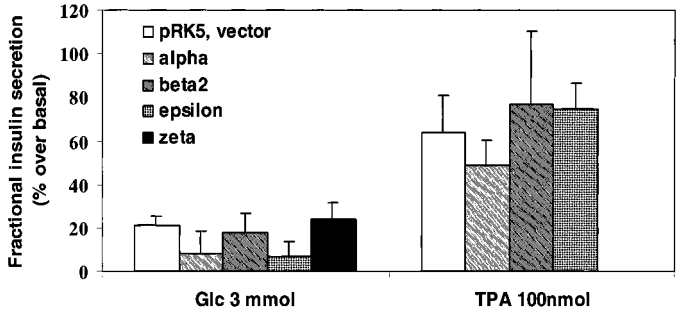

FIG. 2. Fractional insulin secretion in response to glucose and TPA. RIN 1046-38 cells stably overexpressing vector or PKC α, β2, ϵ, and ζ were stimulated with glucose (3 mmol/l, 30 min) or TPA (100 nmol/l, 10 min). At the end of the incubation period the supernatant was collected and total cellular insulin content was determined from lysed cell extracts. Insulin concentration in both, cellular extracts as well as supernatant was determined by RIA. Insulin release was standardized to total cellular insulin content (determined by RIA after cell lysis) and expressed as the percentage of fractional insulin secretion over basal. Data are means ± SE of 6 independent experiments, each carried out in triplicate.

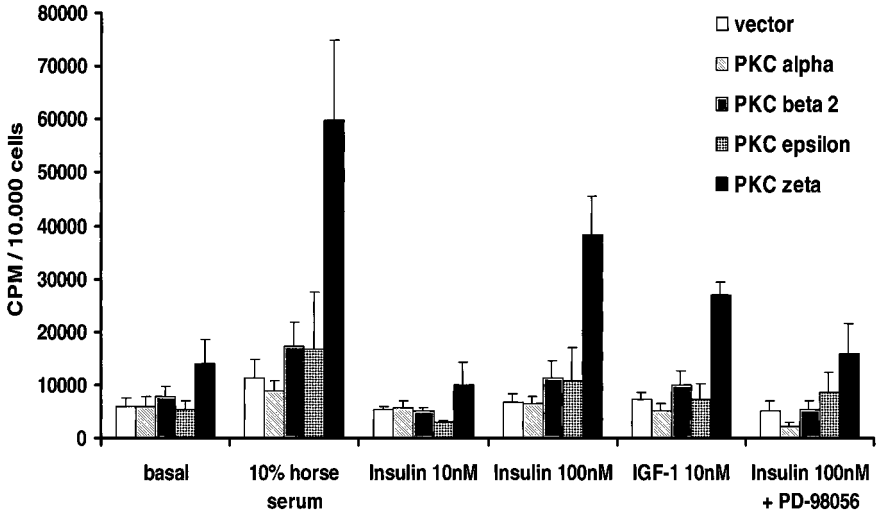

FIG. 3. [^{3}H]Thymidine incorporation rate in RIN 1046-38 cells overexpressing PKC α, β2, ϵ, or ζ. RIN 1046-38 cells overexpressing vector or PKC α, β2, ϵ, and ζ were stimulated with IGF-1 (10 nmol/l) or insulin (10 and 100 nmol/l) with or without PD-98056 (50 μmol/l) for 16 h. Subsequently, cells were incubated for 4 h with 0.5 μCi/ml [^{3}H]thymidine and then washed with TCA. TCA precipitable radioactivity was measured in a liquid scintillation counter. Results are mean values ± SE of 5 experiments, each carried out in duplicate.

β2 or ε compared to vector transfected cells (Fig. 3). This suggests that PKC ζ is specifically important for basal as well as IGF-1- and serum-dependent mitogenic activity in RIN 1046-38 cells.

We have tested whether MAP kinase is necessary for PKC ζ mediated mitogenic activity by using the MAP-kinase inhibitor PD-98056 since there is evidence that insulin and IGF-1 mediate mitogenic activity through the MAP kinase pathway. Figure 3 shows that PD-98056 is able to completely inhibit insulin-dependent thymidine incorporation in PKC ζ expressing cells suggesting that MAP-kinase is involved in insulin/IGF-1-stimulated and PKC ζ-mediated mitogenic activity in RIN 1046-38 cells. The differences of the PKC ζ-mediated mitogenic activity in the different cell preparations were tested for statistical significance using Student's *t* test for paired samples and Bonferroni correction to take multiple comparisons into account.

Among the transcription factors which are involved in β cell differentiation and proliferation PDX-1 (also known as IPF-1, STF-1 and IDX-1) seems to play a critical role in rodent and human pancreas (17–20). Therefore, we tested whether IGF-1-dependent and PKC ζ-mediated mitogenic activity in RIN 1046-38 cells leads to activation of PDX-1 by stimulating cells with or without IGF-1 (10 nmol/l). Activation of PDX-1 was studied by testing the DNA binding activity of nuclear proteins prepared from these cells to the A3 element of the rat insulin II promoter, which is known as a PDX-1 binding site by electrophoretic mobility shift assay (EMSA). Figure 4 shows a representative autoradiogram indicating activation of PDX-1 in PKC ζ overexpressing cells after stimulation with IGF-1 (10 nmol/l). In four independent experiments DNA binding activity to the PDX-1 binding site was significantly increased upon IGF-1 stimulation by 1.54 ± 0.28 fold over basal (n = 4, P = 0.016). There was a slight tendency to increased basal PDX-1 binding activity in PKC ζ transfected cells and a faint increase of IGF-1 stimulated PDX-1 binding in vector transfected cells, however, these effects did not reach statistical significance. Specificity of the enhanced binding to the PDX-1 binding site was confirmed by addition of excessive amounts of unlabeled oligonucleotides, which displaced the specific binding in a dose-dependent manner (Fig. 4b).

DISCUSSION

We have attempted to define the importance of classical, novel as well as atypical PKC isoforms for glucose- and TPA -dependent insulin secretion as well as mitogenic activity in the rat β cell line RIN 1046-38 using stable overexpression of the PKC isoforms α, β2, ϵ, and ζ. In agreement with earlier studies we observed increased glucose and TPA induced insulin release in RIN 1046-38 cells. Our results do not support a role of PKC α, β2, ε, and ζ for glucose- or TPA-dependent insulin secretion in RIN 1046-38 cells, although activation of PKC α, β2, and ϵ has been clearly demonstrated by TPA-dependent translocation/activation in confocal laser microscopy. This does not exclude that other PKC isoforms are involved. It is possible that TPA stimulates insulin release mainly via PKC δ being the only TPA dependent PKC isoform, which is endogenously expressed at considerable amounts in the parental cell line. Another reason for the failure of a further increase of TPA-induced insulin release in PKC overexpressing cells could be that TPA-dependent signaling pathways are already maximally challenged by the endogenous PKC isoform δ in RIN

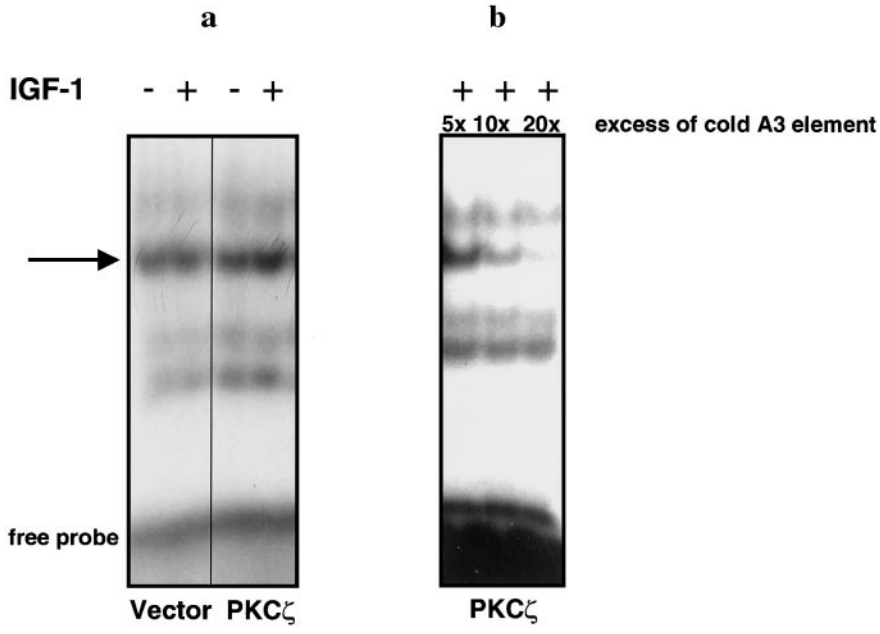

FIG. 4. Effect of IGF-1 on PDX-1 protein binding to the rat insulin promotor A3 element using nuclear extract from RIN 1046-38 cells. Cells were cultured to 70% confluence, serum starved for 24 h and stimulated with IGF-1 (10 nmol/l) for 16 h. (a) 10 μg of nuclear extracts were incubated with 50,000 cpm of the ^{32}P-labeled DNA probe and analyzed by electrophoretic mobility shift assays (EMSA). Arrow indicates enhanced binding of nuclear proteins. (b) The IGF-1-enhanced DNA binding could be displaced in a specific and dose-dependent manner by excessive unlabeled probe of the A3 element of the rat insulin II promotor.

1046-38 cells and due to this PKC-overexpression might not cause a further increase of insulin release after treatment with TPA.

While no effects of PKC overexpression have been found on glucose or TPA dependent insulin secretion, our data suggest a specific role of the PKC isoform ζ for mitogenic activity of RIN 1046-38 cells. This result is in agreement with studies from non-β cell lines showing mitogenic activity of PKC isoform ζ (21) and with an earlier study suggesting that unidentified PKC might be involved in β cell mitogenesis of rat islets (2). It can be suggested that mitogenic activity triggered by insulin is mainly mediated through the IGF-1 receptor since RIN 1046-38 cells express predominantly IGF-1 and not insulin receptors at the mRNA level (data not shown). The dose-dependent effects of insulin and IGF-1 further support this assumption. While IGF-1 stimulated [^{3}H]thymidine incorporation already at 10 nmol/l, insulin exerts its effect only at 100 nmol/l. Lower insulin concentrations, which do not cross-stimulate the IGF-1 receptor showed no increase. This suggests that the effects of insulin on mitogenic activity are mediated rather through IGF-1 than through insulin receptors. Insulin- and IGF-1-dependent stimulation of mitogenic activity involves activation of the MAP kinase pathway (22–24). In RIN 1046-38 cells MAP-kinase activation seems to be necessary for PKC ζ mediated mitogenic activity as well since the MAP-kinase inhibitor PD-98065 suppresses this effect.

It is believed that β cell mass underlies a permanent degree of turnover induced by differentiation of pancreatic progenitor cells as well as proliferation and apoptosis (14). Although not a highly proliferative tissue, β cells in adult subjects still show neogenesis through replication and differentiation of progenitor cells (14, 15, 18–20, 25, 26). Furthermore, a role of the IGF-family has been suggested for β cell development and DNA synthesis in rat islet cells (27, 28). In agreement with these results we demonstrate IGF-1-dependent stimulation of DNA synthesis and activation of PDX-1. Our data strongly suggest that IGF-1 induces mitogenic effects via a PKC ζ and MAP-kinase-dependent pathway in RIN 1046-38 cells. A role of PKC ζ for mitogenesis in β cells could theoretically offer interesting approaches for β cell expansion. However, to substantiate such speculations further studies have to show whether this pathway exists in pancreatic islets as well. Unfortunately, endogenous expression levels of these signaling elements are very low and therefore these mechanisms are difficult to analyze in primary β cells.

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