Possible Involvement of Atypical Protein Kinase C (PKC) in Glucose-Sensitive Expression of the Human Insulin Gene: DNA-Binding Activity and Transcriptional Activity of Pancreatic and Duodenal Homeobox Gene-1 (PDX-1) Are Enhanced via Calphostin C-Sensitive but Phorbol 12-Myristate 13-Acetate (PMA) and Gö 6976-Insensitive Pathway

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Abstract. Pancreatic and duodenal homeobox gene-1 (PDX-1) is a transcription factor which regulates the insulin gene expression. In this study, we tried to elucidate the role of PDX-1 in the glucose-induced transcriptional activation of the human insulin gene promoter in MIN6 cells. Electrophoretic mobility shift assay (EMSA) and chloramphenicol acetyltransferase (CAT) assay demonstrated that both DNA-binding activity and transcriptional activity of PDX-1 were increased with 20 mmol/l glucose more than with 2 mmol/l glucose. The DNA-binding activity of PDX-1 induced by high glucose was blocked by phosphatase treatment, suggesting the involvement of PDX-1 phosphorylation in this event. In an in vitro phosphorylation study, PDX-1 was phosphorylated by protein kinase C (PKC), but not by cAMP dependent protein kinase (PKA) or mitogen-activated protein kinase (MAPK). Furthermore, increased PDX-1 function induced by high glucose was blocked by calphostin C, an inhibitor of all PKC isoforms, but unaffected by phorbol 12-myristate 13-acetate (PMA), an activator of classical and novel PKC, or Gö 6976, an inhibitor of classical and novel PKC, which suggested that the PKC family which activated PDX-1 in MIN6 cells was atypical PKC. Western blot and immunocytochemical studies with anti-PKC ζ antibody confirmed the presence of PKC ζ, one of the isoforms of atypical PKC, in MIN6 cells. Furthermore, PKC ζ activity was significantly increased by glucose stimulation. These results suggest that high glucose increased DNA-binding activity of PDX-1 by activating atypical PKC including PKC ζ, resulting in transcriptional activation of the human insulin gene promoter.

Key words: MIN6 cell, Insulin gene expression, Pancreatic and duodenal homeobox gene-1 (PDX-1), Atypical protein kinase C

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Insulin gene expression has been shown to be dependent on the interaction between trans-acting factors and cis-acting elements located within 350 base pairs upstream from the transcription start site [7-11]. In this region, there are two different types of cis-acting elements, the A element with a TAAT sequence motif [8, 12], and the E element with a CANNTG sequence motif [8, 13]. In the human insulin gene promoter, there are four A elements (A1–A3, A5) and two E elements (E1, E2). The A element has been reported to be essential for glucose sensitivity in the human and rat insulin I gene promoters [14, 15]; other investigators have reported the E element to be important for glucose-sensitive rat and human insulin gene expression [16-20].

Several nuclear proteins which bind to A or E elements have been identified. One of these, pancreatic and duodenal homeobox gene-1 (PDX-1), which contains homeodomain and binds to the A element, has been reported to be important for β cell-specific insulin gene expression [11, 12, 21-23], glucose-sensitive insulin gene expression [14, 15, 24-26], and pancreatic islet development [11, 19, 27-31]. Several investigators showed that glucose increased the binding activity of PDX-1 to the A element of the rat and human insulin genes in isolated rat and human islets [14, 15, 26]. It was reported that in HIT cells chronic exposure to high glucose medium could decrease insulin mRNA levels, insulin content and insulin secretion [32], these decreases accompanied by decreased binding of PDX-1 to the A element of the insulin gene promoter [24, 25]. Recently, Macfarlane et al. showed that phosphorylation of PDX-1 was involved in the glucose-induced increase in DNA-binding of PDX-1 [26], and that this phosphorylation was activated indirectly by stress-activated protein kinase 2 (SAPK2, also termed the p38 mitogen-activated protein kinase [MAPK]) pathway [33], but how PDX-1 was phosphorylated at high glucose was not clarified.

To further clarify the mechanisms of glucose-induced phosphorylation of PDX-1, we analyzed the phosphorylation status of PDX-1 and tried to identify the kinase involved in the phosphorylation at a high glucose concentration in the mouse insulinoma cell line, MIN6 cells.

**Materials and Methods**

**Materials**

MIN6 cells, a mouse pancreatic β cell line, which retains glucose-dependent insulin secretion [34-36], and pAC-LacZ plasmid [37] were kindly provided by Dr. Miyazaki. Purified cAMP dependent protein kinase (PKA), protein kinase C (PKC) and recombinant mitogen-activated protein kinase (MAPK) (recombinant extracellular signal-regulated kinase-1 [Erk-1], an isoform of MAPK, Upstate Biotechnology [Lake Placid, NY, U.S.A.]) were kindly provided by Drs. Yamamoto, Fukunaga and Miyamoto [36, 38]. Phorbol 12-myristate 13-acetate (PMA), calphostin C, propidium iodide (PI) and myelin basic protein (MBP) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Go 6976 was from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.). Potato acid phosphatase, protein A sepharose beads and nitrocellulose filters were from Boehringer Mannheim Biochemicals (Penzberg, Germany), Pharmacia Biotech (Uppsala, Sweden) and Schleicher and Schuell (Dassel, Germany), respectively. Exonuclease III and mung bean nuclease were from New England Biolabs, Inc. (Beverly, MA, U.S.A.), and the SAPK2 assay kit (p38/RK/Mpk2 Assay Kit) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and the DEAE-dextran Kit were purchased from Nissui (Tokyo, Japan), Intergen Company (Purchase, NY, U.S.A.) and Stratagene (La Jolla, CA, U.S.A.), respectively. [γ-32P] ATP and [125I] protein A were from NEN Research Products (Boston, MA, U.S.A.) and ICN Biomedical Inc. (Costea Mesa, CA, U.S.A.), respectively.

**Antibodies**

Anti-PDX-1 antiserum was prepared as described below. The peptide corresponding to amino acid residues 1-13 from the N-terminus of mouse PDX-1 [27], Met-Asn-Ser-Glu-Glu-Gln-Tyr-Tyr-Ala-Ala-Thr-Gln-Leu-Cys, was synthesized (A Chiron Company, Clayton, Victoria, Australia). The conjugate was emulsified with complete Freund’s adjuvant and injected subcutaneously into New
Zealand rabbits. Subcutaneous boosted injections of the antigen emulsified in incomplete Freund's adjuvant were given every two weeks. Serum was collected after 8 weeks. We also used another anti-PDX-1 antiserum raised against amino acid residues 269–282 of the PDX-1 peptide and kindly provided by Dr. Kajimoto [39]. Anti-PKC \( \zeta \) antibody, anti-PKC \( \lambda \) antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Cell culture and purification of nuclear extract

MIN6 cells were cultured in 10-cm plates in DMEM supplemented with 10% FBS and 20 mmol/l glucose. When the cells reached approximately 80% confluence, they were washed twice with phosphate-buffer saline (PBS) (pH 7.4, 37 °C) and preincubated for 24 h in DMEM supplemented with 10% FBS and 2 mmol/l glucose. The cells were then incubated for 24 h with 2 mmol/l or 20 mmol/l glucose in DMEM supplemented with 10% FBS. The nuclear extract was purified as previously described by Dignam et al. [40].

Construction of the human insulin gene promoter CAT gene fusion plasmids

Promoter activity of the human insulin gene was analyzed with a low background promoterless chloramphenicol acetyltransferase (CAT) plasmid, pSV001CAT [41, 42]. The 12 kilo base pair fragment containing the 5' flanking region of the human insulin gene was cloned from a human genomic library as described previously [43] with primers designed from the human insulin gene [44] (sense primer \([-24 \text{ to } +6, 5'\text{-AGGCCAGCGGGGCCC-ACAGCCCTCAGCCC-3'}}\] and anti-sense primer \([+237 \text{ to } +266, 5'\text{-GCGCCAGGCCGCTCCGACCAGCC-3'}}]\)). Human insulin gene promoter (\(-2184 \text{ to } +236\)) was subcloned into pSV001CAT plasmid, and the resultant plasmid was termed pIPA-CAT. The pSV001CAT plasmids which contained \(-230 \text{ to } +236\) and \(-201 \text{ to } +236\) regions in the human insulin gene were made by partial excision of pIPA-CAT with exonuclease III and mung bean nuclease, and the resultant plasmids were termed pIPC-CAT and pIPD-CAT, respectively.

Transfection and CAT assay

Transfections were carried out with a DEAE-dextran Kit and 10 \( \mu \text{g} \) pIPC-CAT plasmid, pIPD-CAT or phIR-CAT [43], which contained human insulin receptor gene promoter as a control, and 2 \( \mu \text{g} \) of pAC-LacZ [37], which contained the LacZ gene under the control of chicken \( \beta \)-actin promoter, as an internal control. After 4 h, the culture medium was replaced by medium containing either 2 mmol/l or 20 mmol/l glucose with or without the reagent indicated (PMA, calphostin C or Gö 6976). The cells were further cultured for 12 h for PMA, 24 h for calphostin C or Gö 6976, harvested and subjected to CAT assay, as described previously [45]. All experiments were performed 3–5 times each in duplicate. CAT activity was normalized with respect to \( \beta \)-galactosidase activity and expressed as a percentage of each CAT plasmid in MIN6 cells cultured in 2 mmol/l glucose (Fig. 6) or 0 nmol/l PMA medium (Fig. 4A).

Electrophoretic mobility shift assay (EMSA)

A fragment corresponding to the sequence \(-230 \text{ to } -201 (5'\text{-GCCCTGTTAAGACTCTAATG-ACCCGCTG-3'}}\) in the human insulin gene promoter which contained the A3 element was used as a radiolabeled probe or a non-labeled competitor. Probes were end-labeled with \([\beta^{32}\text{P}]\) ATP and T4 polynucleotide kinase. A mutated competitor, in which the TAAT sequence in the A3 element [8] was changed to TCCT, was designed as follows: \(5'\text{-GCCCTGTTAAGACTCTCCTGACCCGCTG-3'}}\). EMSA employing probes, competitors and nuclear extract was performed as described previously [42]. In some experiments, 5 \( \mu \text{g} \) of nuclear extract was treated with 60 mU of potato acid phosphatase or heat-inactivated potato acid phosphatase (80 °C for 15 min) for 30 min at 37 °C, then EMSA was performed. The intensity of each band was measured with a Bio Image Analyzer BA100 (Fuji Photo Film Co., Ltd. [Tokyo, Japan]), and expressed as the mean of six independent experiments.

UV crosslink assay

EMSA was performed with \(1.0 \times 10^4 \text{ cpm of}\)
radiolabeled probe and 5 µg of nuclear extract prepared from MIN6 cells cultured with high glucose. The gel was exposed to UV light (266 nm) for 20 min and autoradiographed overnight. The retarded bands were excised from the gel and each DNA-protein complex was eluted in 0.5 x TBE buffer, followed by ethanol precipitation. The pellet was eluted in Laemmli sample buffer (2.5 % SDS, 2.5 mmol/l Tris-HCl, pH 6.8, 10% glycerol, 0.025% bromophenol blue, 100 mmol/l dithiothreitol [DTT]), and loaded onto a 10% SDS-PAGE. The gel was dried and autoradiographed with an intensifying screen for 24 h at -70 °C.

Immunoprecipitation and Western blot

100 µg of nuclear extract was incubated with 1:100 dilution of anti-PDX-1 antiserum raised against the N-terminus of the PDX-1 in immunoprecipitation buffer (50 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 5 mmol/l EDTA, 5 mmol/l EGTA, 20 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, 20 mmol/l NaF and 10 µmol/l p-a-phenylmethylsulfonyl fluoride [PMSF]) for 2 h at 4 °C. Protein A sepharose beads (40 µl) were added, and incubated for 1.5 h at 4°C. After centrifugation, the precipitate was eluted in Laemmli sample buffer, boiled, and subjected to SDS-PAGE. The electrotransfer of proteins from gel to a nitrocellulose filter was performed for 45 min at a constant current (2 mA/cm²). The filter was preincubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk, 20 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 0.1% Tween 20), and incubated for 1 h at room temperature with the same anti-PDX-1 antiserum used in immunoprecipitation diluted 1:1000 in wash buffer (20 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.1% Tween 20). The filter was then washed for 25 min in wash buffer, incubated with 2.4 µCi/ml of [32P] ATP (3000 cpm / pmol) and 1 µg/ml bovine serum albumin (BSA) in a final volume of 25 µl. After incubation for 60 min at 30 °C, each reaction was stopped by adding 5 µl of Laemmli sample buffer. The samples were boiled, subjected to SDS-PAGE, and followed by autoradiography. To calculate each kinase activity, we performed kinase assay with synapsin 1 as the substrate [36, 38] in the same conditions, and the sample was subjected to SDS-PAGE or liquid scintillation counting. The specific activity of each kinase used was 144 cpm/ µg/min.

Immunocytochemical study

MIN6 cells cultured in 35-mm plates were washed three times with cold PBS, and fixed with 100% cold methanol for 10 min on dry ice. The samples were rehydrated with PBS for 15 min, and then permeabilized by incubation for 15 min with PBS/0.01% Triton X. After rinsing with PBS, the samples were incubated for 30 min with PBS/0.3% BSA to block non-specific binding sites and then incubated with anti-PKC ζ antibody diluted 1:500 in PBS/0.3% BSA for 2 h at room temperature. After rinsing with PBS, the samples were incubated with FITC-conjugated anti-rabbit IgG diluted 1:80 in PBS/0.01% Triton X for 1 h at room temperature, and washed with PBS. To locate nuclei, the samples were incubated with 5 µmol/l of the DNA counterstain propidium iodide (PI) for 3 min at room temperature. After rinsing with PBS, the samples were mounted with PBS/90% glycerol under a glass coverslip. A Confocal Laser Microscopy System (Olympus Co. Ltd. [Tokyo, Japan]) with an argon laser was used to obtain thin optimal slices through the fixed labeled cells.

In vitro phosphorylation

Fifty µg of nuclear extract was immunoprecipitated with anti-PDX-1 antiserum or pre-immune serum, and the precipitate was assayed for phosphorylation. The reaction mixtures for PKA, PKC and MAPK contained: 1 mmol/l EGTA and 100 nmol/l PKA; 25 mmol/l CaCl₂, 5 µg/ml diacylglycerol, 50 µg/ml phosphatidylserine and 100 nmol/l PKC; and 25 mmol/l EGTA and 20 nmol/l MAPK, respectively. All these mixtures also contained 50 mmol/l HEPES (pH 7.5), 10 mmol/l magnesium acetate, 100 mmol/l [γ32P] ATP (3000 cpm / pmol) and 1 µg/ml bovine serum albumin (BSA) in a final volume of 25 μl. After incubation for 60 min at 30 °C, each reaction was stopped by adding 5 μl of Laemmli sample buffer. The samples were boiled, subjected to SDS-PAGE, and followed by autoradiography. To calculate each kinase activity, we performed kinase assay with synapsin 1 as the substrate [36, 38] in the same conditions, and the sample was subjected to SDS-PAGE or liquid scintillation counting. The specific activity of each kinase used was 144 cpm/ µg/min.
**Assay of PKC ζ activity**

800 µg of nuclear extract was immuno-precipitated with anti-PKC ζ antibody, and the precipitate was subjected to the PKC assay [46]. The total assay volume was 50 µl and contained 25 mmol/l Tris-Cl, pH 7.5, 10 mmol/l MgCl₂, 1 mmol/l DTT, 0.5 mmol/l EGTA, 80 nmol/l [γ-³²P] ATP (3000 cpm/pmol) and 0.6 mg/ml MBP. After incubation at 30°C for 5 min, the reaction was stopped by spotting onto phosphocellulose paper, and the paper was washed twice in 500 ml of 5% phosphoric acid and twice in 500 ml water. Incorporation of ³²P was determined by liquid scintillation counting. PKC ζ activity was represented by the mean ± SD from three independent experiments each in duplicate, and expressed as the percentage of PKC ζ activity at 2 mmol/l glucose.

**Assay of SAPK2 activity**

50 µg of whole cell lysate from MIN6 cells cultured in 20 mmol/l glucose with or without 200 nmol/l calphostin C was purified as described previously [47], and was subjected to SAPK2 activity assay with a p38/RK/Mpk2 Assay Kit according to the manufacturer's instructions (Upstate Biotechnology [Lake Placid, NY, U.S.A.]).

**Statistical analysis**

Student’s t-test was used for analysis of statistical significance.

**Results**

**Identification of trans-acting factors which bind to the glucose-responsive element**

To identify the trans-acting factors which bind to the A3 element of the human insulin gene, we performed EMSA with nuclear extract purified from MIN6 cells cultured with 2 mmol/l or 20 mmol/l glucose. We detected two bands, termed C1 and C2, at both concentrations (Fig. 1A). The intensities of C1 and C2 were significantly increased at 20 mmol/l glucose (192.3 ± 22.8% and 153.4 ± 15.6%, respectively, P<0.05) (Fig. 1A, lane 3) as compared with those at 2 mmol/l glucose (100 %) (Fig. 1A, lane 2). In EMSA, with a probe containing CCAAT/enhancer-binding protein (C/EBP)
binding site [45], no difference in the C/EBP band intensity was observed between the two glucose concentrations (data not shown), which suggested that the increase induced by 20 mmol/l glucose stimulation was specific for C1 and C2. C1 and C2 were competed out by adding a 100-fold molar excess of non-labeled wild-type competitor (Fig. 1A, lane 4), but not by adding non-labeled mutated competitor with a mutation in the A3 element (Fig. 1A, lane 5). In the gel supershift assay, anti-PDX-1 antiserum (Fig. 1A, lane 7) but not pre-immune serum (Fig. 1A, lane 6) eliminated or supershifted C1 and C2. This supershifted band was competed out specifically by a 100-fold molar excess amount of wild-type competitor (Fig. 1A, lane 8) but not by a mutated competitor (data not shown). These results indicated that both proteins which composing C1 and C2 were recognized by anti-PDX-1 antibody.

To identify the molecular weight of the proteins composing C1 and C2, we performed a UV crosslink assay. The molecular weight of the protein composing C1 and that composing C2 appeared to be 36 and 48 kDa, respectively (data not shown). The molecular weight of the protein composing C2 was identical to the reported size of PDX-1 on SDS-PAGE (46–49 kDa) [15, 48, 49]. Since the proteins composing C1 and C2 specifically bound to the A3 element, and were recognized by anti-PDX-1 antibody, we considered that the nuclear protein composing C2 could therefore be PDX-1, and the protein composing C1 may be degraded PDX-1.

**Effect of phosphatase treatment of nuclear proteins on DNA-binding activity, and determination of glucose-induced quantitative change in PDX-1**

To determine whether phosphorylation of PDX-1 could alter its binding to the A3 element, we performed EMSA with nuclear proteins treated with potato acid phosphatase. When nuclear extract was treated with potato acid phosphatase, the intensities of C1 and C2 were reduced (Fig. 1B, lane 2) compared with those with binding buffer alone (Fig. 1B, lane 1), but were not affected by inactivated potato acid phosphatase (Fig. 1B, lane 4). Since we had confirmed that the potato acid phosphatase used in this experiment did not dephosphorylate the end-labeled probe, this finding indicated that the DNA-binding activity of PDX-1 depended on its phosphorylation status.

To further study the quantitative change in PDX-1 in response to glucose, nuclear extracts of MIN6 cells cultured with 2 mmol/l or 20 mmol/l glucose were immunoprecipitated with anti-PDX-1 antiserum, and the immunoprecipitate was subjected to Western blot with anti-PDX-1 antiserum (Fig. 2). When immunoprecipitate was blotted with anti-PDX-1 antiserum, no significant difference in PDX-1 content was observed between the two glucose concentrations (Fig. 2A, lanes 1 and 2, Fig. 2B). The same result was obtained in

![Graph](A)

![Graph](B)

*Fig. 2.* Determination of glucose-induced quantitative change in PDX-1. (A) Each nuclear extract was purified from MIN6 cells cultured with 2 mmol/l (lane 1) or 20 mmol/l (lane 2) glucose, immunoprecipitated with anti-PDX-1 antiserum raised against the N-terminus of the PDX-1, and immunoblotted with the same antiserum. Representative results are shown. (B) The intensity of each band was measured with a Bio Image Analyzer BA100. The values represent the mean ± SD (n=6) of six independent experiments and are calculated relative to that with 2 mmol/l glucose. Open and closed columns indicate 2 mmol/l glucose and 20 mmol/l glucose, respectively.
Western blot analysis with whole nuclear extract blotted with anti-PDX-1 antiserum (data not shown).

In vitro phosphorylation of PDX-1 by protein kinases

To determine the protein kinase(s) which phosphorylate PDX-1, we performed in vitro phosphorylation analysis of PDX-1 with purified PKA and PKC, and recombinant MAPK. Although synapsin 1 was phosphorylated by each kinase (Fig. 3, lanes 7–9), PDX-1 was phosphorylated only by PKC (Fig. 3, lane 4) but not by PKA (Fig. 3, lane 2) or MAPK (Fig. 3, lane 3). Phosphorylation of PDX-1 was not detected in immunoprecipitate with pre-immune serum (Fig. 3, lane 5).

Effect of PMA, calphostin C and Gö 6976 on glucose-induced increases in DNA-binding activity and transcriptional activity of PDX-1

We studied whether the increases in transcriptional activity and DNA-binding activity of PDX-1 were induced by PMA, a specific activator of classical and novel PKC. We first analyzed the promoter activity of the human insulin gene by CAT assay in MIN6 cells treated with various concentrations (0, 10, 100 and 500 nmol/l) of PMA for 12 h. The CAT activities were unchanged by PMA treatment (Fig. 4A). We then performed EMSA with nuclear extract prepared from MIN6 cells treated with various concentrations of PMA (0, 10, 100 and 500 nmol/l). The DNA-binding activity of PDX-1 was not affected at any concentrations of PMA (Fig. 4B), and it was not altered by incubation with 500 nmol/l PMA for any incubation period (0, 10, 30 and 60 min) (data not shown). By PKC assay with MBP as the substrate, we confirmed that the PKC activity in whole cell lysate of MIN6 cells cultured with 500 nmol/l PMA for 60 min was doubled compared with that without PMA (data not shown). These results suggested that glucose-induced increases in transcriptional activity and DNA-binding activity of PDX-1 were unaffected by PMA treatment.

We next determined the effect of calphostin C, an inhibitor of all PKC isoforms, and Gö 6976, a specific inhibitor of classical and novel PKC, on the DNA-binding activity of PDX-1. In a control study, 20 mmol/l glucose or calphostin C did not affect the binding of C/EBP to its consensus sequence in MIN6 cells (data not shown), but the intensity of C2 cultured in 20 mmol/l glucose was significantly increased (153.4 ± 15.6%, P<0.05) (Fig. 5, lane 2) compared with that with 2 mmol/l glucose (100%) (Fig. 5, lane 1). This increased binding was blocked by calphostin C (46.5 ± 9.6%, P<0.05) (Fig. 5, lane 3), but the glucose-induced
increase in PDX-1 DNA-binding activity was not blocked by adding Gö 6976 (132.8 ± 14.3%) (Fig. 5, lane 4).

We then determined the effect of calphostin C or Gö 6976 on the transcriptional activity of the human insulin gene promoter. MIN6 cells were incubated either with 2 mmol/l glucose, 2 mmol/l glucose plus 200 nmol/l calphostin C, 20 mmol/l glucose, 20 mmol/l glucose plus 200 nmol/l calphostin C or 20 mmol/l glucose plus 200 nmol/l Gö 6976 for 24 h, and were subjected to CAT assay. At 20 mmol/l glucose, the transcriptional activity of the pIPC-CAT, which contained −230 to +236 of human insulin gene promoter including A3 element, was increased by 3 fold compared with that with 2 mmol/l glucose. The increase in the transcriptional activity induced by 20 mmol/l glucose was partially blocked by calphostin C, but not by Gö 6976. In 2 mmol/l glucose condition, the transcriptional activity of the pIPC-CAT was not altered by adding calphostin C. The transcriptional activities of the human insulin receptor gene promoter (as a control) [43] or −201 to +236 of human insulin gene promoter which lacked the A3 element were not altered by 20 mmol/l glucose, calphostin C or Gö 6976 (Fig. 6B). 

Taking together the results of EMSA and the CAT assay, we speculated that glucose-induced increases in DNA-binding activity of PDX-1 and transcriptional activity of the human insulin gene promoter were mediated by a calphostin C-sensitive, but PMA and Gö 6976-insensitive pathway, probably by atypical PKC.

Identification of atypical PKC and measurement of PKCζ activity in the nucleus of MIN6 cells

The presence of PKCζ, or λ, members of the atypical PKC isoforms, in nuclei of MIN6 cells was confirmed by Western blot analysis. Nuclear
extracts purified from MIN6 cells were subjected to Western blot analysis with anti-PKC \( \zeta \) or PKC \( \lambda \) antibody. A band of 72 kDa which corresponded to PKC \( \zeta \) (Fig. 7A, lanes 2 and 3) was detected at the same position as PKC purified from rat brain (Fig. 7A, lane 1) [36, 38]. The identity of the second band was unknown, but the band of PKC \( \lambda \) was not detected in the nuclear extract of MIN6 cells (data not shown). To further confirm the localization of PKC \( \zeta \) to the nucleus, immunocytochemical analysis was performed (Fig. 7B). PKC \( \zeta \) was detected with an FITC-conjugated secondary antibody recognizing anti-PKC \( \zeta \) antibody (Fig. 7B, a and c, shown in green). The nucleus was visualized with propidium iodide (PI) which specifically binds to DNA (Fig. 7B, b and c, shown in red). PKC \( \zeta \) localized in the nucleus is shown in yellow (green + red) (Fig. 7B, c).

To further examine the activity of PKC \( \zeta \) in the nucleus of MIN6 cells treated with 2 mmol/l or 20 mmol/l glucose in the absence or presence of

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**Fig. 5.** Effect of calphostin C and Gö 6976 on glucose-induced DNA-binding activity of PDX-1 in MIN6 cells. 5 µg of nuclear extract prepared from MIN6 cells cultured with 2 mmol/l glucose (lane 1), 20 mmol/l glucose (lane 2), 20 mmol/l glucose plus 200 mmol/l calphostin C (lane 3) and 20 mmol/l glucose plus 200 mmol/l Gö 6976 (lane 4) was subjected to EMSA with a probe containing A3 element. Arrows indicate C1, C2 and free probe, respectively.

**Fig. 6.** Effect of calphostin C and Gö 6976 on glucose-induced transcriptional activation of the human insulin gene promoter in MIN6 cells. (A) Structures of pIPC-CAT and pIPD-CAT plasmids. Schematic structure of the human insulin gene promoter is shown at the top. The structures of pIPC-CAT and pIPD-CAT are shown below. A-elements (closed boxes) and E-elements (hatched boxes) in the human insulin promoter are indicated as A1, A2, A3 or A5, and E1 or E2, respectively. C-element, G-element and cAMP responsive element are indicated as C1, G1 and CRE or CRE/CCAAT, respectively [8]. (B) CAT assay with pIPC-CAT plasmid (closed column), pIPD-CAT plasmid (hatched column) or phIR-CAT plasmid containing human insulin receptor gene promoter (open column). CAT activity in MIN6 cells transfected with either pIPC-CAT, pIPD-CAT or phIR-CAT, and cultured with 2 mmol/l glucose is arbitrarily defined as 100%. The relative CAT activities are shown, with each value representing the mean of five independent experiments each in duplicate (mean ± SD, n=10). Asterisks indicate significant difference: *P<0.05.*
calphostin C, we measured the PKC activity in the immunoprecipitate with anti-PKC ζ antibody. PKC ζ activity was increased by 2-fold with 20 mmol/l glucose (P<0.05) compared with that with 2 mmol/l glucose, and this increase was completely inhibited by calphostin C treatment (P<0.05) (Fig. 7C).

Effects of calphostin C on the PDX-1 DNA-binding activity induced by heat shock

It has been reported that the DNA-binding activity of PDX-1 purified from heat-treated pancreatic β cells (cultured at 45 °C for 30 min) was increased, and that this increased binding was mediated by the activation of SAPK2 [33]. To clarify the correlation between atypical PKC and SAPK2, we purified nuclear extract from MIN6 cells cultured for 30 min in 2 mmol/l glucose with or without 200 nmol/l calphostin C before heat-treatment (45°C for 30 min), and performed EMSA. The intensity of the C2 band tended to be increased by heat shock (128.3 ± 16.3%, Fig. 8, lane 2) compared with that at 37 °C (100%, Fig. 8, lane 1), but this increase was not inhibited by calphostin C treatment (123.4 ± 15.8%, Fig. 8, lane 3). To determine the effect of PKC on SAPK2 activity, we measured the SAPK2 activity of MIN6 cells cultured with or without 200 nmol/l calphostin C. The activity of SAPK2 was not changed by calphostin C treatment (data not shown).

Discussion

In this study, we used MIN6 cells established from an insulinoma obtained by targeted expression of the simian virus 40 T antigen gene in a transgenic mouse [34]. MIN6 cells show morphological characteristics of pancreatic β cells, and exhibit glucose-inducible insulin secretion. Therefore, MIN6 cells could be an appropriate cell line to analyze glucose-induced insulin gene expression [36, 50].

Several cis-acting elements in the insulin gene promoter, for example, A or E elements, and trans-acting factors which bind to these elements, have
been reported to play an important role in glucose-induced insulin gene expression [14–20, 24–26, 39, 51]. As shown in Fig. 6, the ratio of CAT activities treated with high and low glucose (CAT activity with high glucose/CAT activity with low glucose) of pIPD-CAT which lacked an A3 element was significantly decreased compared with that of pIPC-CAT which contained an A3 element, indicating that the A3 element was the major cis-acting element for glucose-sensitive expression of the human insulin gene. These results were in accordance with previous reports stating that the A3 element was essential for glucose sensitivity in the human and rat insulin I gene promoters [14, 15]. Although other investigators have reported the importance of the E2 element for glucose-sensitive rat insulin I gene expression [17], the ratio of CAT activities with high and low glucose of pIPC-CAT which lacked the E2 element was not decreased as compared with that of the CAT plasmid with the E2 element (which contained −361 to +236 region in the human insulin gene) in our experiments (unpublished observation). It was reported that PDX-1 also bound to the A1 element in the rat insulin I gene [22], but because the CAT activity of pIPD-CAT was not significantly increased by 20 mmol/l glucose treatment, we considered that CRE, A2, C1, E1 or the A1 element was not the major element responsible for the glucose-induced human insulin gene expression. Nevertheless, because it was reported that PDX-1 could not function independently as a mediator of the glucose responsiveness of the human glucokinase gene β-cell-type promoter [39], and other investigators reported cooperative interactions between the PDX-1 and the helix-loop-helix factor E47 which bound to the E element [51], it was possible that PDX-1 acted synergistically with other trans-acting factor(s) which bound to cis-acting element(s) including CRE, A2, C1, E1 or A1 element.

We then focused on the mechanism whereby glucose could induce insulin gene expression through PDX-1. We evaluated the quantitative and qualitative changes in PDX-1 induced by a high glucose concentration in MIN6 cells. The amount of PDX-1 was not affected by glucose as shown in the Western blot, suggesting that the change in the binding activity of PDX-1 could be caused by a modification of PDX-1. Previous reports showed that several trans-acting factors were both positively and negatively regulated by phosphorylation, and that phosphorylation of trans-acting factors could be exerted both on DNA-binding activity and transactivation function [52–56]. As it has also been reported that the loss of PDX-1 DNA-binding activity at low glucose concentration was prevented by incubation of the islets with non-specific phosphatase inhibitors [26], we examined the effect of the phosphorylation status of PDX-1 on its DNA-binding activity. As a result, we found that the DNA-binding activity of PDX-1 treated with potato acid phosphatase was decreased. Similar results with potato acid phosphatase and rat islet were
also reported by Macfarlane et al. [26].

It has been reported that some protein kinases, for example, PKA, PKC and MAPK, could phosphorylate trans-acting factors through exogenous stimuli [55], and that in pancreatic β cells, PKC and PKA were considered to be activated through glucose metabolism [57]. We therefore tried to identify the protein kinase which phosphorylates PDX-1. After assessing the activity of each kinase by pre-kinase assay with synapsin 1 as the substrate under the same conditions as for in vitro phosphorylation assay, we performed in vitro phosphorylation assay of PDX-1, and found that PKC but not PKA or MAPK could phosphorylate PDX-1 in vitro. In this assay, we used two different anti-PDX-1 antiserums which recognized the N or C terminus of mouse PDX-1, and we obtained the same results, suggesting that no inhibitory effect of anti-PDX-1 antibody on PDX-1 phosphorylation occurred. We next focused on the role of PKC in vivo, and we could confirm that PKC affected DNA-binding activity and transcriptional activity of PDX-1 in MIN6 cells by EMSA and CAT assay with calphostin C.

Several isoforms of PKC have been identified, and these are divided into three families, designated classical PKC, novel PKC and atypical PKC, respectively. The classical PKC isoforms are induced by Ca²⁺ and phorbol ester/diacylglycerol (DAG). The novel PKC isoforms are phorbol ester/DAG dependent but do not require Ca²⁺. In contrast, the atypical PKC isoforms are not activated by either phorbol ester/DAG or Ca²⁺ [58-60]. Since it has been reported that pancreatic islet cells contain at least six PKC isoforms, classical PKC, α, β, novel PKC, δ, ε and atypical PKC, ζ, τ [61, 62], we tried to identify the PKC family responsible for DNA-binding activity and transcriptional activity of PDX-1 by employing PMA, calphostin C and Gö 6976. We could confirm that PMA did not affect DNA-binding activity of PDX-1, which was in accordance with the results of Macfarlane et al. [26], but, interestingly, glucose-induced increases in DNA-binding activity and transcriptional activity of PDX-1 were significantly decreased by calphostin C, but not by Gö 6976. These results suggested that the PKC which affected the glucose-induced DNA-binding activity and transcriptional activity of PDX-1 was atypical PKC.

By Western blot with anti-PKC ζ antibody, we could detect PKC ζ in nuclear extract of MIN6 cells. Since it was reported that the anti-PKC ζ antibody used in this experiment could cross-react with PKC λ, we further performed Western blot with anti-PKC λ antibody, but we could not detect PKC λ in MIN6 cells. Moreover, we confirmed the presence of PKC ζ in the nucleus of MIN6 cells in an immunocytochemical study, and found that the activity of PKC ζ in nuclear extracts of MIN6 cells was increased with 20 mmol/l glucose more than with 2 mmol/l glucose. From these results, we speculated that the atypical PKC isoform which affected PDX-1 in MIN6 cells could be PKC ζ. To confirm the role of PKC ζ in the activation of PDX-1, it could be interesting to study the effect of the dominant negative PKC ζ in MIN6 cells [63, 64].

Recently, because involvement of the SAPK2 pathway in the transactivation of PDX-1 has been reported [33], to clarify whether activation of atypical PKC is related to activation of SAPK2, we studied the effect of calphostin C on the increase in PDX-1 DNA-binding activity induced by SAPK2. As a result, the increase in PDX-1 DNA-binding caused by heat shock was found to be not blocked by calphostin C. Moreover, SAPK2 activity in MIN6 cells was also not blocked by pretreatment with calphostin C. Taking these results together, we speculate that the atypical PKC does not exist either on the downstream or upstream SAPK2 pathway, and these signal pathways might be independent.

The physiological activators of atypical PKC isoforms have not been clearly identified. It has been suggested that product of phosphatidylinositol 3-kinase (PI3K), phosphoinositol 3, 4, 5-triphosphate could induce PKC ζ activity [65], but in our unpublished observation of PI3K assay with anti-PI3K (p85 subunit) antibody (Upstate Biotechnology [Lake Placid, NY, U. S. A.]), PI3K activity was not changed at 20 mmol/l glucose compared with that at 2 mmol/l glucose in MIN6 cells. We therefore concluded that the PKC ζ activation mediated by a high glucose concentration might not be associated with the PI3K pathway. Arachidonic acid, a product of cellular phospholipase A₂, has been reported to activate atypical PKC [59, 66], and phospholipase A₂ is activated through glucose metabolism [67, 68]. In view of our results and previous reports, we speculated that phosphatidic acids produced by phospholipase A₂, which was activated through glucose metabolism, might activate atypical PKC in MIN6 cells.

In conclusion, we suggest that a high glucose
concentration induced an increase in DNA-binding activity and transcriptional activity of PDX-1 by activation of atypical PKC, which resulted in activation of the human insulin gene promoter. More detailed analysis of glucose-induced modification of PDX-1 could provide us with a better understanding of the mechanisms of insulin gene expression regulated by glucose metabolism.

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