Protein Kinase Cζ Activation Mediates Glucagon-Like Peptide-1–Induced Pancreatic β-Cell Proliferation

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Glucagon-like peptide-1 (GLP-1), an insulinotropic and glucoincretin hormone, is a potentially important therapeutic agent in the treatment of diabetes. We previously provided evidence that GLP-1 induces pancreatic β-cell growth nonadditively with glucose in a phosphatidylinositol-3 kinase (PI-3K)–dependent manner. In the present study, we investigated the downstream effectors of PI-3K to determine the precise signal transduction pathways that mediate the action of GLP-1 on β-cell proliferation. GLP-1 increased extracellular signal-related kinase 1/2, p38 mitogen-activated protein kinase (MAPK), and protein kinase B activities nonadditively with glucose in pancreatic β(INS 832/13) cells. GLP-1 also caused nuclear translocation of the atypical protein kinase C (aPKC) ζ isoform in INS as well as in dissociated normal rat β-cells as shown by immunolocalization and Western immunoblotting analysis. Tritiated thymidine incorporation measurements showed that the p38 MAPK inhibitor SB203580 suppressed GLP-1–induced β-cell proliferation. Further investigation was performed using isoform-specific pseudosubstrates of classical (α, β, and γ) or ζ aPKC isoforms. The PKCζ pseudosubstrate suppressed the proliferative action of GLP-1, whereas the inhibitor of classical PKC isoforms had no effect. Overexpression of a kinase-dead PKCζ acting as a dominant negative protein suppressed GLP-1–induced proliferation. In addition, ectopic expression of a constitutively active PKCζ mutant stimulated tritiated thymidine incorporation to the same extent as GLP-1, and the glucoincretin had no growth-promoting action under this condition. The data indicate that GLP-1–induced activation of PKCζ is implicated in the β-cell proliferative signal of the insulinotropic hormone. The results are consistent with a model in which GLP-1–induced PI-3K activation results in PKCζ translocation to the nucleus, which may play a role in the pleiotropic effects (DNA synthesis, metabolic enzymes, and insulin gene expression) of the glucoincretin. *Diabetes* 50: 2237–2243, 2001

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Received for publication 16 November 2000 and accepted in revised form 29 June 2001.

aPKC, atypical protein kinase C; BSA, bovine serum albumin; CA, constitutively active; cPKC, classical protein kinase C; DN, dominant-negative; DTT, dithiothreitol; ERK, extracellular signal-related kinases; GLP-1, glucagon-like peptide-1; MAPK, mitogen-activated protein kinase; MEK, mitogenic-extracellular signal-regulated kinase; MOI, multiplicity of infection; NFκB, nuclear-factor κB; PBS, phosphate-buffered saline; PDK, phosphoinositide-dependent kinases; PDX-1, pancreatic and duodenal homeobox gene-1; PI-3K, phosphatidylinositol-3 kinase; PKB, protein kinase B; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; WT, wild-type.

Glucagon-like peptide-1 (GLP-1)-(7-36) amide, a potent glucoincretin hormone (1,2), is secreted by the intestinal L-cells in response to fat meals and carbohydrates (3,4). It is a potentially important drug in the treatment of diabetes in view of its ability to improve insulin secretion in both patients with impaired glucose tolerance and type 2 diabetes (5,6). GLP-1 is also an insulinotropic agent through its ability to stimulate insulin gene expression and proinsulin biosynthesis (7) and acts as a potent β-cell growth factor (8). GLP-1 increases the expression level of the β-cell specific transcription factor pancreatic and duodenal homeobox gene-1 (PDX-1) (8,9). In addition, the glucoincretin increases β-cell proliferation nonadditively with glucose in a phosphatidylinositol-3 kinase (PI-3K)–dependent manner in β(INS-*1*) cells (8) as well as islet mass in mouse pancreas (9). However, the precise signal transduction pathway that mediates the proliferative action of GLP-1 is not completely elucidated.

PI-3K is a family of proteins known to be activated in response to various growth factors in different cell types (10). Many downstream effectors of PI-3K mediate proliferative signals. Extracellular signal-related kinases (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK) are in some instances downstream targets of PI-3K (11), mediating the proliferative response of a variety of external signals (12). ERK 1/2 and p38 MAPK also promote cell growth by being involved in antiapoptotic processes (13). Glucose activates p38 MAPK in pancreatic β-cell, an action that may be causally implicated in insulin gene induction by the sugar via phosphorylation of the transcription factor PDX-1 (14). However, the involvement of p38 in PDX-1 activation was challenged recently (15). Among other downstream effectors of PI-3K are phosphoinositide-dependent kinases (PDK), which in turn activate protein kinase B (PKB) (also named Akt) (16). PKB participates in proliferative signals in response to many stimuli in different cell types possibly via the activation of the mammalian target of rapamycin and p70s6 kinase (17). Other targets of PDK that could play a role in cell growth regulation include the atypical isoform ζ of protein kinase C (PKC) (18,19). PKC is a multigene family divided into three classes depending on their cofactor requirements: classical PKCs (cPKCs), which are sensitive to calcium/diacyl-

glycerol and tumor-promoting phorbol esters; novel PKCs, which are sensitive to diacylglycerol and tumor-promoting phorbol esters only; and atypical PKCs (aPKCs), which are insensitive to all three regulators (20).

We report here that GLP-1 increases the PI-3K downstream targets ERK 1/2, p38 MAPK, and PKB activities nonadditively with glucose in INS(832/13) cells. GLP-1 also causes PKCζ nuclear translocation. However, only PKCζ and p38 MAPK are likely to be involved in GLP-1–induced proliferation as revealed by tritiated thymidine incorporation measurements in the presence of specific inhibitors. The implication of the aPKC isoform ζ in the proliferative action of GLP-1 is demonstrated using recombinant adenoviruses, which allow expression of various PKCζ constructs.

RESEARCH DESIGN AND METHODS

Reagents. Pharmacological inhibitors (SB203580, PD98059, LY294002, KN-93, myristoylated PKCζ, and cPKC [20–28] peptide inhibitors) were purchased from Biomol (Plymouth Meeting, PA). Human GLP-1 fragment 7–36 amide was obtained from Sigma (St. Louis, MO). The anti-PKCζ antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The anti-insulin antibody was from Sigma. RPMI-1640 and the supplements, including fetal calf serum, were purchased from Gibco BRL (Burlington, Ontario, Canada). Methyl [^{3}H]-thymidine was from ICN (Costa Mesa, CA).

Cell culture and incubation. INS(832/13) (21) cells (passages 36–70) were grown in monolayer cultures as described previously (22) in regular RPMI-1640 medium supplemented with 10 mmol/l HEPES, 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified (5% CO_2, 95% air) atmosphere. This clone (832/13) of INS-1 cell was used because it shows better differentiation characteristics in term of glucose-stimulated insulin secretion than the original INS-1 cells (21). When cells reached 80% confluence after ~7 days, they were washed with phosphate-buffered saline (PBS) and preincubated at 37°C for 90 min in a Krebs-Ringer bicarbonate HEPES medium containing 1 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3$, 25 mmol/l HEPES (pH 7.4) supplemented with 3 mmol/l glucose and 0.1% defatted bovine serum albumin (BSA) (Fraction V; Sigma). Cells were then washed with PBS and incubated for the indicated times in the same supplemented Krebs-Ringer bicarbonate HEPES medium containing the substances to be tested.

In vitro kinase assays. In vitro kinase activities were evaluated using MAPK (ERK 1/2), p38 MAPK, and PKB/Akt kinase assay kits from New England Biolab (Beverly, MA) according to the manufacturer's protocol. In brief, cells that were cultured as described above were homogenized in lysis buffer (1% SDS, 60 mmol/l Tris-HCl [pH 6.8], 10% glycerol). The different kinases (ERK 1/2, p38 MAPK, and PKB/Akt) were then immunoprecipitated from 200 μg of cell lysate and resuspended in 40 μl of kinase buffer (25 mmol/l Tris [pH 7.5], 5 mmol/l β-glycerophosphate, 2 mmol/l dithiothreitol [DTT], 0.1 mmol/l sodium-orthovanadate, and 10 mmol/l $MgCl_2$) supplemented with 200 μmol/l ATP. Specific substrates (respectively Elk-1, ATF-2, and GSK3a for ERK 1/2, p38 MAPK, and PKB) were added, and the reactions were stopped after 30 min by adding SDS sample buffer containing 62.5 mmol/l Tris (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/l DTT, and 0.1% bromophenol blue. Twenty microliters of samples were loaded on SDS-PAGE gels for Western immunoblottings. Substrate phosphorylation was detected by incubation of the membranes with phospho-specific antibodies.

Immunofluorescence and confocal microscopy. INS cells were cultured overnight on polyornithine-coated coverslips and stimulated as described in the legend to Fig. 2. After washing with PBS, the cells were fixed with 3.7% paraformaldehyde/PBS for 15 min at room temperature before incubation for 5 min with 0.1 mol/l glycine/PBS and permeabilization with 0.2% Triton X-100 in PBS for 2 min. For immunofluorescence, cells were blocked with 1% BSA/PBS for 10 min, incubated with PKCζ primary antibodies at 10 μg/ml for 1 h, washed three times with PBS, stained with a goat anti-rabbit fluorescein secondary antibody (Pierce, Rockford, IL) for 1 h, and washed three times with PBS. Image acquisition was performed using a LSM-410 confocal microscope (Carl Zeiss).

Rat islets were isolated from 200-g Wistar rats as described previously (23) and trypsinized to obtain dissociated islet cells (24). Dissociated islet cells (corresponding to 100 islets per condition) were seeded on polyornithine-coated coverslips in six-well plates and cultured in regular RPMI for 24 h. Cells were then washed with PBS, incubated in the absence and presence of GLP-1, and subsequently fixed as described above for INS cells. For immunofluorescence, cells were blocked with 1% BSA/PBS for 10 min, incubated for 1 h with both a polyclonal PKCζ (10 μg/ml) and a monoclonal mouse anti-insulin (10 μg/ml) primary antibody, washed three times with PBS, incubated for 1 h with both a goat anti-rabbit fluorescein secondary antibody (Pierce) and a rhodamine-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA), and finally washed three times with PBS. Image acquisition was performed using an LSM-410 confocal microscope (Carl Zeiss).

Preparation of nuclear extracts and immunoblot analysis of PKCζ. Nuclear extracts were isolated using a published procedure (25). Briefly, cells (40×10^6 per condition) previously grown in 225 cm^2 Petri dishes were harvested with a rubber policeman in cold PBS, sedimented at 3,500 g for 4 min, and lysed in 1 ml of ice-cold buffer A (15 mmol/l KCl, 2 mmol/l $MgCl_2$, 10 mmol/l HEPES [pH 7.4], 0.1% phenylmethylsulfonyl fluoride [PMSF], and 0.5% Nonidet P-40). After a 10-min incubation on ice, nuclei were collected by centrifugation (1,000*g* for 5 min) and washed with buffer A without Nonidet P-40. Nuclei were lysed in a buffer containing 2 mmol/l KCl, 25 mmol/l HEPES (pH 7.4), 0.1% EDTA, and 1 mmol/l DTT. After a 15-min incubation period on ice, a dialysis buffer (25 mmol/l HEPES [pH 7.4], 1 mmol/l DTT, 0.1% PMSF, 2 μg/ml aprotinin, 0.1 mmol/l EDTA, and 11% glycerol) was added to the nuclei preparations. Samples were centrifuged (16,000*g* for 20 min), and the supernatants containing the nuclear proteins were used for protein determinations, subsequently aliquoted (50 μl), and kept frozen at −70°C for subsequent immunoblot analysis. Lysates were subjected to electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were probed with a PKCζ primary antibody and subsequently with peroxidase-conjugated goat anti-rabbit IgG. Signals were visualized by chemiluminescence, using ECL reagent (Amersham Pharmacia, Buckinghamshire, England).

Tritiated thymidine incorporation assay. A previously described procedure was used (8,26). In brief, INS-1 cells were seeded 2 days before use in 96-well plates (8×10^4 cells/well) and cultured in regular RPMI medium as described above. Cells were then washed with PBS and preincubated for a period of 24 h in minimal RPMI medium, i.e., without serum and glucose but with 0.1% BSA. They were then incubated for 24 h in minimal RPMI medium with various test substances. Proliferation was determined by incorporation of [^{3}H]-thymidine (1 μCi/well) during the final 4 h of the 24-h incubation period. Cells were then harvested with a PHD cell harvester (Cambridge Technology, Watertown, MA), and the radioactivity retained on the dried glass fiber filters was measured.

Adenovirus constructs. PKCζ cDNA was a gift from H. Mischak (Laboratory of Genetics, National Cancer Institute, Bethesda, MD). Dominant-negative (DN) kinase defective PKCζ was generated by PCR mutagenesis using the pAlter system (Promega, Madison, WI) to give a K281-W substitution within the ATP-binding site. This mutation results in a DN mutant of PKCζ, which has been shown to inhibit PKCζ-stimulated nuclear-factor κB (NFκB) reporter gene activity in NIH 3T3 fibroblasts (27) and PKCζ-dependent mitogenic activity in oocytes and fibroblasts (28,29). An adenoviral shuttle plasmid (pXCMV) was generated by subcloning the NruI/*Dra*III digested and blunted expression cassette from pRcCMV (Invitrogen, Carlsbad, CA) into *Xba*I digested and blunted pXCX3 (pXCX3 was derived from pXCX2). The PKCζ constructs (wild-type [WT], DN, and constitutively active [CA]) were then subcloned into the *Eco*RV site of pXCMV. Recombinant adenoviruses were prepared essentially as described by Graham and Prevec (30). Cesium chloride–purified plasmids that contained the pXCMV PKCζ gene cassettes were cotransfected with pJM17 (a circular form of the adenovirus genome) in HEK 293 cells at a ratio of 1:1 using calcium phosphate. Control virus MX17, which does not contain a gene cassette, was constructed by recombination between pXCX2 and pJM17. Once transfected, the HEK 293 cells were maintained in 0.5% agarose and 1× culture medium. Recombinant viruses were isolated 1–2 weeks later as single plaques and amplified by reinfecting confluent monolayers of HEK 293 cells. Recombinant viruses were prepared from lysates of cytopathic cells 3–7 days after the second round of infection. Medium from the 35-mm dish was used in further rounds of amplification to generate viral stocks that were purified by CsCl gradient. Plaque assays were performed in HEK 293 cells to determine the titer (pfu/ml) of these stocks, which were then used to infect INS(832/13) cells at the designated multiplicity of infection (MOI). CA PKCζ was generated as described previously (31) by an A119E mutation in the pseudosubstrate site of PKC, a substitution that frees the catalytic region from the inhibitory constraint of being bound to the regulatory region.

Infections. INS(832/13) cells were seeded 2 days before use in six-well plates (4×10^6 cells/well) and cultured in regular RPMI medium as described above. Cells were then incubated with different PKCζ adenoviral constructions at an

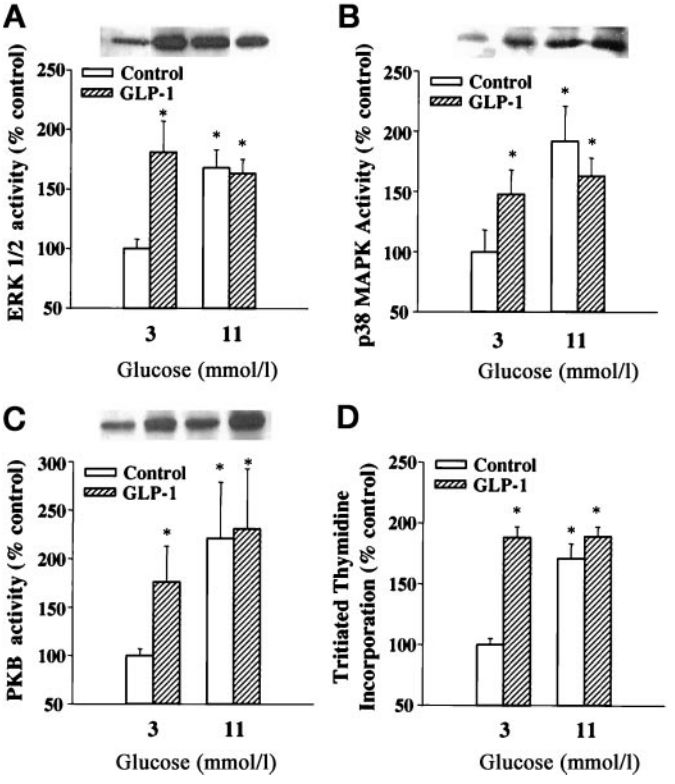

FIG. 1. Effects of GLP-1 and glucose on ERK ½ (*A*), p38 MAPK (*B*), PKB (*C*), and DNA synthesis (*D*) in INS(832/13) cells. In vitro kinase activities were measured at 3 or 11 mmol/l glucose in the presence or absence of 10 nmol/l GLP-1. DNA synthesis was determined by tritiated thymidine incorporation. Cells were cultured in serum-free RPMI medium containing 3 or 11 mmol/l glucose in the presence or absence of GLP-1 for 24 h. One μCi/well of tritiated thymidine was added during the final 4 h of the 24-h incubation period. Representative immunoblots for each kinase activity measurement are shown. Data are mean ± SE of four independent experiments for kinase activities and four independent experiment each comprising three or four wells for cell proliferation assay. *$P < 0.05$.

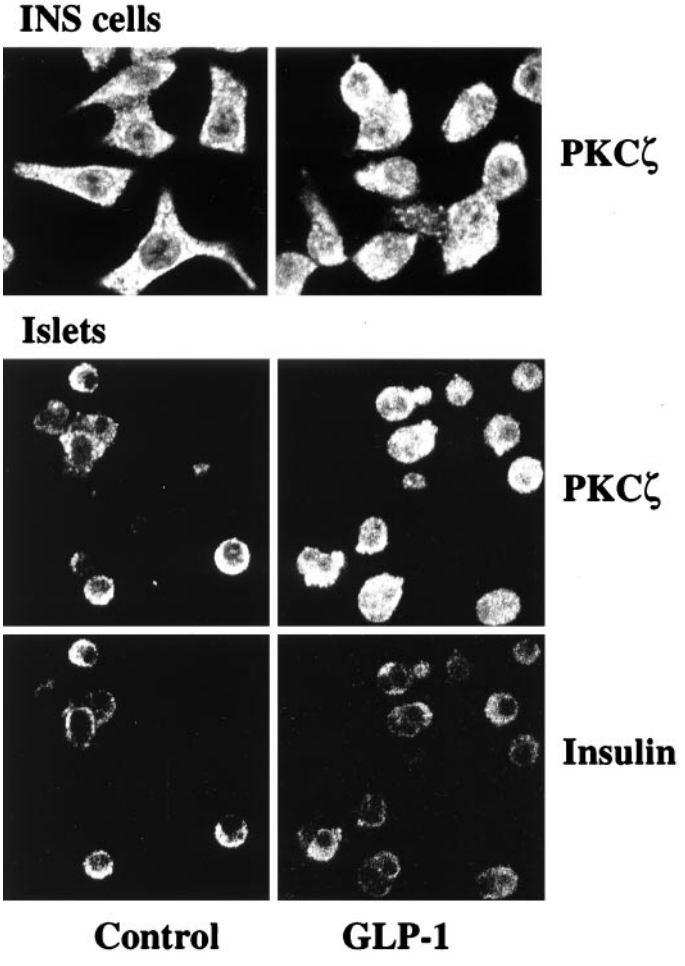

FIG. 2. GLP-1 causes nuclear translocation of PKCζ in INS(832/13) and dissociated normal rat β-cells. INS(832/13) cells (top two panels) and dissociated rat islet cells (lower four panels) were incubated at 3 mmol/l glucose with or without 100 nmol/l GLP-1 for 5 min (INS cells) or 2 min (islet cells). Cells were examined using a confocal microscope (630× magnification with oil immersion) for their immunofluorescence staining as described in RESEARCH DESIGN AND METHODS. Dissociated rat islet cells were coimmunostained with anti-PKCζ and mouse anti-insulin antibodies to identify β-cells.

MOI of 10 pfu/cell for 5 h in 0.5 ml of complete RPMI medium. Fresh RPMI medium (1.5 ml) was then added to each well still containing 0.5 ml of media with viruses. Seven hours later, cells were trypsinized and plated in 96-well plates as described above to perform a tritiated thymidine incorporation assay after a 24-h incubation in the absence or presence of GLP-1.

Calculations and statistics. Data are presented as mean ± SE. Statistical analyses were performed with the SPSS for Windows system. Differences between two conditions were assessed with Student's *t* test for related samples. Differences were deemed to be significant at $P < 0.05$.

RESULTS

Activation of signal transduction pathways downstream of PI-3K by GLP-1. ERK 1/2, p38 MAPK, and PKB are three potential downstream effectors of PI-3K. We wanted to determine whether their activities in the presence of GLP-1 correlate with cell growth measurements as evaluated with the tritiated thymidine incorporation assay. In vitro ERK 1/2, p38 MAPK, and PKB activities were studied after 1 h of incubation of INS(832/13) cells at 3 or 11 mmol/l glucose with or without 10 nmol/l GLP-1 (Fig. 1). The results indicate that GLP-1 at a maximal effective concentration of 10 nmol/l caused activation of ERK 1/2, p38 MAPK, and PKB at low (3 mmol/l) glucose. Elevated (11 mmol/l) glucose also increased the activity of the same kinases. The action of GLP-1 and glucose was not additive. GLP-1 caused a rise in tritiated thymidine incorporation in INS(832/13) cells to an extent similar to that of 11 mmol/l glucose, and the actions of both agents were not additive. The threshold half-maximum and maximum concentrations of GLP-1 at 3 mmol/l glucose on PKB activation were 0.01 and 10 nmol/l, respectively (data not shown). These values were similar to that obtained for INS(32/13) cell proliferation (data not shown). Overall, the results indicate that both GLP-1 and glucose activate ERK 1/2, p38 MAPK, and PKB and that the action of GLP-1 on the activation of these signaling pathways correlates with the proliferative response of the glucoincretin.

GLP-1 induces PKCζ nuclear translocation. PKCζ, an atypical isoform of PKC, is an additional downstream target of both PI-3K and PDK (19,32). It is translocated from the cytoplasm to the nucleus after its activation by various stimuli (32). We therefore investigated whether GLP-1 causes nuclear translocation of PKCζ in INS(832/13) cells as well as in dissociated normal rat β-cells. Figure 2 shows that a 5-min exposure of INS cells to 10 nmol/l GLP-1 caused PKCζ translocation to the nucleus as observed by confocal microscopy in association with immunofluorescence (Fig. 2, top two panels). It is interesting that GLP-1 caused a change in cell shape of INS cells,

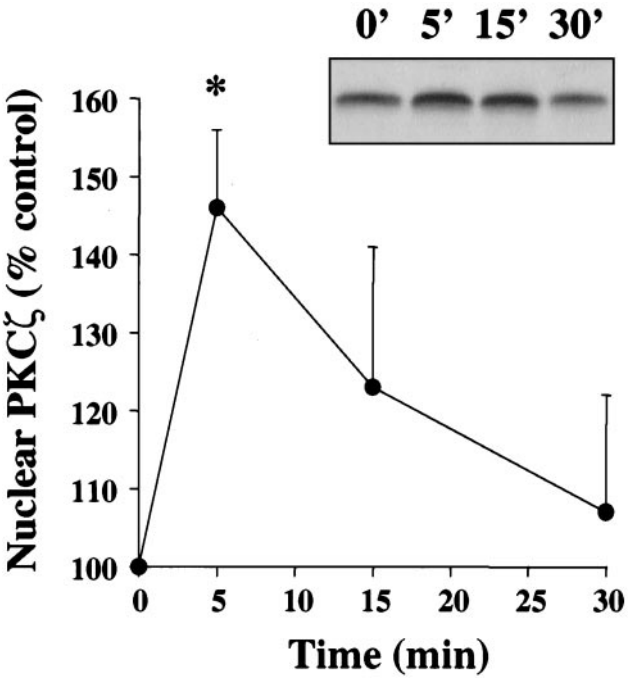

FIG. 3. Time-dependent increase in PKCζ immunoreactivity in nuclear fractions of INS-cells. INS cells were incubated at 3 mmol/l glucose in the presence or absence of 10 nmol/l GLP-1 for the indicated time periods. Nuclear proteins were extracted to perform Western blot analysis. Immunoreactivity was quantified by densitometry. Inset, a representative PKCζ immunoblot is shown. Data are mean ± SE of four different experiments. *$P < 0.05$.

which appeared less flat and rounder than controls. PKCζ nuclear translocation was also observed in dissociated rat β-cells (identified by insulin staining) after a 2-min treatment with 10 nmol/l GLP-1 (Fig. 2, lower four panels). Figure 3 shows a time-dependent increase in PKCζ immunoreactivity in nuclear fractions of INS-cells, with a maximum effect observed at 5 min and a return to basal value after a 30-min incubation period. Consistent with a causal implication of PI-3K in PKCζ activation by GLP-1, the PI-3K inhibitor LY294002 (50 μmol/l) suppressed the GLP-1–induced PKCζ nuclear translocation (data not shown).

Specific inhibitors for PKCζ and p38 MAPK suppress GLP-1–induced β-cell proliferation. To obtain further insight into the signal transduction pathways implicated in the proliferative action of GLP-1, we used specific inhibitors for different kinases known to mediate cell proliferation in response to diverse stimuli. The inhibitors were tested at concentrations at which they are known to be effective without displaying major cytotoxicity in a variety of cell systems (33). Cellular proliferation was evaluated with the tritiated thymidine assay, as described before in INS cells (8,26). Figure 4 shows that the calmodulin-dependent kinase II inhibitor KN-93 and the mitogenic-extracellular signal-regulated kinase (MEK) inhibitor PD98059 did not affect significantly GLP-1–induced proliferation, suggesting that calmodulin-dependent kinase II and ERK 1/2 are not involved in the β-cell growth effect of the glucoincretin. The p38 MAPK inhibitor SB203580 suppressed GLP-1–induced proliferation while strongly affecting basal thymidine incorporation as well. Further investigation was performed using isoform-specific pseudosubstrates to inhibit classical (α, β, and γ) or the ζ atypical isoforms of PKC. The PKCζ pseudosubstrate blocked GLP-1–induced INS(832/13)-cell proliferation, whereas the inhibitor of cPKC enzymes did not. The p38 MAPK, PKCζ, and cPKC inhibitors reduced proliferation observed at basal glucose in the absence of GLP-1 (Fig. 4). However, no apparent cytotoxicity was observed as evaluated by morphologic examination of the cells under the microscope. Although we cannot exclude some cytotoxicity, we favor the view that the corresponding pathways are involved in cell proliferation under nonstimulated conditions. These observations provide pharmacological evidence for the implication of both PKCζ and p38 MAPK in the cell growth–promoting action of GLP-1. Because pseudosubstrate peptides are highly specific enzyme inhibitors, the pharmacological evidence is particularly strong for PKCζ.

GLP-1 induced β-cell proliferation is altered by overexpressing PKCζ mutants. A molecular approach was used to document further the implication of PKCζ in GLP-1–induced proliferation. Thus, adenoviral constructs were used to increase the expression level of various PKCζ proteins in INS(832/13) cells. Western blot studies showed that the WT and kinase-dead DN constructs were expressed ~20-fold over basal PKCζ at an MOI of 10 pfu/cell (data not shown). Overexpressing WT PKCζ slightly but significantly enhanced basal proliferation without affecting maximum thymidine incorporation in the presence of GLP-1. The DN construct reduced GLP-1–induced proliferation by ~60%. CA PKCζ increased β(INS 832/13)-cell proliferation in the absence of GLP-1 to an extent similar to that occurring in the presence of the glucoincretin. In addition, GLP-1 did not further enhance thymidine incorporation under this condition (Fig. 5). Adenoviral infection by itself did not affect INS-cell proliferation because there was no significant difference in tritiated thymidine incorporation between uninfected cells and cells overexpressing β-gal after infection with a β-gal adenoviral construct (data not shown).

DISCUSSION

GLP-1, a potent glucoincretin hormone and a potentially important drug in the treatment of diabetes (26,34), was described recently as a growth factor in the β(INS-1)-cell line (8) as well as in mouse islet tissue (9). However, the exact mechanism by which GLP-1 exerts its growth-promoting action remains to be defined.

Pharmacological and biological evidence has suggested that PI-3K plays a central role in the transduction of the GLP-1–induced proliferative signal (8). The results of the present study indicate that GLP-1 and glucose activate nonadditively ERK 1/2, p38 MAPK, and PKB, three potential downstream targets of PI-3K, as they do for β-cell growth (8). PKCζ, an atypical isoform of PKC and downstream target of PDK, is also activated by the glucoincretin as evidenced from its translocation from the cytoplasm to the nucleus after GLP-1 treatment of INS(832/13) cells and dissociated rat β-cells. The transient nuclear translocation of PKCζ induced by GLP-1 in β-cells is similar to that caused by nerve growth factor in PC12 cells in terms of intensity and duration (35). We therefore studied the effect of specific inhibitors of these signaling pathways activated by the glucoincretin on GLP-1–induced tritiated thymidine incorporation. On the one hand, the MEK inhibitor

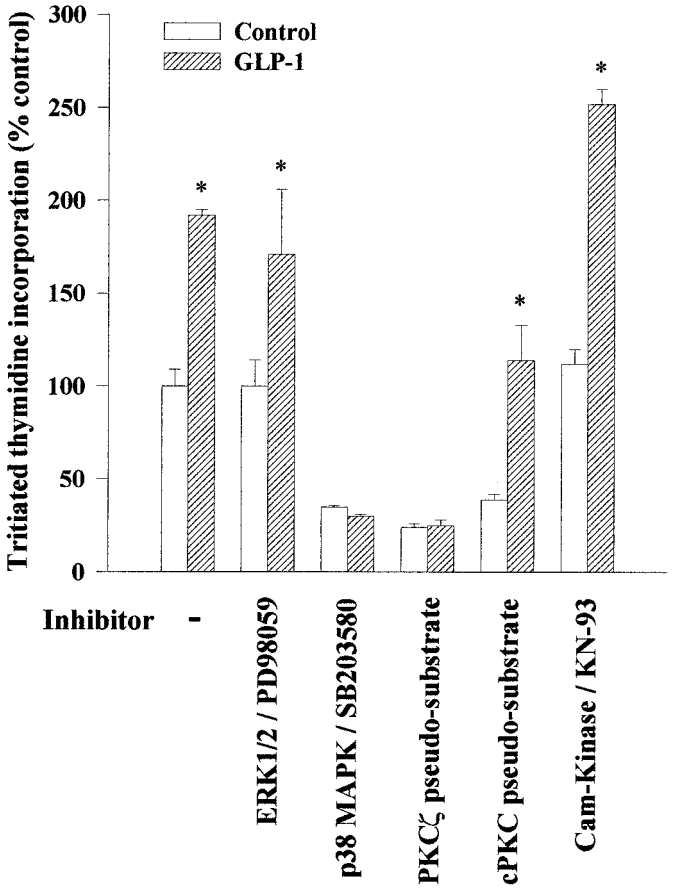

FIG. 4. PKCζ and p38 MAPK inhibitors suppress GLP-1–induced DNA synthesis in INS(832/13) cells. Cells were cultured at 3 mmol/l glucose for 24 h in serum-free RPMI medium in the presence or absence of 10 nmol/l GLP-1 and with or without different pharmacological inhibitors (50 μmol/l PD98059, 10 μmol/l SB203580, 50 μmol/l PKCζ pseudosubstrate, 50 μmol/l cPKC pseudosubstrate, and 1 μmol/l KN-93). Data are mean ± SE of three separate experiments, each comprising four wells. Cam-Kinase, calmodulin-dependent protein kinase. *$P < 0.05$.

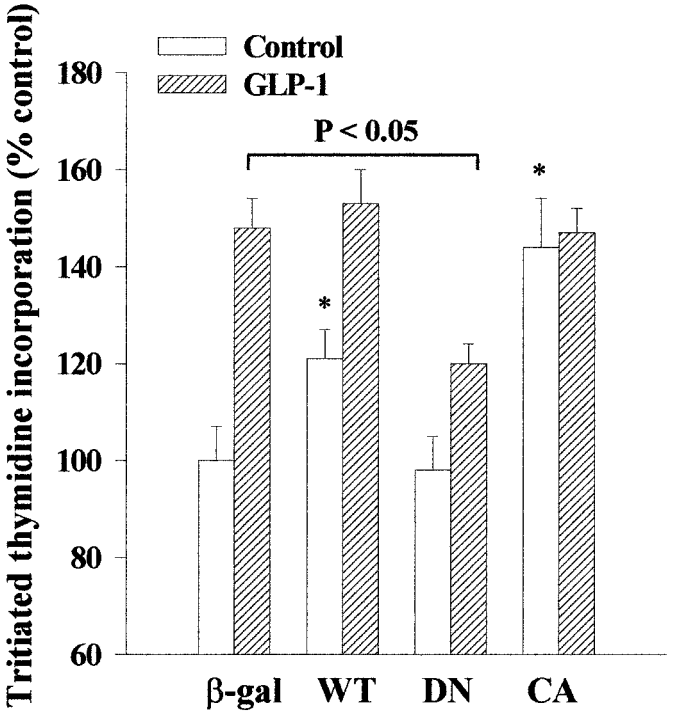

FIG. 5. Effects of adenoviral overexpression of various PKCζ constructs on GLP-1–induced proliferation in INS(832/13) cells. Cells were infected for overexpression of WT, DN, and CA PKCζ constructs as described in RESEARCH DESIGN AND METHODS. Cells were then cultured at 3 mmol/l glucose for 24 h in serum-free RPMI medium in the presence or absence of 10 nmol/l GLP-1 to perform tritiated thymidine incorporation assay as described in Fig. 1. Data are mean ± SE of three separate experiments, each comprising four wells. *$P < 0.05$.

PD98059 did not suppress GLP-1–induced DNA synthesis. MEK is an upstream kinase and activator of ERK 1/2, thus indicating that the ERK 1/2 pathway is unlikely to be involved in this proliferative process. On the other hand, both the p38 MAPK inhibitor SB203580 and the PKCζ pseudosubstrate suppressed the GLP-1–induced growth response, thus providing pharmacological evidence for the implication of these particular pathways in this process. Moreover, overexpression of a DN PKCζ in INS cells reduced the GLP-1–induced tritiated thymidine incorporation, and increasing the expression level of a CA PKCζ was sufficient to cause the proliferation of INS cells to the same extent as GLP-1. The combined pharmacological and molecular biology approaches allow the conclusion that PKCζ activation is an important step in the proliferative signaling pathway(s) of the glucoincretin.

Recent studies suggested a role for PKCζ nuclear translocation in the transduction of proliferative signals in response to various stimuli in different cell types (36–38). However, the precise way by which PKCζ might activate cell growth is unclear because little is known about the targets of PKCζ. An interesting candidate is NFκB, whose activation, which is followed by its nuclear translocation, is known to be modulated by PKCζ (39). Thus, NFκB activation is an antiapoptotic signal in some cells (40). It is interesting that another candidate target of PKCζ is the transcription factor PDX-1, which is also induced by GLP-1 (8,41). PDX-1 is a key β-cell–specific transcription factor (42) that regulates the development of the endocrine pancreas (43,44) as well as a number of β-cell genes, including those encoding insulin, glucokinase, and the glucose transporter GLUT2 (45,46). Moreover, PDX-1 expression has been shown to correlate with the proliferation of β(INS) cells (8) and pancreatic β-cell regeneration (47).

GLP-1 was already known to activate p38 MAPK in Chinese hamster ovary cells and rat insulinoma cells (RIN 1046-38) (48). In the present study, we showed that GLP-1 activates p38 MAPK in INS(832/13) cells as well and that p38 MAPK inhibition suppresses the proliferative action of GLP-1. Evidence has been obtained for a role for p38 MAPK along with ERK 1/2 in the mitogenic response of MIN6 β-cells to serum (49). Because p38 MAPK phosphorylates numerous transcription factors and induces several immediate-to-early response genes involved in cell growth/apoptosis control (50,51), it can be postulated that p38 MAPK is also implicated in the growth-promoting action of GLP-1. The hypothesis that p38 MAPK could cross-talk with the PKB cascade to mediate a proliferative event is a possibility that requires evaluation in the β-cell in view of a recent publication that documented such an effect in muscle cells (39).

In conclusion, our results indicate that GLP-1 increases

ERK 1/2, p38 MAPK, and PKB activities in INS-1 cells nonadditively with glucose. GLP-1 also causes the translocation of PKCζ, a downstream target of PI-3K, from the cytoplasm to the nucleus. However, utilization of specific kinase inhibitors reveals that only p38 MAPK and PKCζ activation are likely to play a role in the GLP-1–induced proliferative response. The use of recombinant adenoviruses to express various PKCζ constructs has allowed the demonstration of the implication of PKCζ in the GLP-1–induced increase in DNA synthesis in INS(832/13) cells. The results are consistent with a model in which GLP-1–induced PI-3K activation results in PKCζ translocation to the nucleus, which may play a role in the long-term pleiotropic effects (DNA synthesis, metabolic enzymes, and insulin gene expression) of the glucoincretin.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institute of Health Research and the Canadian Diabetes Association (to M.P.).

M.P. is a Canadian Institute of Health Research Scientist.

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