Original Article

Mechanisms of Glucose-Induced Secretion of Pancreatic-Derived Factor (PANDER or FAM3B) in Pancreatic β-Cells

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Pancreatic-derived factor (PANDER) is an islet-specific cytokine present in both pancreatic α- and β-cells, which, in vitro, induces β-cell apoptosis of primary islet and cell lines. In this study, we investigated whether PANDER is secreted by pancreatic α- and β-cells and whether PANDER secretion is regulated by glucose and other insulin secretagogues. In mouse-derived insulin-secreting β-TC3 cells, PANDER secretion in the presence of stimulatory concentrations of glucose was 2.8 ± 0.4-fold higher ($P < 0.05$) than without glucose. Insulin secretion was similarly increased by glucose in the same cells. The total concentration of secreted PANDER in the medium was ~6–10 ng/ml (0.3–0.5 nmol/l) after a 24-h culture with glucose. L-Glucose failed to stimulate PANDER secretion in β-TC3 cells. KCl stimulated PANDER secretion 2.1 ± 0.1-fold compared with control without glucose. An L-type Ca^{2+} channel inhibitor, nifedipine, completely blocked both glucose- or KCl-induced insulin and PANDER secretion. In rat-derived INS-1 cells, glucose (20 mmol/l) stimulated PANDER secretion 4.4 ± 0.9-fold, while leucine plus glutamine stimulated 4.4 ± 0.7-fold compared with control without glucose. In mouse islets overexpressing PANDER, glucose (20 mmol/l) stimulated PANDER secretion 3.2 ± 0.5-fold ($P < 0.05$) compared with basal (3 mmol/l glucose). PANDER was also secreted by α-TC3 cells but was not stimulated by glucose. Mutations of cysteine 229 or of cysteines 91 and 229 to serine, which may form one disulfide bond, and truncation of the COOH-terminus or NH_2-terminus of PANDER all resulted in failure of PANDER secretion, even though these mutant or truncated PANDERs were highly expressed within the cells. In conclusion, we found that *1*) PANDER is secreted from both pancreatic α- and β-cells, *2*) glucose stimulates PANDER secretion dose dependently in β-cell lines and primary islets but not in α-cells, *3*) PANDER is likely cosecreted with insulin via the same regulatory mechanisms, and *4*) structure and conformation is vital for PANDER secretion. *Diabetes* 54:3217–3228, 2005

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CCH, carbachol; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; KRBB, Krebs-Ringer bicarbonate buffer; PANDER, pancreatic-derived factor (or FAM3B); TBST, Tris-buffered saline with Tween; TNF, tumor necrosis factor.

Type 1 diabetes is an autoimmune disease resulting from progressive destruction of insulin-secreting β-cells of pancreatic islets (1). It has been established that cytokines (mainly interleukin [IL]-1β, tumor necrosis factor [TNF]-α, and interferon [IFN]-γ) play vital roles in β-cell dysfunction and death and in the development of type 1 diabetes (2–6). Recently, it was shown that glucose causes islet β-cells to produce IL-1β, while the released IL-1β has a deleterious effect on human pancreatic islets (7,8). These studies suggest that production and release of cytokines from pancreatic islet cells are involved in β-cell dysfunction and death in hyperglycemia. To date, although several cytokines have been shown to be involved in β-cell dysfunction and death, the precise mechanisms of type 1 diabetes are still incompletely understood, suggesting that other potential factors may be involved.

Pancreatic-derived factor (PANDER), also known as FAM3B (9,10), is one of four members of a new cytokine family recently identified using the algorithm ostensible recognition of folds (11) while searching for novel cytokines based on their predicted secondary structure. The rationale for this approach is that the secondary structure of cytokines is highly conserved through evolution. Many cytokines, such as IL-2, -3, -4, -5, -6, -7, -9, -10, and -13, and leptin share similar four-helix bundle secondary structures with a typical up-up-down-down typology (12,13). The PANDER gene encodes a 235–amino acid protein with a secretion signal peptide (aa1-29) and four cysteines (C63, C69, C91, and C229), which may form two putative disulfide bridges (9). The PANDER gene is strongly expressed in pancreatic islets and slightly expressed in the prostate and small intestine. The tissue-specific expression implies that PANDER may regulate islet cell function as a locally produced cytokine. Our previous studies showed that PANDER protein is present in α- and β-cells of pancreatic islets. Furthermore, recombinant PANDER protein induces apoptosis of α- and β-cells of mouse, rat, and human islets in a dose- as well as time-dependent manner (10). Treatment with IFN-γ or a combination of IL-1β, TNF-α, and IFN-γ upregulates PANDER gene expression in mouse islets and in insulin secreting β-cell lines in a time- and dose-dependent manner (14), revealing that PANDER may function as a downstream signal in cytokine-mediated β-cell dysfunction and death. The PANDER promoter contains glucose-responsive elements and has a robust glucose response in pancreatic β-cell lines and in primary

islet cells (45). This is similar to the insulin promoter (15), implying a potential role of PANDER in glucose homeostasis. In addition, we find that the PANDER protein is located in the insulin secretory granules of pancreatic β-cells (14); a key question of this study was to determine whether glucose could induce PANDER secretion. Therefore, we evaluated the effects of various insulin secretagogues and inhibitors on PANDER secretion in pancreatic β- and α-cells. We also showed a correlation between insulin and PANDER secretion and the relationship between PANDER structure and PANDER secretion. Due to the low secretion level of endogenous PANDER and low sensitivity of Western blot analysis, the present project was carried out in β-cell lines overexpressing PANDER and confirmed in mouse islets.

RESEARCH DESIGN AND METHODS

α-TC3, β-TC3, and INS-1 cell culture. α-TC3, β-TC3, and INS-1 cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37°C in 5% CO_2, 95% air. The cells were split weekly and the medium changed twice weekly.

Construction of mutant PANDERs. Before mutagenesis, wild-type mouse PANDER cDNA (670 bp) (9) was cloned into a kanamycin-resistant 4.1-kb pShuttle plasmid (BD Biosciences) containing a cytomegalovirus promoter with *Apa*I and *Not*I restriction enzymes, creating wild-type PANDER plasmid. Wild-type PANDER plasmid was used as a template for all PANDER mutant constructions. PCR primers with the desired mutation were synthesized. All PCR were done with QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene) with the following conditions: 95°C (1 min), followed by 30 cycles of 95°C (1 min), 55°C (1 min), and 65°C (10 min) (2 min/kb plasmid length). When the PCR was completed, the methylated template was completely digested by *Dpn*I and the single mutant strand added to ultracompetent *Escherichia coli*. The transformed cells were plated on a Luria-Bertani medium plate containing 50 μg/ml kanamycin and incubated at 37°C overnight. Positive clones were first identified by the predicted molecular weights of plasmid digestion products using restriction enzymes *Apa*I and *Not*I, followed by DNA sequencing for definitive confirmation. To construct M1 PANDER, a stop codon was engineered before helix D (aa 177-235 was truncated). M2 PANDER was generated by mutating Ser176 of M1 PANDER to serine. In M5 PANDER, helix A and most of the AB loop (aa31-90) was truncated, and the signal peptide (aa1-29) was reconnected to the new NH_2-terminus. M6-PANDER was generated by mutating Cys91 of M5-PANDER to serine. C91S (mutation of C91 to serine), C91-229S (mutation of both C91 and C229 to serine), A29E30-G29G30 (mutation of Ala29 and Glue30 to glycine), and Y26L27-G26G27 (mutation of Tyr26 and Leu27 to glycine) PANDERs were also constructed. For large-scale preparation of plasmids needed for transfection, the plasmid was proliferated in *E. coli* and purified using Endo-free Plasmid Maxi Kit (Qiagen). The DNA was precisely quantified by ultraviolet absorbance before transfection. To eliminate the variation from plasmid purification, at least three independent plasmid preparations were prepared for subsequent transfection.

Transfection of cells with wild-type and mutant PANDER plasmids. β-TC3 cells ($7–8 \times 10^5$) were seeded into each well of a 12-well plate in 1 ml antibiotic-free RPMI-1640 (11 mmol/l glucose and 10% FBS) 30 min before transfection. For transfection, 1.6 μg (0.8 μg for α-TC3 cells) of plasmid was diluted into 100 μl prewarmed Opti-MEM medium (antibiotic and serum free) and mixed gently. At the same time, 3.2 μl (1.6 μl for α-TC3 cells) Lipofectamine 2000 (Invitrogen) was diluted into 100 μl Opti-MEM medium. The diluted DNA and Lipofectamine 2000 mixtures were preincubated at room temperature for 5 min, then combined to generate the transfection complex (room temperature, 20 min). After incubation, the transfection complex (200 μl) was added into each well (total transfection volume 1.2 ml). The plate was rocked back and forth gently before incubating at 37°C in 5% CO_2 overnight. INS-1 cells were transfected in attachment, and the plasmid and Lipofectamine were reduced by half, since they were more sensitive to Lipofectamine, and then incubated in 37°C for 4 h. After incubation, the transfection medium was replaced with normal RPMI-1640 and cells cultured overnight.

PANDER secretion from cell lines: 4-h incubation protocol. Transfected cells were washed twice with 2 ml prewarmed Krebs-Ringer bicarbonate buffer (KRBB) (115 mmol/l NaCl, 24 mmol/l $NaHCO_3$, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, 25 mmol/l Hepes [pH 7.4], and 0.1% BSA) before incubating in 2 ml KRBB containing 3 mmol/l glucose for 1 h, then incubated in 2 ml KRBB containing the indicated insulin secretagogues or inhibitors for 4 h at 37°C in 5% CO_2. After incubation, all media was moved into 2-ml microcentrifuge tubes and centrifuged at 4°C at 13,000 rpm for 10 min. The supernatant was kept for insulin and PANDER analysis. The cells were washed twice with 1 ml ice-cold PBS and then lysed in freshly prepared Roth lysis buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 1% Triton X-100, 5 mmol/l EDTA, 5 mmol/l EGTA, 20 mmol/l Na Pyrophosphate, 20 mmol/l NaF, 0.2 mg/ml PMSF, 0.01 mg/ml leupeptin, and 0.01 mg/ml aprotinin, pH 7.4). The lysate was transferred into 1.5-ml tubes and centrifuged at 4°C at 13,000 rpm for 10 min. The pellet was disposed and protein concentration in the supernatant determined by bicinchoninic acid assay to equalize the amount of protein in all conditions.

Twenty-four–hour incubation protocol. The following morning after transfection, the transfection medium was replaced with 2 ml RPMI-1640 (1% FBS) with the indicated conditions, and the cells were cultured at 37°C in 5% CO_2 for 24 h. After incubation, the medium and cells were treated as above. PANDER in the medium and cell lysate were assayed by Western blot assay.

Insulin secretion measurement. The supernatant was submitted to the Radioimmunoassay Core of the Institute of Diabetes, Obesity, and Metabolism of the University of Pennsylvania for insulin content analysis.

Secretion of PANDER from mouse islets. Mouse islets were prepared as detailed previously (16). Mouse islets used in this study were isolated from a transgenic mouse (B6SJLF/J) model overexpressing mouse PANDER specifically in pancreas islets under the control of pancreatic duodenal homeobox-1 promoter (C.E.R. and B.A.W., unpublished observations). Only male mice were used in this study. Before culture, the islets were washed three times with glucose-free RPMI-1640 supplemented with 1% FBS, 2 mmol/l glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Batches of 100 islets with a similar mass were hand-picked under microscope and cultured in 2 ml RPMI-1640 (1% FBS) with 3 or 20 mmol/l glucose at 37°C in 5% CO_2, 95% air, in nontreated 12-well plates for 48 h. Twenty-four hours after incubation, 200 μl medium was collected from each culture condition and centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was stored at −80°C. Forty-eight hours after incubation, all of the islets were moved into a tube and centrifuged at 5,000 rpm at room temperature for 5 min, and the supernatant was kept. The islet pellet was lysed in 40 μl Roth lysis buffer and the protein concentration in the supernatant determined to equalize the amount of protein in all conditions. Insulin and PANDER secretion in the supernatant were assayed.

Western blot assay. The medium and cell lysate were treated with 5× SDS loading buffer (Tris base, 0.313 mol/l; SDS, 10 g; glycerol, 50%; bromophenol blue, 0.05%; pH 6.8) by boiling for 10 min in a water bath. For 15% SDS PAGE, 50 μl treated medium or 10 μg total cell lysate protein was loaded into each lane. Electrophoresis was performed at 170 V. Proteins in the gel were transferred to Hybond-C Extra membrane (Amersham Biosciences) at 120 V for 2 h at 4°C. The membrane was washed once with 10 ml 1× Tris-buffered saline with Tween (TBST) (10 mmol/l Tris base, 100 mmol/l NaCl, 0.1% Tween-20, pH 7.5) before blocking in 20 ml blocking buffer (1× TBST containing 1% BSA) at room temperature for 1 h. The membrane was incubated in 1:1,000 rabbit anti-PANDER polyclonal antibody at 4°C overnight. The anti-PANDER antibody was purified from rabbit antiserum obtained after immunization with recombinant mouse PANDER protein using an antigen-based antibody purification kit (SulfoLink Kit, Cat. no. 44895; Pierce). The membrane was washed five times with 15 ml 1× TBST, incubated in 1:5,000 peroxidase-conjugated donkey anti-rabbit antibodies at room temperature for 1 h before washing as above, and then developed with enhanced chemiluminescence. After the PANDER immunoblot, the membrane was stripped with 0.2 N NaOH and reprobed using 1:500 β-actin antibody (SC-7210; Santa Cruz).

Quantification of PANDER protein. The enhanced chemiluminescence Western blot film was scanned and the relative amount of PANDER protein determined using software ImageQuant (version 5.2; Amersham Biosciences). All data were normalized to the control value. The result of each experiment represented the average of three independent scans. The data were collected from at least three independent experiments. Linearity of the assay is shown in Fig. 1*F*.

Statistical analysis. Data are presented as means ± SEM. Statistical significance of differences between groups was analyzed by unpaired Student's *t* test or by one-way ANOVA when more than two groups were compared.

RESULTS

Glucose stimulates PANDER secretion dose-dependently in β-TC3 cells. To evaluate the effects of glucose, the main physiological insulin secretagogue, on PANDER

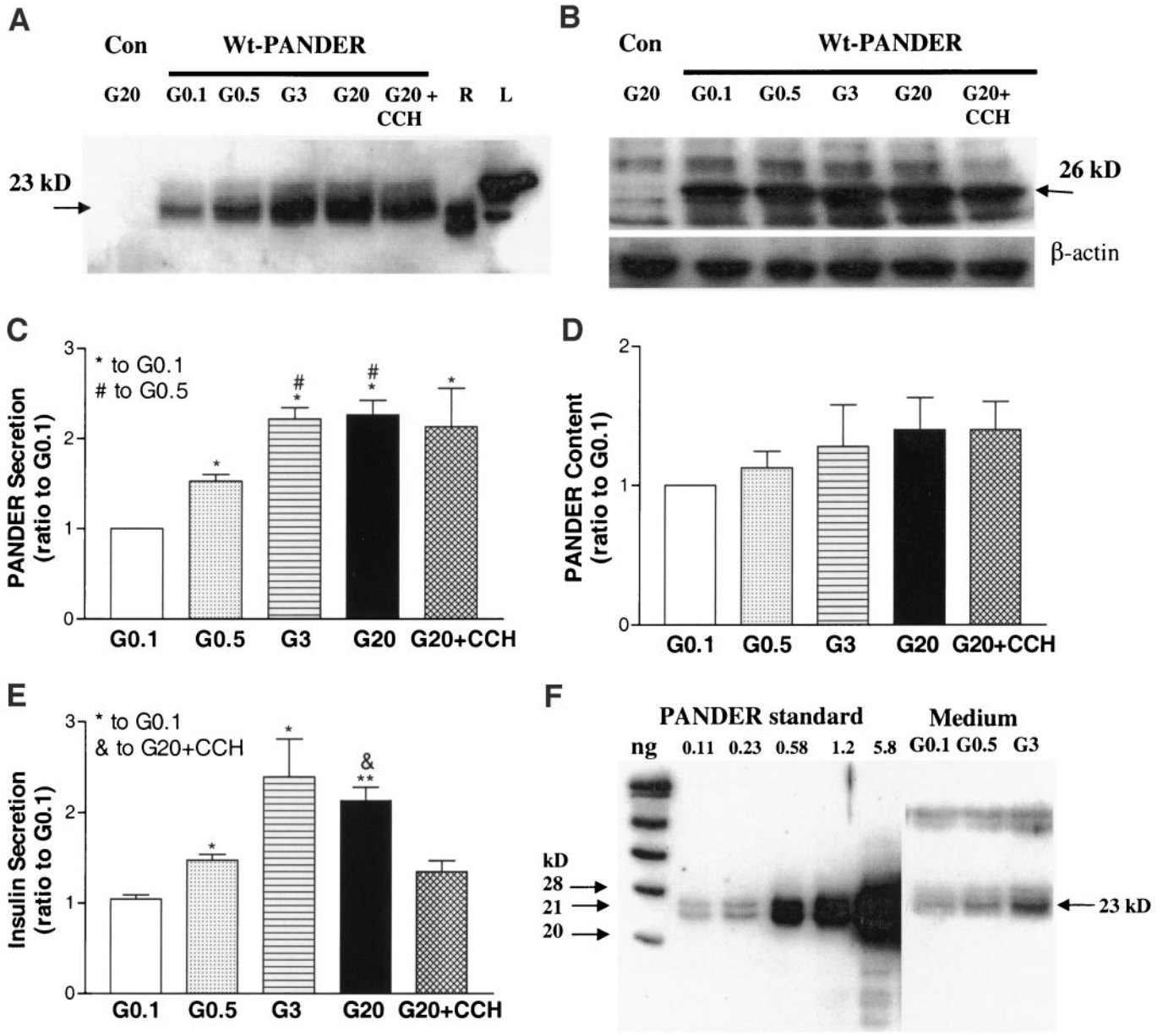

FIG. 1. Glucose stimulated PANDER secretion using a 24-h incubation protocol in β-TC3 cells. On the morning after transfection, the transfection medium was replaced with 2 ml RPMI-1640 (1% FBS) containing 0.1 mmol/l glucose (G0.1), 0.5 mmol/l glucose (G0.5), 3 mmol/l glucose (G3), 20 mmol/l glucose (G20), and 20 mmol/l glucose (G20) + 0.5 mmol/l CCH, and then the cells were cultured for 24 h. The samples were treated and Western blot performed as described in RESEARCH DESIGN AND METHODS. *A*: Representative Western blot of secreted PANDER. R, recombinant PANDER protein; L, cell lysate. *B*: Representative Western blot of PANDER content. *C*: Quantification of *A* with data normalized to the G0.1 value. *D*: Quantification of *B* with data normalized to G0.1 value. *E*: Insulin secretion (from medium of panel *A*) normalized to G0.1 value, which was 540 ± 60 ng insulin/well. *F*: Determination of the absolute secreted PANDER concentration in the medium by recombinant PANDER standard curve. Con, control cells transfected with pShuttle vehicle. Wt-PANDER, cells transfected with pShuttle containing wild-type PANDER gene. The data were collected from at least five independent experiments. *$P < 0.05$ vs. control G0.1 level.

secretion, the overexpressing β-TC3 cells were challenged with different glucose concentrations for different lengths of time, and then PANDER and insulin secretion were assayed. Green fluorescent protein containing pShuttle plasmid transfection indicated that the transfection efficiency in our system is ~30–40% (data not shown). The preliminary experiments indicated that 4 h of incubation is the shortest time needed to detect secreted PANDER using Western blot (data not shown). After 4 h of incubation, glucose stimulated PANDER secretion. PANDER secretion at 3 or 20 mmol/l glucose was 2.1 ± 0.2- ($P < 0.05$) or 2.8 ± 0.4-fold ($P < 0.05$) that of the control value without glucose, respectively. A combination of 20 mmol/l glucose and 0.5 mmol/l carbachol (CCH) stimulated PANDER secretion 1.6 ± 0.5-fold compared with control ($P > 0.05$) (Fig. 2*A* and *C*). Glucose or glucose + CCH had no effect on PANDER content within the cells (1.1 ± 0.2-, 1.2 ± 0.2-, and 1.1 ± 0.3-fold of that without glucose for 3 and 20 mmol/l glucose and 20 mmol/l glucose + CCH, respectively) (Fig. 2*B* and *D*). The insulin secretion in the same medium was similarly increased by glucose (1.8 ± 0.2- [$P < 0.05$], 2.0 ± 0.2- [$P < 0.05$], and 1.2 ± 0.1-fold [$P > 0.05$] of that without glucose for 3 and 20 mmol/l glucose and 20 mmol/l glucose + CCH, respectively). To further evaluate glucose-stimulated PANDER secretion, we tested the effects of 24-h glucose incubation on PANDER and

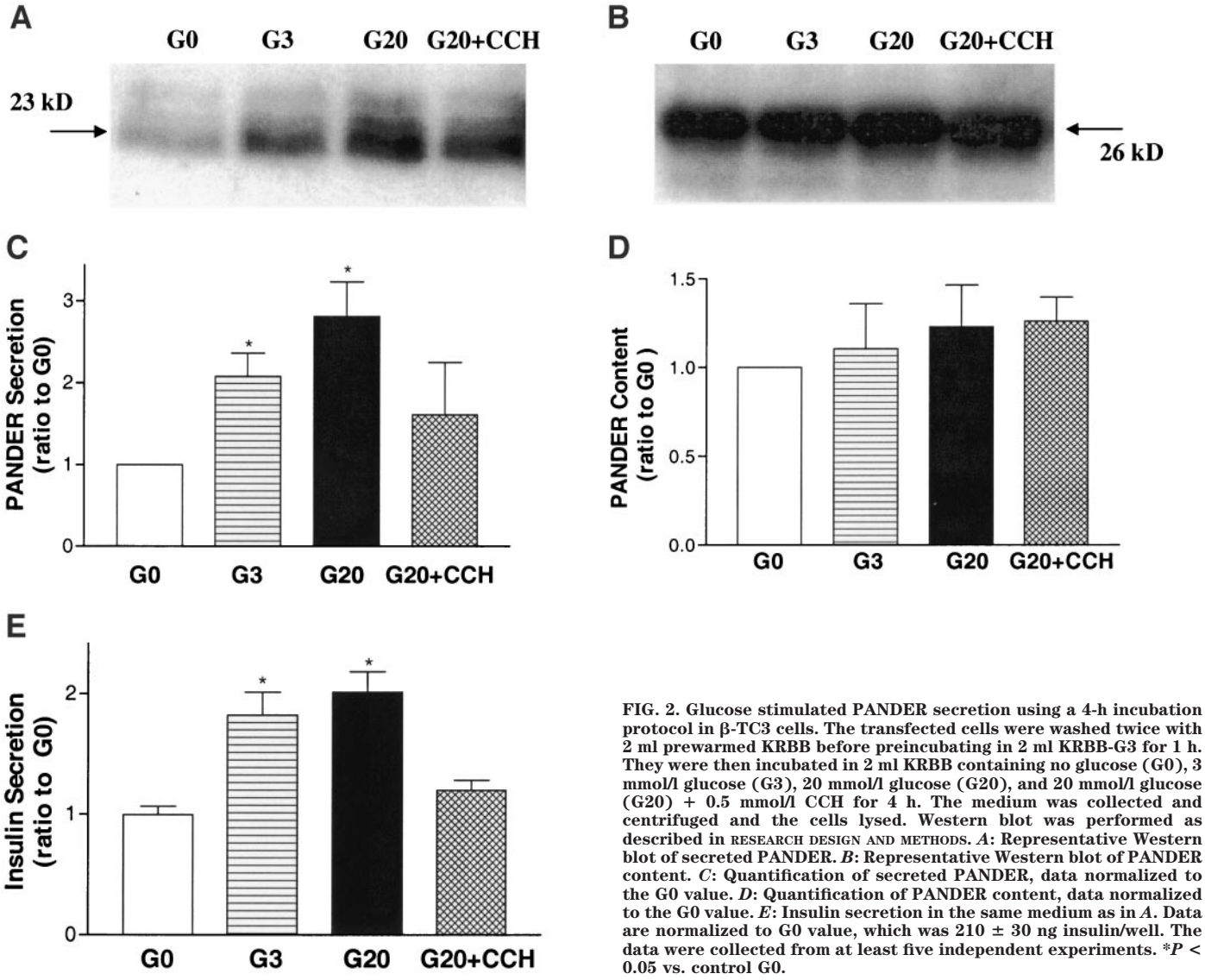

FIG. 2. Glucose stimulated PANDER secretion using a 4-h incubation protocol in β-TC3 cells. The transfected cells were washed twice with 2 ml prewarmed KRBB before preincubating in 2 ml KRBB-G3 for 1 h. They were then incubated in 2 ml KRBB containing no glucose (G0), 3 mmol/l glucose (G3), 20 mmol/l glucose (G20), and 20 mmol/l glucose (G20) + 0.5 mmol/l CCH for 4 h. The medium was collected and centrifuged and the cells lysed. Western blot was performed as described in RESEARCH DESIGN AND METHODS. *A*: Representative Western blot of secreted PANDER. *B*: Representative Western blot of PANDER content. *C*: Quantification of secreted PANDER, data normalized to the G0 value. *D*: Quantification of PANDER content, data normalized to the G0 value. *E*: Insulin secretion in the same medium as in *A*. Data are normalized to G0 value, which was 210 ± 30 ng insulin/well. The data were collected from at least five independent experiments. **P* < 0.05 vs. control G0.

insulin secretion. In the 24-h incubation protocol, we used 0.1 mmol/l glucose, rather than no glucose, as the control group due to the low threshold of insulin secretion in the β-TC3 cell line and to provide the necessary glucose for survival of the cells. After 24 h of incubation, PANDER secretion at 0.5, 3, and 20 mmol/l glucose and 20 mmol/l glucose + 0.5 mmol/l CCH was 1.5 ± 0.1- ($P < 0.05$), 2.2 ± 0.2- ($P < 0.05$ for control vs. 0.5 mmol/l glucose), 2.4 ± 0.3- ($P < 0.05$ for control vs. 0.5 mmol/l glucose), and 1.8 ± 0.4-fold ($P < 0.05$ for control vs. 0.5 mmol/l glucose) that of glucose at 0.1 mmol/l. The PANDER content for 0.5, 3, and 20 mmol/l glucose and 20 mmol/l + CCH, respectively, was 1.1 ± 0.2- ($P > 0.05$), 1.3 ± 0.2- ($P > 0.05$), 1.4 ± 0.2- ($P > 0.05$), and 1.4 ± 0.2-fold ($P > 0.05$) that of the control value (0.1 mmol/l) without significant changes (Fig. 1*B* and *D*). For 0.5, 3, and 20 mmol/l glucose and 20 mmol/l glucose + 0.5 mmol/l CCH, respectively, insulin secretion in the same medium was 1.5 ± 0.1- ($P < 0.05$), 2.4 ± 0.4- ($P < 0.05$ for control vs. 0.5 mmol/l glucose), 2.1 ± 0.2- ($P < 0.05$ for control vs. 0.5 mmol/l), and 1.3 ± 0.2-fold ($P > 0.05$) that of control at 0.1 mmol/l glucose (Fig. 1*E*). The results indicated that the effects of glucose on PANDER secretion as well as insulin secretion reached a maximum at 3 mmol/l (Figs. 2*C* and *E* and 1*C* and *E*) in β-TC3 cells. To determine the absolute concentration of secreted PANDER after 24 h of culture, a recombinant PANDER standard curve was established (Fig. 1*F*). The total concentration of secreted PANDER is ~5–10 ng/ml, equaling 0.3–0.5 nmol/l at 3 mmol/l glucose, which is about one order lower than its effective dose of killing cells as documented previously (4 nmol/l) (10) (Fig. 1*F*). At 3 mmol/l glucose, PANDER secreted into the medium after 8, 12, and 24 h of incubation was 1.2 ± 0.1-, 2.3 ± 0.3- ($P < 0.05$), and 2.8 ± 0.3-fold ($P < 0.05$) over the amount of PANDER secreted at 4 h incubation. L-Glucose failed to stimulate PANDER and insulin secretion in β-TC3 cells (data not shown). In summary, glucose stimulated PANDER secretion dose-dependently with maximal effects at 3 mmol/l. PANDER secretion is correlated strongly with insulin secretion in β-TC3 cells.

Nifedipine inhibits glucose- and KCl-induced PANDER secretion. To determine whether voltage-dependent Ca^{2+} channel activity was required for PANDER secretion, nifedipine was used to inhibit glucose-induced insulin secretion in β-TC3 cells (17,18). Without glucose, PANDER secretion was unchanged in the absence (1.0) or presence (0.96 ± 0.1, $P > 0.05$) of 5 μmol/l nifedipine. PANDER secretion was significantly stimulated by 20

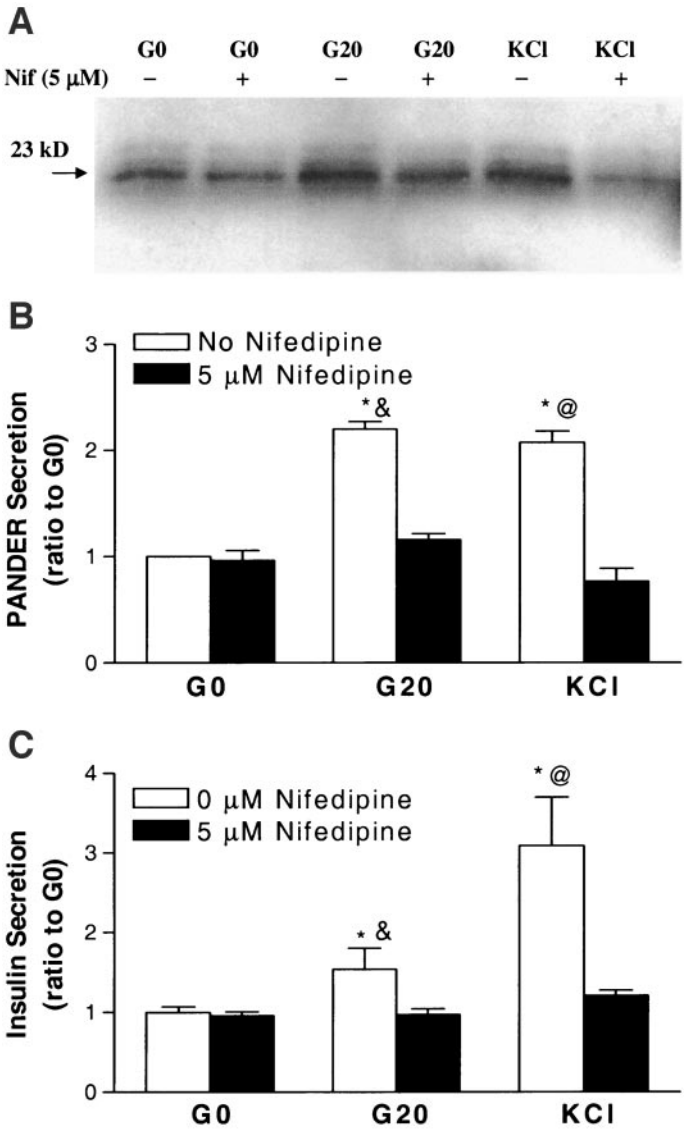

FIG. 3. Nifedipine blocked glucose- and KCl-induced PANDER and insulin secretion in β-TC3 cells. The cells were incubated in 2 ml KRBB with no glucose (G0), G0 + 5 μmol/l nifedipine, 20 mmol/l glucose (G20), G20 + 5 μmol/l nifedipine, 30 mmol/l KCl, and 30 mmol/l KCl + 5 μmol/l nifedipine for 4 h. Western blot was performed as described. *A*: Representative Western blot of secreted PANDER. *B*: Quantification of *A* with data normalized to G0 value. *C*: Insulin secretion (from *A*) normalized to G0 value, which was 174 ± 14 ng insulin/well. Data were collected from at least five independent experiments. **P* < 0.05 vs. G0, &*P* < 0.05 vs. G20 + nifedipine, @*P* < 0.05 vs. KCl + nifedipine.

mmol/l glucose in the absence (2.2 ± 0.2, $P < 0.05$ vs. no glucose) but not in the presence of nifedipine (1.2 ± 0.1, $P < 0.05$ vs. 20 mmol/l glucose without nifedipine, $P > 0.05$ vs. no glucose). KCl at 30 mmol/l induced PANDER secretion by 2.1 ± 0.1-fold ($P < 0.05$ vs. no glucose) compared with no glucose (control) in the absence of nifedipine, but its effect was completely blocked in the presence of nifedipine, which was 0.8 ± 0.2-fold that of the no glucose control ($P < 0.05$ vs. KCl alone, $P > 0.05$ vs. no glucose) (Fig. 3*A* and *B*). There was no difference in PANDER content among the six groups (data not shown). In the same medium, insulin secretion at 0 mmol/l glucose was the same in the absence (1.0) or presence (1.0 ± 0.1, $P > 0.05$) of nifedipine. Glucose at 20 mmol/l significantly stimulated insulin secretion only in the absence (1.6 ± 0.2, $P < 0.05$ vs. no glucose [control]) but not in the presence of nifedipine (0.9 ± 0.1, $P < 0.05$ vs. 20 mmol/l glucose, $P > 0.05$ vs. no glucose). Nifedipine also inhibited KCl-induced insulin secretion completely, at 3.1 ± 0.5-fold without nifedipine ($P < 0.05$ vs. no glucose) and 1.2 ± 0.1-fold ($P < 0.05$ vs. KCl without nifedipine, $P > 0.05$ vs. no glucose) of the control value with nifedipine (Fig. 3*C*). Thus, nifedipine completely inhibited both glucose- and KCl-induced PANDER secretion and insulin secretion, revealing that PANDER secretion and insulin secretion share a similar Ca^{2+} channel–dependent mechanism in pancreatic β-cells.

Glucose fails to stimulate PANDER secretion in α-TC3 cells. Since PANDER protein is also present in the α-cells of pancreatic islets (10), we tested whether glucose stimulated PANDER secretion in α-TC3 cells. Preliminary experiments showed that α-TC3 cells were more sensitive to PANDER, and overexpressing α-TC3 cells had lower PANDER secretion levels than β-TC3 cells (data not shown), so we reduced the plasmid dose by half and chose the 24-h incubation protocol. PANDER secretion at 0.5, 3, and 20 mmol/l glucose was 1.1 ± 0.2- ($P > 0.05$ vs. 0.1 mmol/l glucose), 1.2 ± 0.3- ($P > 0.05$), and 1.2 ± 0.1-fold ($P > 0.05$) that at 0.1 mmol/l glucose (Fig. 4*A* and *B*). Glucose did not change PANDER content significantly (0.9 ± 0.2-, 0.8 ± 0.3-, and 0.7 ± 0.2-fold that of the control at 0.1 mmol/l glucose for 0.5, 3, and 20 mmol/l glucose, respectively) (Fig. 4*C*).

Glucose stimulates PANDER secretion in isolated mouse islets. To demonstrate the physiological relevance of glucose-simulated PANDER secretion, we used isolated mouse islets overexpressing PANDER. After 24 h of stimulation, PANDER secretion at 20 mmol/l glucose was 2.8 ± 0.4-fold ($P < 0.05$) that of the control value at 3 mmol/l glucose. After 48 h of stimulation, PANDER secretion at 20 mmol/l glucose was 3.2 ± 0.5-fold ($P < 0.05$) that at 3 mmol/l glucose (Fig. 5*A* and *B*). After 48 h of incubation, PANDER content at 20 mmol/l glucose was slightly higher (1.4 ± 0.1, $P < 0.05$) than the control value at 3 mmol/l glucose. In the same medium, insulin secretion was 3.2 ± 0.2 (20 mmol/l glucose, $P < 0.05$) versus 1.0 ± 0.1 (3 mmol/l glucose) after 24 h of incubation, and 3.1 ± 0.4 (20 mmol/l glucose, $P < 0.05$) versus 1.0 ± 0.1 (3 mmol/l glucose) after 48 h of incubation (Fig. 5*C*). The results indicated that glucose stimulated PANDER secretion in primary islet cells and that PANDER secretion is strongly correlated with insulin secretion in islet cells.

Mutant or truncated PANDERs are not released from β-TC3 cells. To test the hypothesis that the correct structure and conformation is important for PANDER secretion, a series of constructs containing truncated or mutant PANDER genes were expressed in β-TC3 cells. To ensure a sufficient amount of released protein for Western blot detection in the medium, mutant PANDER secretions were assayed using a 24-h incubation protocol at 3 mmol/l glucose. The entire helix D (the fourth helix) was truncated in M1 (176 aa), which caused the absence of secreted PANDER protein in the medium (Fig. 6*A*). M2 had an additional cysteine at the new COOH-terminus of M1 but did not restore secreted PANDER in the medium (Fig. 6*A*). Both M1 and M2 produced PANDER proteins of 16 and 19 kDa within the cells. The 19-kDa band is the truncated PANDER with the signal peptide. The 16-kDa band in these cell lysate is presumably the truncated PANDERs without the signal peptides (Fig. 6*A*), implying that the signal peptide of truncated PANDER can be cleaved successfully. In the NH_2-terminus including the

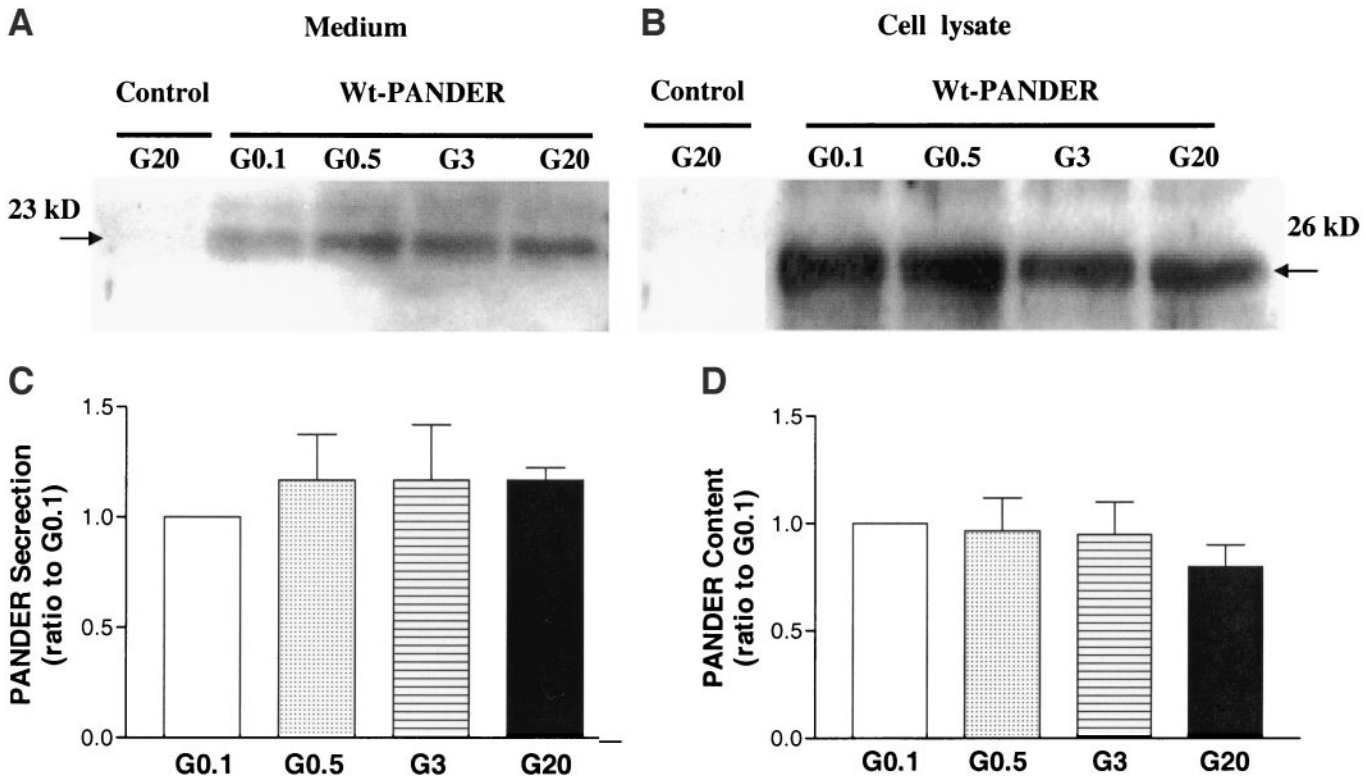

FIG. 4. Glucose failed to stimulate PANDER secretion in β-TC3 cells. The cells were stimulated in 2 ml RPMI-1640 (1% FBS) containing 0.1 mmol/l glucose (G0.1), 0.5 mmol/l glucose (G0.5), 3 mmol/l glucose (G3), and 20 mmol/l glucose (G20) for 24 h. Western blot was performed as described. *A*: Representative Western blot of secreted PANDER. *B*: Representative Western blot of PANDER content. *C*: Quantification of *A* with data normalized to G0.1 level. *D*: Quantification of *B* with data normalized to G0.1 level. The data were collected from at least three independent experiments. There was no significant difference among the four groups.

entire helix A (the first helix), the first two cysteines (Cys63 and Cys69) were truncated in M5 (175 aa) with the signal peptide reconnected to the new NH_2-terminus, which did not produce any secreted PANDER (Figs. 6*A* and 7). M6 was generated by mutating Cys91 to serine of M5, which did not restore the PANDER secretion (Fig. 6*A*). Cells transfected with M5 and M6 both produced PANDER proteins within the cells (Fig. 6*A*). Similarly, the 19-kDa band is with signal peptide, while the 16-kDa band is without (Fig. 6*A*).

Two other mutants, C229S (mutation of Cys229 to Ser) and C91–229S (mutation of both Cys91 and Cys229 to Ser), also did not produce any secreted PANDER in the medium, but both produce PANDER proteins within the cell (Fig. 6*B*). Two bands of mutant proteins were detected with similar affinity as wild-type PANDER (Fig. 6*B*). The heavier band (26 kDa) is presumably with the signal peptide, while the lighter one (23 kDa) is without. In A29E30-G29G30 PANDER, Ala29 and Glu30 were mutated to Gly, which is predicted as the cleavage site of the signal peptide (9). A29E30-G29G30 PANDER protein was secreted from the cells (Fig. 6*B*). Two bands were also detected in both the medium and the cells. The heavier band (26 kDa) is presumably PANDER with the signal peptide, while the lighter one (23 kDa) is without. For Y26L27-G26G27, Tyr26 and Leu27 were mutated to Gly, in case the predicted cleavage site shifts left. Y26L27-G26G27 PANDER protein was secreted from the cells. Interestingly, an enhanced secretion was observed in Y26L27-G26G27 PANDER (two- to fivefold) when compared with wild-type PANDER under the same condition (Figs. 6*B* and 7).

Glucose stimulates PANDER secretion in INS-1 cells. We also used INS-1 cells to extend our finding in another β-cell line. Wild-type and YL-GG PANDERs were expressed in INS-1 cells, and secretion was assayed using a 4-h incubation protocol. For wild-type PANDER, 3 and 20 mmol/l glucose stimulated 1.9 ± 0.1- ($P < 0.05$) and 4.4 ± 0.9-fold ($P < 0.05$) that of secretion compared with control without glucose. The secretion of YL-GG PANDER at 0, 3, and 20 mmol/l glucose was 3.5 ± 0.8- ($P < 0.05$), 3.9 ± 0.7- ($P < 0.05$), and 7.7 ± 1.4-fold ($P < 0.05$) that of the wild-type control without glucose (Fig. 8*A* and *B*). Insulin secretion at 3 and 20 mmol/l glucose was 1.3 ± 0.1- ($P > 0.05$) and 2.2 ± 0.1-fold ($P < 0.05$) that of the control without glucose for wild-type PANDER–overexpressing cells, respectivley. In YL-GG PANDER–expressing cells, insulin secretion at 0, 3, and 20 mmol/l glucose was 1.3 ± 0.2-, 1.8 ± 0.4-, and 2.4 ± 0.2-fold ($P < 0.05$) that of wild-type control without glucose (Fig. 8*C*). Similarly, as in β-TC3 cells, C229S and C91–229S PANDERs were not released from INS-1 cells (data not shown). In summary, glucose stimulated PANDER secretion dose-dependently in INS-1 cells, and YL-GG PANDER mutant had an enhanced secretion level at various glucose concentrations compared with wild-type PANDER.

Leucine plus glutamine significantly stimulates PANDER secretion in INS-1 cells. In the absence of glutamine, 10 mmol/l leucine (Leu10) and KIC (KIC10) stimulated PANDER secretion 2.0 ± 0.3- ($P < 0.05$) and 1.7 ± 0.4-fold ($P > 0.05$) when compared with control without glucose, while they stimulated PANDER secretion 4.4 ± 0.7- ($P < 0.05$) and 2.0 ± 0.3-fold ($P < 0.05$) in the presence of 4 mmol/l glutamine, respectively. Glucose at

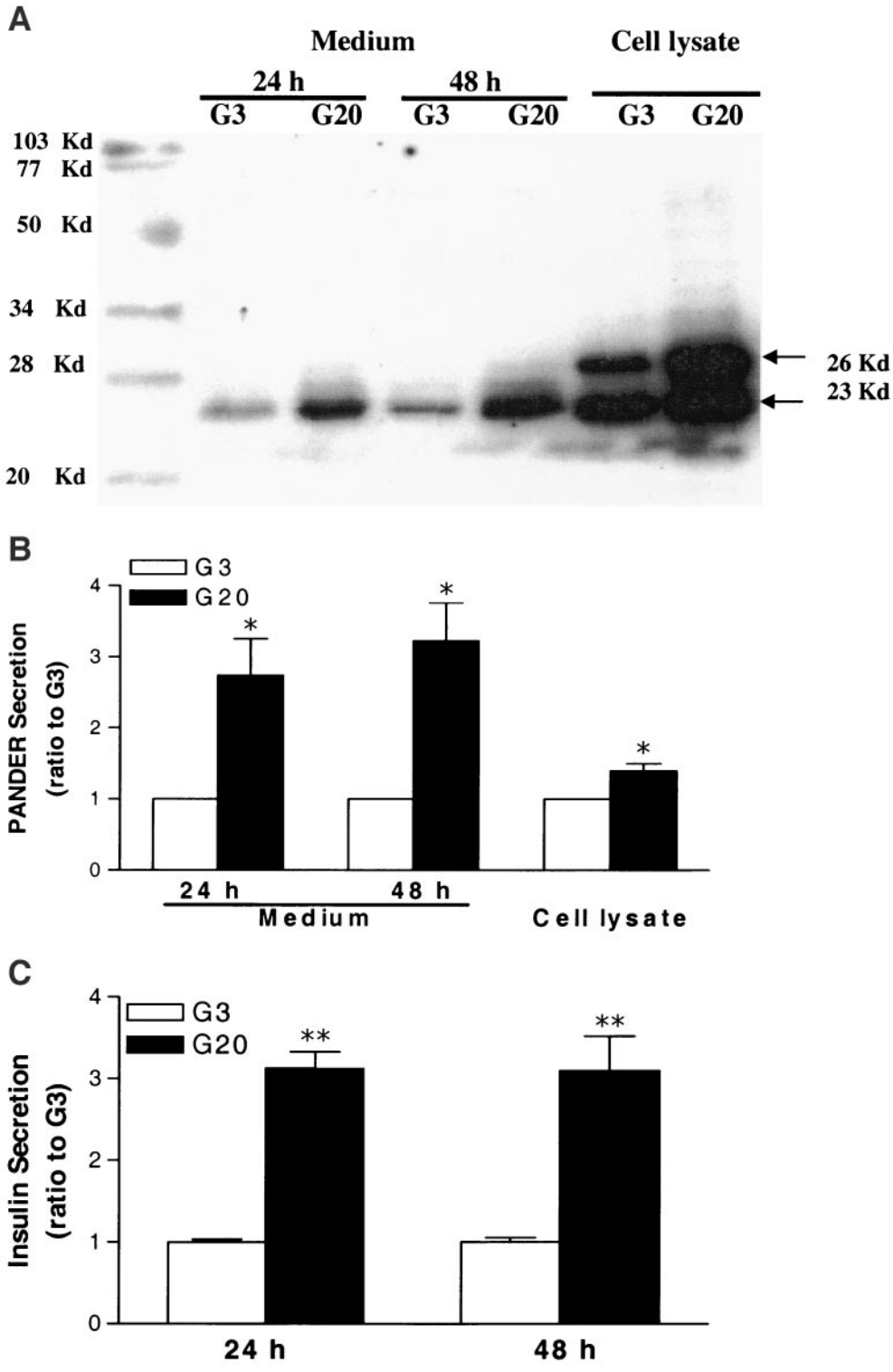

FIG. 5. Glucose-stimulated PANDER secretion in primary islet cells. Batches of 100 islets were cultured in 2 ml RPMI-1640 (1% FBS) containing 3 mmol/l glucose (G3) or 20 mmol/l glucose (G20) for 48 h. At the 24-h point, 200 μl medium was collected from culture condition. At 48 h, the medium was collected and islets lysed as described in RESEARCH DESIGN AND METHODS, and Western blot was performed as described. *A*: Representative Western blot of secreted PANDER. *B*: Quantification of *A* with all data normalized to G3 value at each time-point. *C*: Insulin secretion (from panel *A*) normalized to G3 value at each time-point (1,530 ± 100 ng insulin/well after 24 h and 2,770 ± 340 ng insulin/well after 48 h culture, respectively). Data were collected from four independent experiments. *$P < 0.05$, **$P < 0.01$ vs. corresponding control G3 value.

20 mmol/l stimulated PANDER secretion 5.9 ± 0.8-fold ($P < 0.05$) compared with control without glucose (Fig. 9*A*). In the same medium, insulin secretion with Leu10, KIC10, Leu10 + glutamine, KIC10 + glutamine, and 20 mmol/l glucose was 1.4 ± 0.1- ($P < 0.05$), 1.5 ± 0.1- ($P < 0.05$), 3.5 ± 0.3- ($P < 0.01$), 1.9 ± 0.1- ($P < 0.05$), and 5.4 ± 0.4-fold ($P < 0.01$) that of control values with no glucose, respectively (Fig. 9*B*). There was no difference in PANDER content among the six groups (data not shown). In summary, these results further confirm that PANDER and

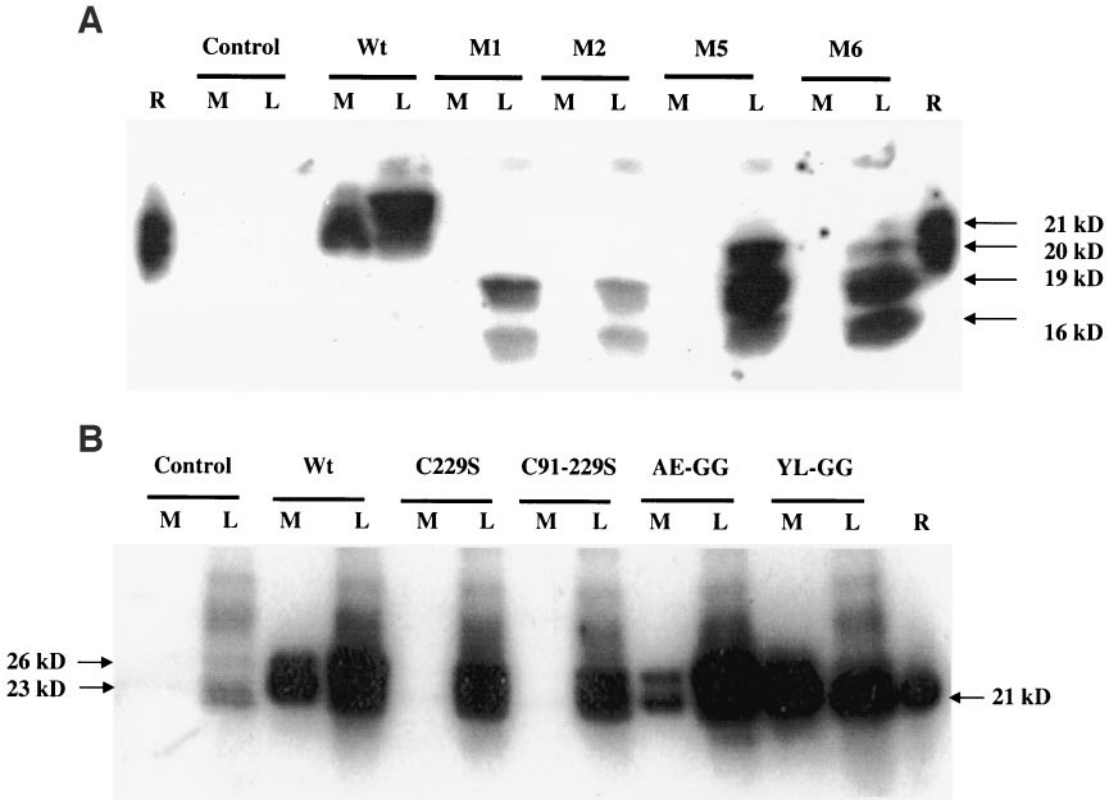

FIG. 6. Structure and conformation is vital for PANDER secretion in β-TC3 cells. The cells were transfected with mutant or wild-type PANDERs and secretion was assessed at 3 mmol/l glucose using the 24-h incubation protocol. *A*: Representative Western blot of PANDER secretion of truncated-PANDERs. *B*: Representative Western blot gel of PANDER secretion of single-mutant PANDERs. AE-GG, A29E30-G29G30 PANDER; C229S, C229S PANDER; C91–229S, C91–229S PANDER; Control, pShuttle vehicle; L, cell lysate; M, medium; M1, M1-PANDER; M2, M2-PANDER; M5, M5-PANDER; M6, M6-PANDER; R, recombinant PANDER; Wt, wild-type PANDER; and YL-GG, Y26L27-G26G27 PANDER. Results from at least three independent experiments are shown.

insulin are likely cosecreted via similar mechanisms in pancreatic β-cells.

DISCUSSION

PANDER is cosecreted with insulin. Recently, it has been reported that glucose-induced cytokine production and release from pancreatic islet β-cells are involved in pancreatic β-cell dysfunction and death in hyperglycemia (7,8). In that context, this study addresses the following questions. How is PANDER secreted? Is PANDER secreted in response to glucose? In our previous studies, we demonstrated that PANDER is colocalized to the insulin-containing granules as demonstrated by immunoelectron microscopy and confocal microscopy (14). In the present study, we demonstrated that PANDER is secreted by β-cells of primary islets and by β-cell lines. In this study, all conditions that stimulate insulin secretion also stimulate PANDER secretion, while all conditions that inhibit or fail to stimulate insulin secretion also inhibit or fail to stimulate PANDER secretion. The secretion of insulin and PANDER is stimulated by glucose after 4 or 24 h. The secretion of insulin and PANDER depends on Ca^{2+} influx through L-type Ca^{2+} channel and can be induced by membrane depolarization with high potassium. Comparable results were obtained using murine-derived β-TC3, rat-derived INS-1 cell lines, and primary mouse islets. These observations are consistent with PANDER being secreted from insulin-containing granules with a similar regulatory control as that for insulin exocytosis from pancreatic β-cells. The ratio of insulin to PANDER molecules in each secretory granule is ~180–300 on average in β-TC3 cells, after PANDER overexpression. To our knowledge, this is the first demonstration of a cytokine cosecreted with insulin from the same secretory granules. Since PANDER is apoptotic to β-cells, at least in vitro, the increased PANDER secretion induced by glucose stimulation may cause apoptosis of the nearby β-cells and thus play a role in glucose-induced β-cell toxicity (19–21). Since PANDER protein is also present in α-cells of pancreatic islets (10), we tested whether glucose stimulated PANDER secretion in α-TC3 cells. However, glucose failed to affect PANDER secretion in α-TC3 cells. Thus, glucose-dependent PANDER secretion is β-cell specific.

Cytokines including IL-β, TNF-α, IFN-γ, IL-6, IL-13, and IL-18 are released at a very low level (~1–100 pg/ml in circulation or medium) in vitro and in vivo, which can only be effectively detected by highly sensitive radioimmunoassay or enzyme-linked immunosorbent assay (sensitivity 0.1–10 pg/ml) (22–25). With our current PANDER antibody, the sensitivity of Western blot analysis in this study is ~1–2 ng/ml. Hence, our currently available methodology is not sensitive enough to detect endogenously secreted PANDER if it is released at levels similar to those of other circulating cytokines. In addition, our previous study (10) indicated that β-cell lines had a lower PANDER expression than primary islets. All these limitations make overexpression necessary and the only option to obtain insight into the mechanisms of PANDER secretion. In addition, in studies of APP secretion from primary neurons (26–30) more than a decade ago, a similar overexpressing strategy

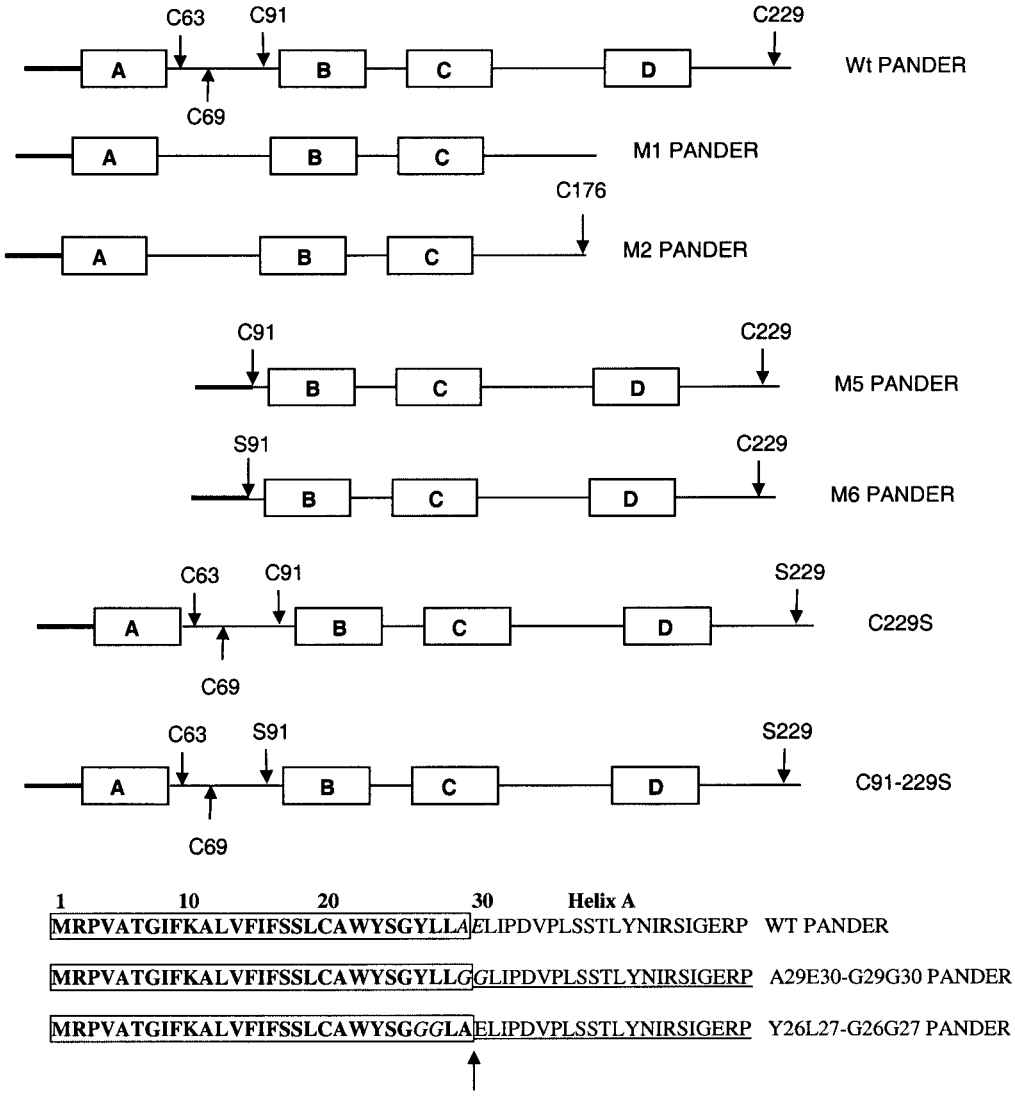

FIG. 7. Mutant PANDERs. The signal peptide of wild-type PANDER is shown in bold, while the four helices are indicated by the letters A, B, C, and D. C63, C69, C91, and C229 are the four cysteines in wild-type PANDER. The arrow indicates the cleavage site between the signal peptide and helix A. M, mutant, Wt, wild type.

was successfully used to demonstrate the mechanisms of production of amyloid β-peptide (Aβ) from the amyloid-β precursor protein (APP) and accumulation of Aβ as amyloid in neural cell lines.

Relationship between insulin and PANDER. Amylin (islet amyloid polypeptide), a 37–amino acid peptide, is another protein also cosecreted with insulin from β-cells (31). Amylin inhibits glucagon secretion, delays gastric emptying, and inhibits food intake (32). Co-administration of insulin and a human amylin agonist, pramlintide, which will not deposit as amyloid, has been reported in human clinical therapy to have a better therapeutic effect on decreasing serum HbA_{1C}, inhibiting glucagon secretion, and controlling blood glucose than administration of insulin alone (33–35). Other reports indicate that deposition of amyloid from amylin in islets is involved in type 2 diabetes (36), and transgenic mice or rats expressing human amylin develop diabetes (37,38). In addition, human amylin added in an aqueous solution induces apoptosis of β-cells of mouse and human islets (39,40). Amylin is thus multifunctional. Another multifunctional protein is uncoupling protein-2, which on one hand prevents islet cells from being damaged by superoxide during lipotoxic insult or other stressed conditions and on the other hand impairs ATP synthesis and insulin secretion of islet β-cells (41,42). Therefore, although in vitro PANDER overexpression or treatment with recombinant PANDER protein induces apoptosis of pancreatic α- and β-cells, the pancreas may not be its only target tissue. We speculate that PANDER, secreted in response to glucose or other fuel secretagogues, may have metabolic effects on other target tissues. The availability of transgenic and knockout PANDER mouse models will help to address this fundamental question.

Correlation between structure and secretion of PANDER. In a previous study of the PANDER structure-function relationship, we observed that secreted PANDER analogues were barely detectable in the medium of cells overexpressing truncated or mutated PANDERs even though PANDER proteins were produced within the cells (43). We had suggested three potential explanations: *1*) structure and conformation is important for PANDER secretion, thus the levels of secreted mutant PANDERs are very low; *2*) our polyclonal antibody recognizes and binds

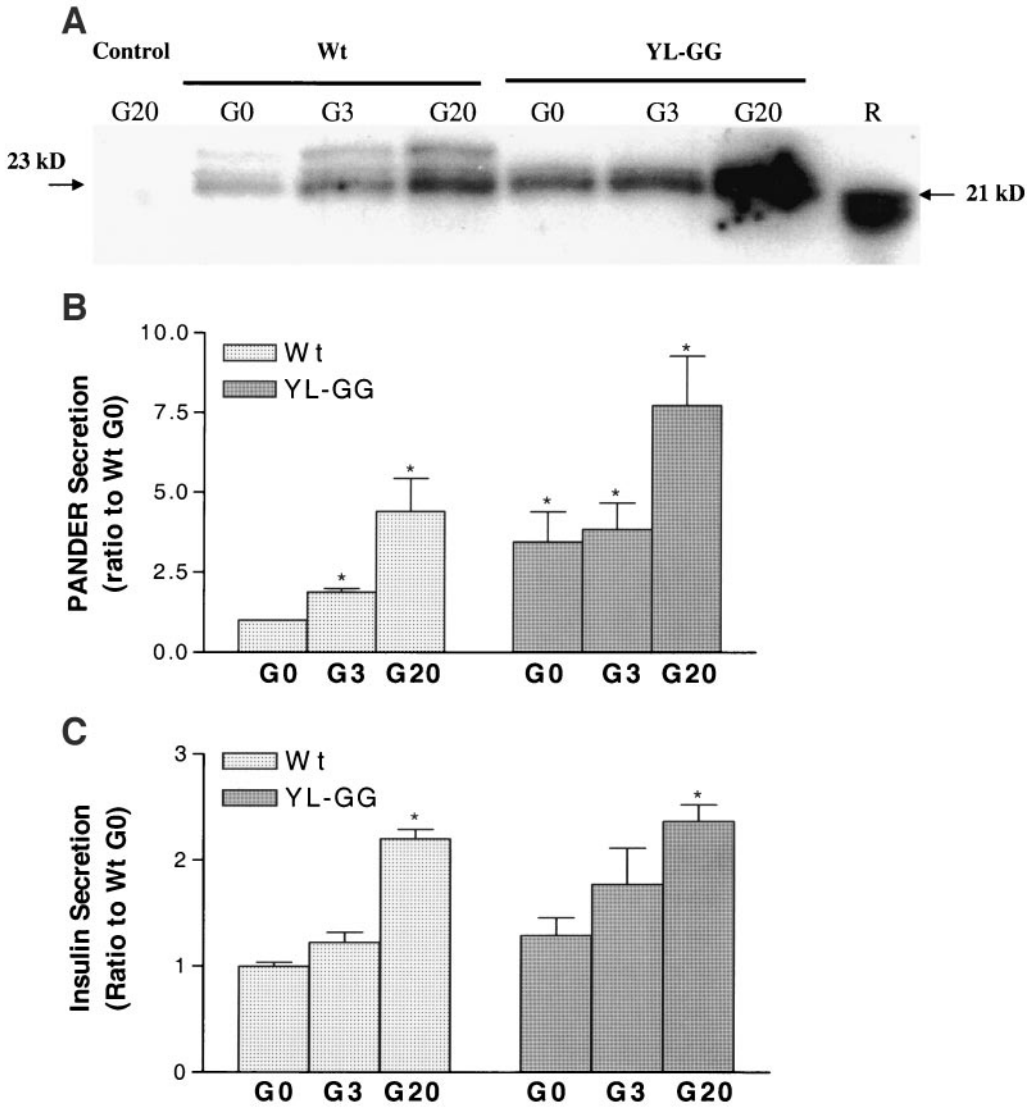

FIG. 8. Glucose-stimulated PANDER secretion in INS-1 cells. The cells were stimulated in 2 ml KRBB containing no glucose (G0), 3 mmol/l glucose (G3), and 20 mmol/l glucose (G20) for 4 h. Western blot was performed as described in RESEARCH DESIGN AND METHODS. *A*: Representative Western blot of secreted PANDER. *B*: Quantification of secreted PANDER with data normalized to the wild-type G0 value. *C*: Insulin secretion (from panel *A*) normalized to the wild-type G0 value, which was 79 ± 5 ng insulin/well. R, recombinant PANDER; Wt, wild-type PANDER; YL-GG, YL-GG PANDER. The data were collected from at least four independent experiments. *$P < 0.05$ vs. control wild-type G0.

to the truncated PANDERs at lower affinities; and 3) a large fragment truncation decreases the stability of the secreted PANDER analogue. In the present study, we further tested the hypothesis that structure and conformation are important for PANDER secretion and provide novel data on PANDER processing in pancreatic β-cells. PANDER mutants M1, M2, M5, and M6 and C229S PANDERs were constructed previously (43), while the remainder were constructed in this study. The details of all PANDER mutants are shown in Fig. 7.

In our previous work, we demonstrated that C91 and C229 form one disulfide bond, which is functionally important for PANDER's cytotoxicity (43). We eliminated this disulfide bond by mutating C229 alone or both C91 and C229 to serine. This strategy is likely to change the structure and conformation of PANDER only, without damaging its antigen epitopes, so that the polyclonal antibody will recognize and bind to the proteins with a similar affinity as wild-type PANDER. We failed to detect any secreted PANDER fragment in the conditioned medium or in the concentrated conditioned medium of cells expressing M1, M2, M5, and M6 PANDERs (Fig. 7), although all of them were highly expressed within the cells. We also failed to detect any secreted fragment in the medium of cells expressing C229S and C91–229S PANDERs, though they were similarly expressed within the cells as wild type. The results also indicated that the polyclonal antibody recognized and bound to these two mutants with the similar affinity as wild-type PANDER. Similar results were obtained in both mouse-derived β-TC3 cells and in rat-derived INS-1 cells, even with prolonged stimulation for 48 h. These observations strongly indicate that the absence of secreted PANDER protein is not due to the lower affinity for antibody. It is likely that these mutant PANDERs are released at too low a level to be detected by Western blot assay or cannot be released at all. The fact that M1, M2, and M6 maintain most of wild-type PANDER's cytotoxicity on β-cells and that M5 is as fully active as wild-type PANDER indicates that they have preserved the key structure and stability of wild-type

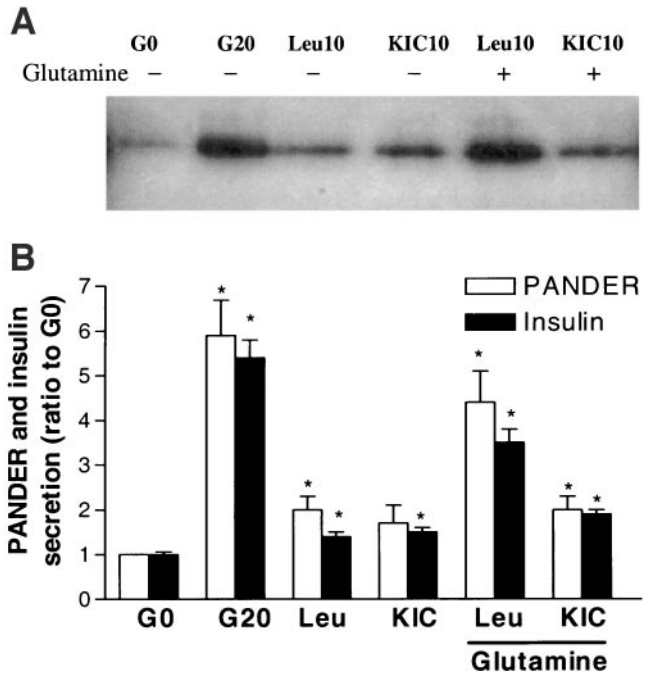

FIG. 9. Effects of leucine and KIC on PANDER secretion in INS-1 cells. The cells were stimulated in 2 ml KRBB with no glucose (G0), 20 mmol/l glucose (G20), 10 mmol/l leucine (Leu10), 10 mmol/l α-ketoisocaproic acid (KIC10), Leu10 + 4 mmol/l glutamine, and KIC10 + 4 mmol/l glutamine for 4 h. Western blot was performed as described in RESEARCH DESIGN AND METHODS. *A*: Representative Western blot of secreted PANDER. *B*: Quantification of secreted PANDER and insulin secretion (from *A*) with data normalized to the G0 value, which was 41 ± 3 ng insulin/well. The data were collected from at least four independent experiments. **P* < 0.05 vs. control G0.

PANDER, which is supported by the observations that all of these mutants are significantly accumulated within the cells (43).

Mutation at the predicted cleavage site (A29-E30) failed to interrupt the cleavage of the signal peptide and PANDER secretion, revealing the existence of multiple cleavage sites of the signal peptide in pancreatic β-cells. This is supported by observations that the secreted wild-type PANDER consisted of various fragments. Interestingly, mutation of Phe26 and Leu27 to glycine increased PANDER secretion by approximately two- to fivefold in both β-TC3 and INS-1 cell lines under the same conditions. This observation is similar to Saunders's observations in studying the secretion of a 34–amino acid fragment of human parathyroid hormone using a *Bacillus amyloliquefaciens* neutral protease signal (44). They found that the secretion efficiency of this hybrid depended on the amino acid sequence near the signal sequence cleavage site, and replacement of alanine with glycine at the COOH-terminus of the signal peptide increased the secretion (44). Our further experiments in INS-1 cells indicated that enhanced secretion in YL-GG PANDER is probably achieved through sorting more PANDER molecules into each insulin secretory granule. Y26L27-G26G27 PANDER provides a new model for studying the biological functions of PANDER in the future. In summary, *1*) PANDER with correct structure is recognized and sorted by some unknown mechanism(s) into insulin secretory granules; *2*) the signal peptide of the sorted PANDER will be cleaved within the insulin granules during secretion, resulting in various secreted analogues (~23–26 kDa) due to incomplete digestion of the signal peptide and the existence of multiple cleavage sites; and *3*) PANDER with an incorrect structure and conformation will not be sorted into the insulin secretory granule, but its signal peptide can be cleaved within the cells. It should also be noted that the amount of PANDER released from β-TC3 cells after overexpression is an order of magnitude lower than the level of PANDER that effectively kills cells, as documented previously (10) .

In conclusion, our study using an overexpressing strategy demonstrates for the first time that *1*) PANDER is secreted from both pancreatic β- and α-cells; *2*) glucose stimulates PANDER secretion dose dependently only in β-cell lines and in primary islets, not in α-cells; *3*) PANDER is likely cosecreted with insulin via the same regulatory mechanisms; and *4*) structure and conformation is vital for PANDER secretion.

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