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Pituitary Adenylate Cyclase-Activating Polypeptide Prevents Cytokine-Induced Cytotoxicity via Inhibition of Inducible Nitric Oxide Synthase Expression in βTC Cells

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Type 1 diabetes mellitus is an autoimmune disease resulting from apoptotic destruction of pancreatic β-cells. The activation of inducible nitric oxide synthase (iNOS) by inflammatory cytokines is considered a mediator of destruction in β-cells. Recent findings showed that the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), whose distribution was identified in pancreatic neurons, inhibited nitric oxide (NO) production in cytokine-activated macrophages. In the present study, we investigated the cytoprotective effect of PACAP in the cytokine-exposed mice β-cell line, βTC cells. 1×10^{-8} M PACAP inhibited the reduction of cell viability, NO production, expression of iNOS mRNA, and iNOS promoter activity caused by the combination of three proinflammatory cytokines. Selective iNOS inhibitor also showed the cytoprotective effect in βTC cells. These data suggested that PACAP has a cytoprotective effect in cytokine-treated β-cells through inhibition of iNOS transcription. © 2000 Academic Press

Key Words: **PACAP; type 1 DM; cytokine; β cell; βTC cell; iNOS; NO.**

Most type 1 diabetes mellitus is an autoimmune disease resulting from destruction of pancreatic β-cells (1, 2). Apoptosis of these cells is induced by either specific T lymphocyte or proinflammatory cytokine-mediated mechanism (3). Proinflammatory cytokines produced by activated macrophages and monocytes promote iNOS expression in β-cells (3–7). iNOS produces high levels of NO and superoxide, resulting in the destruction of β-cell function and apoptosis (5). Recently, in β-cell line overexpression of manganese superoxide dismutase, an antioxidant enzyme, prevented interleukine 1β (IL-1β)-induced cytotoxicity via a blockade in IL-1β activation of iNOS and NO production (8). Moreover, NO is shown to be the main mediator of β-cell damage in the nonobese diabetic mouse that is considered an animal model of type 1 diabetes mellitus (9).

PACAP, a neuropeptide of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family of peptides, was originally identified in hypothalamus as a stimulator of adenylate cyclase. PACAP exists in two biologically active forms, PACAP38 and PACAP27, which share the same 27 amino-terminal amino acids (10). PACAP is widely distributed in the central nervous system and peripheral tissues such as pituitary, adrenal medulla, testis, gastrointestinal tract, and pancreas (11). PACAP/VIP receptor (PVR) consists of three subtypes that all belong to the GTP-binding protein-coupled receptor family and mediate the accumulation of both c-AMP and intracellular free calcium (12). In mouse pancreas, PACAP was demonstrated to be exclusively localized to the nerves within the islets and in pancreatic ganglia (13), while PACAP receptor mRNA was expressed in islet cells (14). PACAP is shown to be a multifunctional neuropeptide that acts as a neurotrophic factor to protect neuron from apoptosis or necrosis (15–17). PACAP also has anti-inflammatory effects in immune cells, resulting in inhibition of the production of several proinflammatory cytokines (18, 19).

A recent study has demonstrated that PACAP suppresses iNOS transcription in lipopolysaccharide (LPS)/interferon gamma (IFN-γ) stimulated macrophages by inhibiting activation of nuclear factor κB (NF-κB) and IFN regulatory factor 1 (IRF-1) (20). On the other hand, PACAP has been considered to be involved in the neuronal regulation of insulin from the fact that PACAP potentiates insulin secretion (21). However, there are no studies on the anti-inflammatory effects of PACAP in β-cells.

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In the present study, we intended to investigate the cytoprotective effect of PACAP in βTC cells (22) in the presence of proinflammatory cytokines. We showed that PACAP prevented the inhibition of cell viability caused by induction of cytokines via inhibition of NO transcription.

MATERIALS AND METHODS

Materials. PACAP38 was purchased from Peptide Institute, Inc. (Osaka, Japan). Human recombinant IL-1β (1×10^7 U/mg) and murine recombinant IFN-γ (1×10^7 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Murine recombinant TNF-α (2.55×10^6 U/mg) was a generous gift from Dainippon Pharmaceutical Co., Ltd., (Osaka, Japan). N^G-monomethyl-L-arginine (NMMA) was purchased from Sigma.

Cell culture. βTC cells were cultured in Dulbecco's modified Eagle's medium (Dainippon) supplemented with 10% fetal calf serum (Dainippon) and antibiotics (50 μU/ml penicillin and 50 μg/ml streptomycin) at 37°C in 5% CO_2 and 95% air. Culture medium was exchanged every 2–3 days, and cells were passaged at 7-day intervals.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the βTC cells using TRIzol reagent (GIBCO BRL, Gaithersburg, MD), and 2 μg of total RNA was reverse-transcribed to 1st strand cDNA with oligo-dT primer and reverse transcriptase (SuperScript Preamplification System; GIBCO BRL). The 1st strand cDNA was used for PCR. Each subtype of the PVR cDNA was amplified as previously described (12) with rTaq DNA polymerase (Takara Shuzo Co., Ltd., Tokyo, Japan). Mouse iNOS and GAPDH cDNA were amplified by PCR with specific primers. Primers for mouse iNOS cDNA were as follows: sense 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, and antisense 5′-GGC-TGTCAGAG CCTCGTGGCTTTGG-3′ that corresponds to exon 24 and 27 for iNOS genomic DNA, respectively (23, 24). Primers for mouse GAPDH cDNA were as follows: sense 5′-ACCACAGTC-CATGCCATCAC-3′, and antisense 5′-TCCACCACCCTGTTGCT-GTA-3′. PCR was performed for iNOS as follows: 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C for 20 cycles. For GAPDH, 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 20 cycles. Each PCR product was analyzed with 1% agarose gel electrophoresis and analyzed by NIH image software as previously described (25).

Cell viability. βTC cells were plated at a density of 1×10^4/well in 96 well plates and cultured in the DMEM medium 1 day before the assay. The culture medium was then replaced by DMEM medium in the presence or absence of 1×10^{-8} M PACAP and cytokines (100 U/ml, IL-1β, 1000 U/ml IFN-γ and 1000 U/ml TNF-α, the same combination used in other experiments to induce iNOS expression). The cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit; Promega, Madison, WI) according to the manufacturer's instructions.

Measurement of NO production. βTC cells were plated at a density of 2×10^5/well in 96-well plates and cultured in the DMEM medium 1 day before the experiment. Cells were incubated in the presence or absence of 1×10^{-8} M PACAP and cytokines. After each incubation period, supernatant was collected and NO_2 formation was measured using NO_2/NO_3 fluorometric Assay kit (Chemical Dojindo Inc., Kumamoto, Japan).

Construction of iNOS promoter/luciferase fusion gene. The iNOS promoter region (−1588 to +161 bp) (26) was amplified by PCR from mouse genomic DNA using primers that engineered *Kpn*I and *Mlu*I cloning sites, respectively (sense-5′-GA*GGTACC*TATGCTGAAATC-CATAAGC; *Kpn*I, antisense-5′-GG*ACGCGT*CGTGGAGTGAACAA-GAC; *Mlu*I). The PCR fragments were subcloned into multiple cloning sites upstream of the luciferase reporter gene in expression vector pGL2-Basic (Promega, Madison, WI).

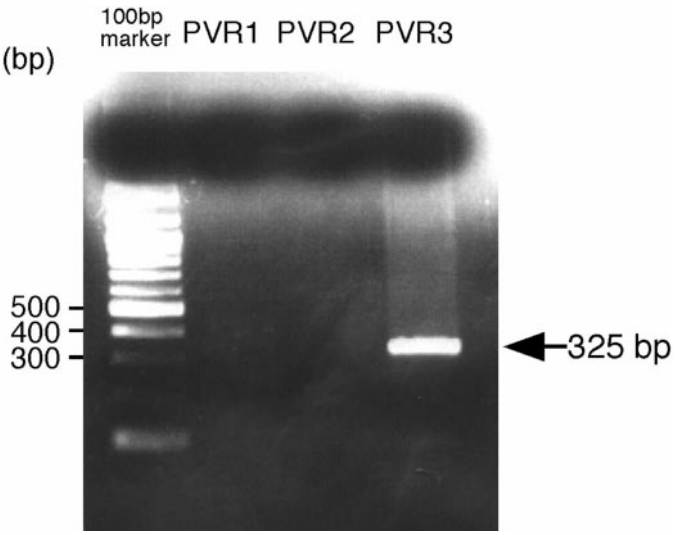

FIG. 1. Expression of the PACAP receptor (PVR) subtypes analyzed by RT-PCR in βTC cells. Photographs of the ethidium bromide-stained products using agarose gel electrophoresis. cDNA produced from a reverse transcription using total RNA from βTC cells was amplified by PCR with oligonucleotide primer pairs specific for PVR1, PVR2, or PVR3. Only a DNA fragment of PVR3 with the predicted length (325 bp) was amplified.

Transient transfection and luciferase assay. βTC Cells were seeded 1 day before transfection at 1×10^6 cells/well in 6-well plates. pGL2-Basic containing iNOS promoter region and expression vector containing β-galactosidase were transfected into βTC cells by lipofection with TransIT LT-2 (Mirus, WI) according to the manufacturer's instructions. After the exposure to the DNA-lipid complex for 4 h, the cells were washed and replaced with culture medium. After 48 h, the cells were washed and incubated in the medium in the presence or absence of 1×10^{-8} M PACAP and cytokines. After each incubation period, cells were harvested and subjected to luciferase assay as previously described (27). β-galactosidase activity was determined as an internal control for each transfection using Galacto-Light plus kit (Tropix, Bedford, MA).

Statistical analysis. Values of cell viability, DNA amount of PCR fragment, NO_2 concentration and luciferase activity were analyzed by ANOVA followed by Fisher's PLSD test for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

RT-PCR Analysis of PACAP Receptor Subtypes in βTC Cells

To identify the subtypes of PVR expressed in βTC cells, RT-PCR analysis was performed using the sets of primers specific for three receptor subtypes. Primers specific for PVR3 produced a band of the predicated size (325 bp), however, no PCR products corresponding to PVR1 or PVR2 were detected (Fig. 1). RT-PCR product for PVR3 was identified by cycle sequencing (data not shown). This finding suggested that PVR3 mRNA was predominantly expressed in βTC cells.

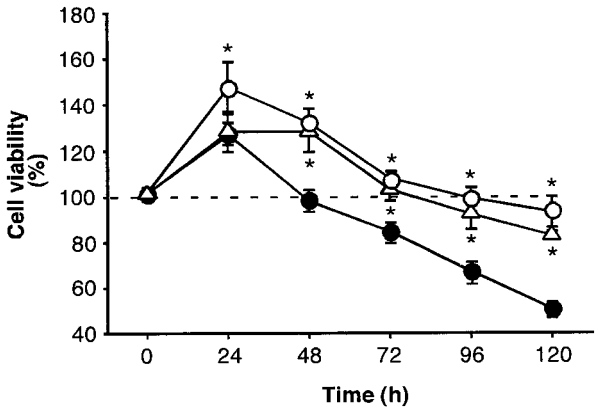

FIG. 2. Effects of PACAP and iNOS inhibitor (NMMA) on cell viability in cytokine-treated βTC cells. βTC cells were incubated with a combination of cytokines (IL-1β 100 U/ml, IFN-γ 1000 U/ml, TNF-α 1000 U/ml) in the absence (closed circle), presence (open circle) of 1×10^{-8} M PACAP or presence of 1×10^{-5} M NMMA (open triangle). Cell viability was determined by MTS assay. The cell viability at the starting point (0 h) was expressed as the basal control (100%), and the cell viability at each incubation period was presented as the percentage of the basal control. Results were expressed as the mean ± standard deviation (S.D.) ($n = 4$). *: $P < 0.05$ vs βTC cells exposed to cytokines alone.

Effects of PACAP and iNOS Inhibitor (NMMA) on Cell Viability in Cytokine-Treated βTC Cells

The effects of cytokines and PACAP on βTC cell viability were determined by MTS assay. The cells were incubated with cytokines for 120 h in the presence or absence of 1×10^{-8} M PACAP. The cell viability in cytokine (IL-1β, TNF-α, IFN-γ)-treated βTC cells was significantly decreased for 48 h, and was reduced to 51% of the basal level for 120 h (Fig. 2). As previously reported (28), we identified DNA fragmentation in βTC cells exposed to cytokines for 48 h (data not shown). PACAP prevented the inhibition of cell viability induced by cytokines after 24 h of incubation (Fig. 2). The cell viability in cytokine- and PACAP-treated βTC cells for 120 h was 92% of the basal level. Moreover, treatment of 1×10^{-5} M NMMA, a specific inhibitor of NOS, prevented the inhibition of cell viability induced by cytokines after 48 h of incubation (Fig. 2). These data, therefore, suggested that PACAP prevented cytokine-induced apoptosis via inhibition of NO production in βTC cells. Though PACAP and NMMA exhibited similar level of cyto-protective effects, PACAP showed the effect 24 h earlier. It might be possible that PACAP have another potency to increase cell viability besides prevention of NO production.

Effect of PACAP on Cytokine-Induced NO_2 Production in βTC Cells

Previous data showed that proinflammatory cytokines (IL-1β, TNF-α and IFN-γ) induced NO production in pancreatic islets (29, 30), and that PACAP blocked NO production induced by cytokines in macrophages (20). In order to examine if PACAP suppressed NO production induced by cytokines in βTC cells, we measured NO_2 accumulation that corresponds with NO production. Cytokines increased NO production for 12 h by 3- to 4-fold the basal level (Fig. 3). PACAP significantly inhibited NO production induced by cytokines by 50% for 12 h and by 40% for 24 h (Fig. 3).

Analysis of iNOS mRNA Expression by Semi-Quantitative RT-PCR in βTC Cells

It is reported that cytokines induce iNOS mRNA expression in β-cell lines (31, 32). To assess the effect of PACAP on the iNOS expression induced by cytokines, we performed RT-PCR of iNOS mRNA. βTC cells were incubated with cytokines for 6 or 24 h in the presence or absence of PACAP, and total RNA was extracted. They were reverse-transcribed and analyzed by semi-quantitative PCR for iNOS cDNA with GAPDH cDNA as an internal control. iNOS mRNA was detected only when βTC cells were exposed to cytokines for 6 and 24 h (Fig. 4A). The treatment of 1×10^{-8} M PACAP significantly reduced iNOS mRNA expression induced by cytokines for 6 and 24 h (Fig. 4B). PACAP inhibited iNOS mRNA expression by 39% for 6 h and by 31% for 24 h, compared to iNOS mRNA expression in cytokine-treated βTC cells.

Effect of PACAP on iNOS Promoter Activity

To determine the effect of PACAP on iNOS transcription, a promoter assay was executed in the βTC cells transiently transfected with a construct containing

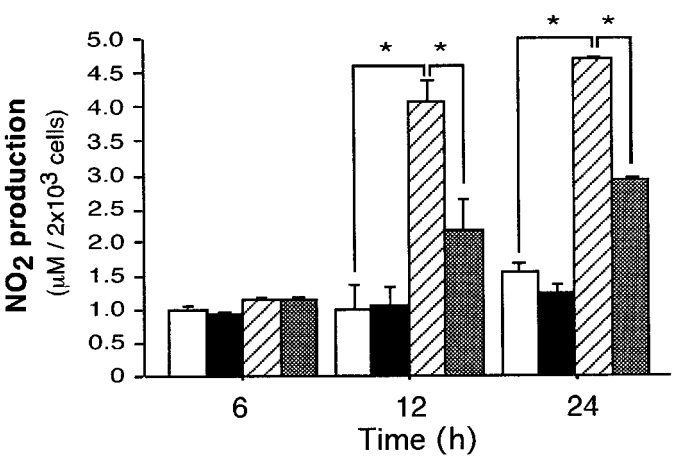

FIG. 3. PACAP inhibits NO in βTC cells exposed to cytokines. Groups of 2×10^5 cells were cultured under the following experimental conditions; control (empty bars), 1×10^{-8} M PACAP (filled bars), cytokines (hatched bars), cytokines and PACAP (crossed bars). Results are expressed as the mean ± S.D. ($n = 6$). *: $P < 0.05$ vs βTC cells exposed to cytokines alone.

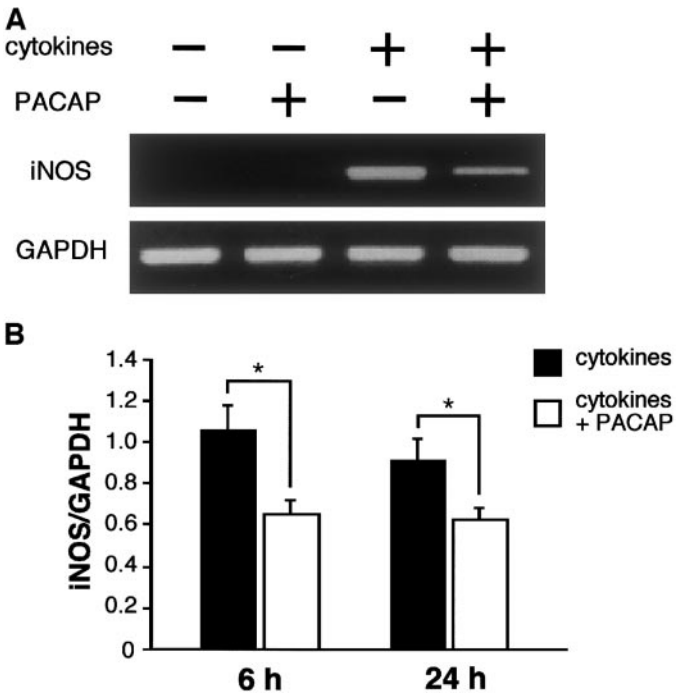

FIG. 4. PACAP inhibits inducible NO synthase (iNOS) mRNA expression in cytokine-exposed βTC cells. βTC cells were harvested after the exposure to cytokines for 6 and 24 h in the presence or absence of 1×10^{-8} M PACAP, and cDNA was amplified using specific iNOS and GAPDH primers. An example of representative result at 6 h is shown in (A). For semiquantification, smaller cycle PCR was performed and GAPDH was used as an internal control. The ratio of iNOS/GAPDH for each experimental group was compared each other. The results of 6 and 24 h incubation are shown in (B). Results are expressed as the mean ± S.D. ($n = 5$). *: $P < 0.05$ vs βTC cells exposed to cytokines.

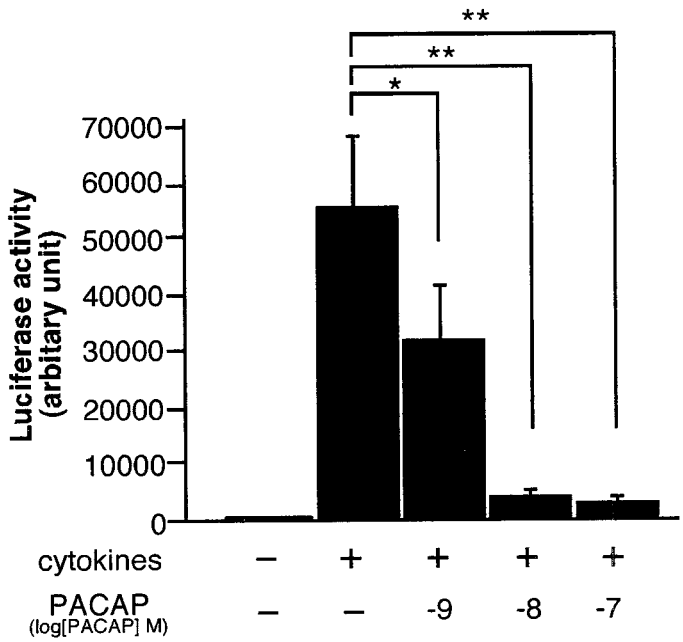

FIG. 5. The dose response effects of PACAP on iNOS promoter activation by cytokines. Cells were exposed to cytokines with PACAP (1 to 100 nM) for 6 h. Results are expressed as the mean ± S.D. ($n = 4$). *: $P < 0.05$, **: $P < 0.01$, vs βTC cells exposed to cytokines.

iNOS promoter region upstream of the luciferase reporter gene. We examined the dose-dependent effect of PACAP for 6 h. PACAP suppressed luciferase activity induced by cytokines in a dose-dependent manner (1×10^{-9} M–10^{-7} M) (Fig. 5). The maximum effect of PACAP was observed in 1×10^{-8} M. We also examined the time course for the iNOS promoter activity in the presence or absence of 1×10^{-8} M PACAP and cytokines (Fig. 6). Cytokines increased luciferase activity at a maximum level for 6 h. However, it decreased to the normal level after 12 h incubation. PACAP significantly inhibited the rise for 6 h (Fig. 6). These results suggested that PACAP blocked cytokine-induced NO production via inhibition of iNOS transcription in βTC cells.

DISCUSSION

In the present study using βTC cells, a mouse pancreatic β tumor cell line, we have demonstrated PACAP significantly blocked cell apoptosis caused by proinflammatory cytokines. IL-1β is considered one of the most important mediators in the process of autoimmune dysfunction in β-cell. However, in βTC cell lines, it is reported that IL-1β was not sufficient to induce apoptosis, and a combination of three cytokines (IL-1β, TNF-α, IFN-γ) showed a more potent cytotoxic effect than by each cytokine alone in βTC cell (28, 33).

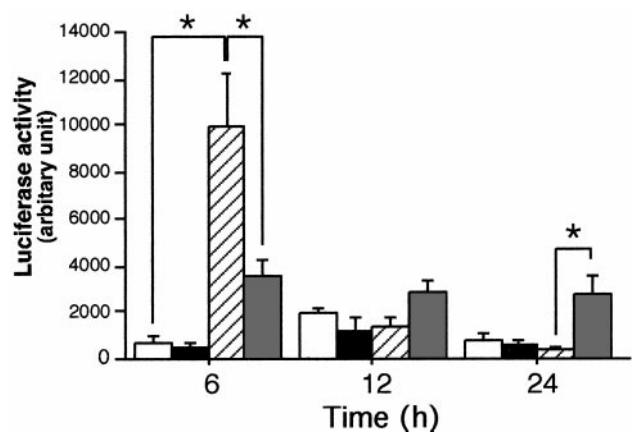

FIG. 6. Time course for the inhibitory effect of PACAP on cytokine-induced iNOS promoter activity. βTC cells (1×10^6 cells) were cultured under the following experimental conditions; control (empty bars), 1×10^{-8} M PACAP (filled bars), cytokines (hatched bars), cytokines and 1×10^{-8} M PACAP (crossed bars). After the each incubation period, cells were harvested, and iNOS promoter activities were determined by luciferase assay. Results are expressed as the mean ± S.D. ($n = 4$). *: $P < 0.05$ vs the value for βTC cells exposed to cytokines.

Therefore, we applied the same combination in this study. NMMA, one of the specific NOS inhibitors, also showed a cytoprotective effect, which suggested that NO produced by cytokines was associated with the damage on βTC cells. We have shown that PACAP reduced NO production through the inhibition of iNOS transcription. These data suggested that the cytoprotective effect of PACAP was mediated, at least partly, by the inhibition of iNOS transcription.

Cytokines increased iNOS promoter activity to the maximum level at 6 h, but the effect terminated at 12 h of incubation (Fig. 6). PACAP significantly suppressed iNOS promoter activity induced by cytokines at 6 h, however, PACAP increased iNOS promoter activity in combination with cytokines after 24 h incubation. This paradoxical result might suggest that PACAP promoted apoptosis at least partly with some factors induced by cytokines. However, PACAP suppressed NO production after 12 h (Fig. 3), suggesting that PACAP inhibits the iNOS promoter activity as a whole.

It has been hypothesized that both β-cell antigen specific and nonspecific immune/inflammatory responses participate in mediating islet β-cell destruction in type 1 diabetic mellitus (34). The antigen-specific immune response results from direct binding of $CD8^+$ cytotoxic T cells to β-cells. On the other hand, the nonspecific inflammatory response consists of β-cell destruction by proinflammatory cytokines and free radicals released from T cell and macrophage. In the latter mechanism, NO is reported to be an important mediator of the proinflammatory cytokines (5, 6). Proinflammatory cytokines from macrophages and T cells induce iNOS transcription through NF-κB and IRF-I activation followed by NO production in β-cells (26), which results in β-cell dysfunction and apoptotic cell death (4, 28).

Although PACAP and its receptor have been identified in pancreatic islets, their biological function is still obscure. From the result of these studies that PACAP promotes insulin secretion from islet cells *in vitro*, PACAP was considered as a neurological regulator of insulin secretion in pancreas (21, 35). In the present study, we showed that PVR3 mRNA was expressed in βTC cells. This result corresponds to the previous report demonstrating PVR3 is expressed in pancreatic islets and insulin-secreting cell lines including MIN6, HIT-T15, and RINm5F (36). We also demonstrated that the inhibitory effects of PACAP in iNOS transcription in βTC cell exposed to proinflammatory cytokines. Recently, PACAP and VIP, which shares PVR3, are reported to have anti-inflammatory effects in various tissues. For example, PACAP is up-regulated after peripheral nerve injury in the dorsal root ganglia neurons (37), and VIP is released from lymphatic tissues in response to endotoxin shock and suppresses cytokine release from macrophages (38). These data suggest the possibility that PACAP may be involved in endogenous agents that have anti-inflammatory effects in pancreatic islet cells.

Based on the finding that NO is associated with autoimmune β-cell apoptosis in animal models, iNOS inhibition is directed to one of the therapeutic approaches to type 1 diabetes. Several pharmacological inhibitors of iNOS have been developed to show potent anti-inflammatory effects (39–42). Therefore, it would be possible that PACAP could be used as an iNOS inhibitor in islet cells. Additionally, considering the various biological effects of PACAP that inhibits cytokine release from macrophage in endotoxin shock mice (43) and that promotes insulin release from islet cell, PACAP itself or its receptor agonists would possibly be beneficial therapeutic agents for autoimmune pancreatitis.

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