Overexpression of PACAP in Transgenic Mouse Pancreatic β-Cells Enhances Insulin Secretion and Ameliorates Streptozotocin-induced Diabetes

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Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the vasoactive intestinal peptide/secritin/glucagon family, stimulates insulin secretion from islets in a glucose-dependent manner at femtomolar concentrations. To assess PACAP’s pancreatic function in vivo, we generated transgenic mice overexpressing PACAP in the pancreas under the control of human insulin promoter. Northern blot and immunohistochemical analyses showed that PACAP is overexpressed in pancreatic islets, specifically in transgenic mice. Plasma glucose and glucagon levels during a glucose tolerance test were not different between PACAP transgenic mice and nontransgenic littermates. However, plasma insulin levels in transgenic mice were higher after glucose loading. Also, increases of streptozotocin-induced plasma glucose were attenuated in PACAP transgenic mice but showed no difference between 12-week-old transgenic and nontransgenic littermates. This is the first time that PACAP has been observed to play an important role in the proliferation of β-cells. Diabetes 52:1155–1162, 2003

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Received for publication 12 December 2001 and accepted in revised form 24 January 2003.

Diabetes is a complex, multifactorial disease that results from defects in insulin secretion and/or insulin action. Although the cause of diabetes is not completely understood, the disease is characterized by hyperglycemia, which can lead to various complications, including cardiovascular disease, kidney disease, neuropathy, and retinopathy. One of the key features of diabetes is the inability of the pancreas to produce enough insulin to maintain normal blood glucose levels. However, the role of PACAP in glucose homeostasis has not been well studied.

PACAP is a neuropeptide that belongs to the glucagon family. It is widely distributed in the nervous system and the endocrine pancreas, and it has been shown to have a variety of biological activities, including the regulation of insulin secretion. Previous studies have suggested that PACAP may play a role in the maintenance of blood glucose levels, but the specific mechanisms by which PACAP affects insulin secretion are not well understood.

In this study, the authors generated transgenic mice overexpressing PACAP in the pancreas under the control of the human insulin promoter. They then assessed the effects of PACAP overexpression on glucose homeostasis in vivo. The results of the study showed that PACAP overexpression led to an increase in plasma glucose and glucagon levels during a glucose tolerance test. However, plasma insulin levels were higher after glucose loading in PACAP transgenic mice compared to nontransgenic littermates. These results suggest that PACAP may have a role in the regulation of insulin secretion.

In summary, this study provides new insights into the role of PACAP in glucose homeostasis. Further research is needed to understand the specific mechanisms by which PACAP affects insulin secretion and to determine the clinical relevance of these findings.
tion, differentiation, and protection on various cell types in physiological and pathophysiological states (1,3). PACAP—but not VIP—stimulates several different signaling cascades, leading to the activation of adenylate cyclase, phospholipase C, extracellular regulated kinase, and p38 mitogen-activated protein (MAP) kinases, and mobilization of calcium (3,13–15). In the pancreatic carcinoma cell line AR4-2J, PACAP—but not VIP—potently stimulates cell proliferation and the MAP kinase (16). However, the relevance of these pharmacological PACAP responses to the actual physiological activities of endogenous PACAP has not been fully addressed, because suitable low-molecular-weight PACAP antagonists have not yet been developed (3). Previously, we cloned the PAC1 receptor cDNA (5,17) and the genes for the PAC1 receptor (18), VPAC1 receptor (19), and the PACAP ligand (20) and analyzed their localization in the nervous system (21–23). Recently, we generated PAC1 receptor exon 2–deficient mice (24). In addition, Jamen et al. (25) reported the generation of PAC1 receptor knockout mice, which are normoglycemic but show a slight feeding hyperinsulinemia and impaired insulinotropic response to glucose. More recently, we generated PACAP-deficient mice, which exhibit high early mortality, marked changes in psychiatric and neurological phenotypes (26), and a slight hypoinsulinemia in response to a glucose load, despite similar basal glucose levels and glucose tolerance compared with control mice (S.T., A.B., unpublished observations). In contrast, Gray et al. (27) reported that targeted deletion of PACAP is associated with an extremely high mortality rate and, in the fasted state, hyperinsulinemia and hypoglycemia. The reason for this variable phenotypic expression is unclear. Because PACAP and/or its receptors are widely distributed in the central nervous system and in peripheral organs (1,3,5,28), the ubiquitous gene inactivation by conventional knockout might make the interpretation of results difficult.

In the present study, we generated transgenic mice overexpressing PACAP in pancreas, specifically under the control of the β-cell–specific insulin promoter. Using these, we hoped to understand the in vivo function of PACAP in pancreatic islet β-cells and endocrine cell lineages.

RESEARCH DESIGN AND METHODS

All animal care and handling procedures were approved by the institutional animal care and use committee of Osaka University.

Construction of transgene. The mouse PACAP precursor cDNA containing all of the region of the open reading frame was isolated by RT-PCR from brain total RNA using forward primer 5′-CAGCGGGATCCAGTTTCTA-3′ and reverse primer 5′-CTTGAGTTAGTTAGGCTGGG-3′ (20). The PCR product was subcloned into pBluescript II KS+ using the EcoRV site and sequenced. The 0.7-kb HindIII-DraI DNA fragment containing the entire coding sequence of the precursor polypeptide was blunted and exchanged to the 0.5-kb EcoRI DNA fragment of the fusion gene, which consisted of the 1.9-kb fragment of the human insulin promoter and the 1.7-kb of the rabbit β-globin gene, as previously reported (29,30) (Fig. 1A).

Generation of transgenic mice. The human insulin promoter/mouse PACAP precursor chimeric gene (Fig. 1A) was microinjected into fertilized eggs of C57BL/6 × DBA/2 F2 mice by standard procedures. Transgenic mice were identified for the presence of the transgene by Southern blot analysis of tail DNA using a 32P-labeled mouse PACAP precursor cDNA probe and were backcrossed to C57BL/6. Serum and pancreatic content of the biologically active mature PACAP isform PACAP38 was analyzed by a radioimmunoassay kit (Peninsula Labs, Belmont, CA). All experiments were conducted with adult (8–12 weeks old) mice, unless otherwise specified.

Northern blot analysis. Total RNA was isolated from lung, brain, pancreas, heart, and liver by the guanidine thiocyanate method (31). Ten micrograms of RNA was separated by electrophoresis through 1.5% agarose-formaldehyde gels and transferred to nitrocellulose membranes. The membranes were hybridized with a 32P-labeled mouse PACAP cDNA probe as a probe. The arrows indicate mRNA from the endogenous mouse PACAP (top) and the PACAP transgene (bottom); lanes 1–5, nontransgenic mice; lanes 6–10, transgenic mice; lanes 1 and 6, lung; lanes 2 and 7, brain; lanes 3 and 8, pancreas; lanes 4 and 9, heart; lanes 5 and 10, liver. The integrity of RNA was assessed by ethidium bromide staining of 18S rRNA band (bottom). C: Overexpression of PACAP in pancreas of transgenic mice. Immunohistochemical analysis was carried out using rabbit anti-PACAP antibody. Fluorescein-conjugated anti-rabbit IgG was used for the second antibody. Left, nontransgenic mice; right, transgenic mice. Scale bar, 100 μm.

**FIG. 1. Generation of PACAP transgenic mice.** A: Schematic representation of the fusion gene used to generate transgenic mice. The fusion gene consists of 1.9 kb of the human insulin promoter (hatched box), fragments of the rabbit β-globin gene (open boxes), and 0.7 kb of the mouse PACAP precursor cDNA (filled box). The lines between them indicate the second intron and 3′-flanking region of the rabbit β-globin gene. Translation initiation (ATG) and termination (TGA) codons are indicated. B: Expression of PACAP precursor mRNA. Northern blot analysis of total RNA isolated from tissues from transgenic and nontransgenic mice was performed using a 32P-labeled mouse PACAP cDNA as a probe. The arrows indicate mRNA from the endogenous mouse PACAP (top) and the PACAP transgene (bottom); lanes 1–5, nontransgenic mice; lanes 6–10, transgenic mice; lanes 1 and 6, lung; lanes 2 and 7, brain; lanes 3 and 8, pancreas; lanes 4 and 9, heart; lanes 5 and 10, liver. The integrity of RNA was assessed by ethidium bromide staining of 18S rRNA band (bottom). C: Overexpression of PACAP in pancreas of transgenic mice. Immunohistochemical analysis was carried out using rabbit anti-PACAP antibody. Fluorescein-conjugated anti-rabbit IgG was used for the second antibody. Left, nontransgenic mice; right, transgenic mice. Scale bar, 100 μm.
RESULTS

Generation of transgenic mice and tissue-specific expression of transgene-encoded PACAP precursor. To investigate the role of PACAP in the pancreas, we constructed several lines of transgenic mice expressing mouse PACAP precursor under the control of the human insulin promoter (Fig. 1A). Twenty-one mice were obtained from eggs microinjected with the fusion gene. Screening of the mice by Southern blot analysis of tail DNA revealed that five animals had been transgened. Breeding of these founders with C57BL/6 established three transgenic lines. Irrespective of their genetic background, transgenic mice were indistinguishable from their nontransgenic littermates with respect to growth, fertility, and apparent behavior in the animal facility.

Insulin tolerance test. Mice were fasted for 2 h with free access to water. Each mouse then received an intraperitoneal injection of insulin (1 unit/kg body wt). Plasma glucose levels were measured as described above.

Streptozotocin treatment. For inducing diabetes, mice aged 8 weeks received injections of streptozotocin (STZ; Sigma, St. Louis, MO) for 5 consecutive days at 70 mg/kg body wt. The STZ was dissolved in 10 mmol/l citrate buffer (pH 4.5) and injected within 5 min of dissolution. The pancreas were made for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and 5-bromo-2-deoxyuridine staining. Forty-two hours after the last dose of STZ, male transgenic and nontransgenic mice received an intraperitoneal injection of 5-bromo-2-deoxyuridine (BrDU; 100 mg/kg body wt; Sigma) dissolved in saline. Six hours after injection of BrDU, the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt), and their tissues were excised, fixed with 4% paraformaldehyde at 4 °C overnight, and embedded in paraffin. Three sets of paraffin sections from each pancreas were made for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and BrDU staining, and each set was cut at intervals of 500 μm. TUNEL staining was performed on paraffin-embedded sections with an in situ apoptosis detection kit (Takara Shuzo, Kyoto, Japan) using horseradish peroxidase–conjugated anti-FITC antibody (DAKO) and DAB substrate kit (Vector Laboratories, Burlingame, CA). BrDU immunoreactivity was visualized with a mouse monoclonal anti-BrDU antibody and peroxidase-conjugated anti-mouse IgG2a using the Cell Proliferation kit (Amersham) and the SG Substrate kit (Vector Laboratories). After staining for TUNEL or BrDU, sections were blocked and incubated with guinea pig polyclonal anti-insulin antibody (1:5,000; DAKO). After 30 min of incubation at 37°C, sections were labeled with biotinylated goat anti-guinea pig IgG (Vector Laboratories) using the ABC method, and positive staining was visualized. After immunostaining, sections were counterstained with methyl green. The number of BrDU+, TUNEL-, and insulin-positive cells was determined from light microscopic images (Provis AX 80 equipped with an HDTV system and a color-chilled three charged coupled device camera; Olympus, Tokyo, Japan) using an image analysis system (Micscope version 2.55; Mitani, Fukui, Japan).

Histomorphometric analysis. Histomorphometric analysis was performed as described previously (32) with minor modifications. The pancreata from 12-week-old or 12-month-old mice were fixed with 10% formalin in 0.1 mol/l phosphate buffer, embedded in paraffin using standard techniques. Sections were prepared and stained with hematoxylin and eosin. Total islet area and pancreatic area were measured by LEICA QWin image processing and analysis software (Leica Microsystems, Buffalo, NY) and NIH Image 1.61, and the number of islets was counted under the microscope in a blind manner.

Basal insulin and glucose levels and glucose tolerance test. Basal plasma insulin and glucose levels were not different between transgenic mice and nontransgenic littermates in the ad libitum–fed state (plasma insulin level, n = 16–18; transgenic, 0.96 ± 0.37 ng/ml; nontransgenic, 1.13 ± 0.23 ng/ml; plasma glucose level, n = 9; transgenic, 164.2 ± 12.0 mg/dl; nontransgenic, 160.8 ± 9.3 mg/dl) and in the fasting state (0 time in Fig. 2).

An intraperitoneal glucose tolerance test was then performed. As shown in Fig. 2A, identical curves were obtained for nontransgenic and transgenic mice at 12 weeks of age (P = 0.85, repeated measures ANOVA), and after the administration of glucose, plasma glucose levels were maximal at 30 min. The curves representing data at 4, 7, or 21 weeks of age were of similar shape (data not shown). In addition, an oral glucose tolerance test showed no difference in plasma glucose levels between nontransgenic and transgenic mice (n = 9–11) at all time points (0, 10, 30, 60, 90, and 120 min; P = 0.61, repeated measures ANOVA; peak at 30 min; transgenic, 428 ± 35 mg/dl; nontransgenic, 420 ± 17 mg/dl). Nevertheless, the insulin levels in trans-
genetic mice were higher than in nontransgenic littermates (P < 0.05, repeated measures ANOVA; Fig. 2B). Similar results were obtained in other transgenic lines. Increased secretion of insulin in transgenic mice during glucose tolerance test seemed not to be quantitatively reflected by total pancreatic insulin content, because pancreatic insulin content was not different between nontransgenic and transgenic mice (transgenic, n = 11, 1.81 ± 0.16 μg/pancreas; nontransgenic, n = 13, 1.69 ± 0.11 μg/pancreas; P = 0.73, Student’s t test).

**Insulin tolerance test.** In the insulin tolerance test, 1 unit/kg body wt of insulin was given intraperitoneally to nontransgenic and transgenic mice. The glucose-lowering effect of insulin was determined in 0, 15, 30, 60, and 120 min after insulin injection (Fig. 3). Glucose values did not differ between nontransgenic and transgenic mice (P = 0.996; repeated measures ANOVA).

**Glucagon levels.** PACAP has been shown to stimulate glucagon release from the isolated rat pancreas (33) and in humans (11), suggesting that PACAP overexpressed in β-cells may stimulate glucagon release in transgenic mice. However, plasma glucagon levels were not different between transgenic mice and nontransgenic littermates during a glucose tolerance test (Fig. 4).

**Plasma glucose levels in mice given STZ injections.** It has been reported that the content of PACAP immunoreactivity in the pancreas was increased in STZ-induced diabetic rats, suggesting a possible protective role in vivo (34). Therefore, in the present study, we examined whether PACAP can protect pancreatic islets against STZ-induced diabetes in vivo.

As shown in Fig. 5A–C, plasma glucose levels were increased in both transgenic and nontransgenic mice that received injections of 70 mg/kg STZ for 5 consecutive days. This multiple low-dose STZ administration induced a mild increase in blood glucose levels without insulitis (Fig. 6). However, the increase in plasma glucose levels in transgenic mice was clearly attenuated compared with nontransgenic littermates (P < 0.001, repeated measures ANOVA). Five days after STZ administration, although there was a significant overlap in the plasma glucose levels in each population, the mean value was significantly lower in transgenic mice than in nontransgenic littermates (P < 0.05), and most nontransgenic mice (94%, n = 18) but only 41% of transgenic mice (n = 17) reached plasma glucose >200 mg/dl (Fig. 5B). Ten days after STZ administration, the mean value was again significantly lower in transgenic mice (P < 0.01); the smallest level of transgenic mice was 208 mg/dl, whereas that of nontransgenic mice was 289 mg/dl, and the range of plasma glucose levels was greater in transgenic mice than in nontransgenic mice (Fig. 5C). The insulin levels 5 days after the last dose of STZ were not significantly different between transgenic mice and nontransgenic littermates (Fig. 5D).

**BrdU labeling and TUNEL assay after STZ injections.** Mitotic and apoptotic cells were then determined by immunocytochemistry for BrdU and TUNEL assay, respectively. After STZ injections, the ratio of TUNEL-positive cells to insulin-positive cells was not significantly different between transgenic mice and nontransgenic littermates (Fig. 5E). The ratio of BrdU-positive cells to insulin-positive cells showed no difference between naïve transgenic and nontransgenic mice; however, after STZ injections, proliferating β-cells (BrdU and insulin double-positive cells) were significantly increased in transgenic mice compared with nontransgenic littermates (Figs. 5F and 6).

**Histologic and histomorphometric analyses.** Histologic examination of the pancreata from aged (12-month-old) transgenic mice suggested that these animals had more than a normal allotment of pancreatic islets (Fig. 7). For determining whether this was in fact the case, a blinded quantitative histomorphometric analysis was performed. As shown in Fig. 8, the mean islet area, islet number per pancreatic area, and islet area per pancreatic area were not significantly different between 12-week-old transgenic and nontransgenic littermate mice. However, in 12-month-old transgenic mice, islet area per pancreatic area tended to increase (1.95-fold compared with 12-week-old nontransgenic mice, P = 0.06; 2.51-fold compared with 12-week-old transgenic mice, P < 0.05), although the difference between 12-month-old transgenic and nontransgenic mice did not reach statistical significance.

**DISCUSSION**

The present study demonstrated that plasma glucose levels in the fed state and during glucose tolerance testing...
were not different between PACAP transgenic mice and nontransgenic littermates. This agrees well with the observation that, despite the potent effect of PACAP on insulin secretion, the peptide does not affect or even increase plasma glucose levels after intravenous administration (2,11). In addition, the present study demonstrated that the PACAP transgene weakly but significantly suppressed the STZ-induced increase in plasma glucose levels. Notably, β-cell proliferation was markedly increased in PACAP transgenic mice after the STZ administration. Furthermore, the total islet mass tended to increase in 12-month-old transgenic mice. These findings suggest, for the first time, that PACAP has a protective role for type 1 diabetes and that PACAP may play an important role in the β-cell proliferation.

The finding that PACAP transgenic mice exhibit enhanced insulin secretion during glucose tolerance test without affecting serum glucose concentration agrees with previous pharmacological studies but is in marked contrast to a previous report of transgenic mice overexpressing VIP in β-cells, in which plasma glucose levels in the fed state and during a glucose tolerance test were significantly reduced (12). Pancreatic β-cells express two PACAP receptor subtypes, the PACAP-preferring PAC₁ receptor, which is coupled to several transduction systems, and the VIP-shared VPAC₂ receptor, which is primarily coupled to adenylyl cyclase (2,3,8,10). Therefore, it is likely that VIP, acting via only VPAC₂ receptor, and PACAP, acting via both PAC₁ and VPAC₂ receptors, differentially affect β-cell functions.

However, the present results might also be explained by increased glycogenolysis resulting from an increase in circulating PACAP and/or catecholamine concentrations, or in glucagon release from pancreatic α-cells, in PACAP-transgenic mice. Indeed, PACAP has been reported to stimulate hepatic glucose output resulting from adrenalin release or by directly acting on hepatocytes (2,35,36). Also, we previously reported that PACAP and PAC₁ receptor are particularly abundant in the sympathoadrenal system (22,23). Although pancreatic PACAP content in transgenic mice was approximately threefold higher than that in nontransgenic mice, both transgenic and nontransgenic mice showed undetectable levels of plasma PACAP38 by radioimmunoassay (data not shown). Such a low level of plasma free PACAP is possibly due to the presence of a PACAP38-specific binding factor ceruloplasmin, whose physiological significance in PACAP-binding activity is currently unknown (37). In VIP transgenic mice, serum VIP levels are 2.5- to 7-fold higher than those of control mice, which is inhibited by glucose load. The present study showed that plasma glucagon levels are not different between transgenic mice and nontransgenic littermates during a glucose tolerance test. This result indicates that PACAP overexpressed in β-cells does not affect glucagon release at high glucose concentrations.

An alternative explanation is that the PACAP transgene causes obesity-induced insulin resistance. However, this seems less likely because there were no significant differences in body weight, food intake, and plasma triglyceride levels between PACAP transgenic and nontransgenic mice (data not shown). In addition, insulin sensitivity measured
by a short insulin tolerance test was not significantly different between transgenic and nontransgenic mice. However, a more precise investigation of insulin sensitivity in transgenic mice is required for this conclusion.

A previous report on freshly isolated rat islets has localized PACAP to islet endocrine cells (8). However, other studies demonstrated that PACAP is expressed in parasympathetic and sensory nerves but not in islet endocrine cells (2). In this study, Northern hybridization showed the low-level expression of intrinsic PACAP mRNA in pancreas of transgenic and nontransgenic mice (Fig. 1B); however, PACAP in islets of nontransgenic mice was undetectable by immunohistochemical analysis (Fig. 1C) that possessed sufficient sensitivity to detect PACAP expression in the brain (data not shown). These observations indicate that endogenous PACAP expression in islet β-cells is very weak, if any.

It has been reported that PACAP reduces a sustained intracellular Ca\(^{2+}\) increase induced by high K\(^{+}\) in pancreatic β-cells (38), which is known to cause impaired responsiveness of β-cells to glucose and induce β-cell apoptosis (39). In STZ-induced diabetic rats, PACAP immunoreactivity has been shown to increase in the pancreas (34). Yada et al. (40) demonstrated that intraperitoneal PACAP administration decreases blood glucose in GK rats and in mice that were on a high-fat diet. In addition, recent genetic linkage studies have revealed linkage between 18p11, where the human PACAP gene resides (41), and a susceptibility locus for type 2 diabetes (42). These findings suggest the implication of PACAP in the development of diabetes. In the present study, PACAP overexpressed in β-cells significantly suppressed the STZ-induced increase in plasma glucose levels, although plasma insulin levels between transgenic and nontransgenic mice showed no significant difference after STZ injections. It is noteworthy that the PACAP transgene causes a marked increase in BrdU-positive β-cells after the STZ injections. Although the mechanisms underlying these effects of PACAP are unclear, the present results provide additional evidence that PACAP has antidiabetic activity.

A computer-aided morphometric analysis of pancreatic islets revealed that the islet area per pancreatic area in 12-month-old transgenic mice tended to be greater compared with nontransgenic littermate mice. It has recently been reported that PACAP and GLP-1, a member of the VIP/secretin/glucagon family (2,3,5), stimulate insulin secretion in a glucose-dependent manner (43) possibly via a similar signal transduction pathway in β-cells (44). GLP-1 and exendin-4, a long-acting analog of GLP-1, induce proliferation of pancreatic AR4–2J cells and the subsequent differentiation of the subpopulation into insulin-positive cells (45). Exendin-4 also stimulates both proliferation and...
neogenesis of β-cells when administered to rats (46). In addition, PACAP stimulates proliferation of AR4–2J cells via activation of MAP kinase cascade (16). Moreover, in many other cell types, PACAP has been shown to play a role in cell proliferation, differentiation, and protection in physiological and pathophysiological states (1,3). These previous findings suggest that the increased β-cell proliferation in the STZ-treated transgenic mice and a tendency toward an increase in β-cell mass in the aged transgenic mice can be explained by increased proliferation and/or differentiation of pancreatic endocrine cells.

That the islet effect of PACAP produced through transgenic overexpression might be different from the normal effect of PACAP, as a neuropeptide in islets, should be noted. In addition to stimulating insulin secretion, long-term effects of PACAP on pancreatic endocrine cells, however, provide the possibility that drugs associated with PACAP-signaling pathways might be of therapeutic value for the treatment of diabetes. Future studies of a genetic cross between PACAP transgenic mice and its knockout mice or established animal models of diabetes will provide further insight into the involvement of PACAP signaling in glucose homeostasis and the molecular pathogenesis of diabetes.

ACKNOWLEDGMENTS
This research was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by grants from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, Taisho Pharmaceutical, AstraZeneca, and The Naito Foundation.

We thank Mayu Yamamoto, Rie Hatanaka, Ayako Ichibori, Tatsuya Ojika, Yuki Kawabata, and Ken-ichi Hama-gami for expert technical assistance.

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