

## Full Paper

**Over-Expression of Pancreatic Pituitary Adenylate Cyclase–Activating Polypeptide (PACAP) Aggravates Cerulein-Induced Acute Pancreatitis in Mice**

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**Abstract.** Development of human chronic pancreatitis is associated with intrapancreatic accumulation of pituitary adenylate cyclase–activating polypeptide (PACAP) accompanied with an altered inflammatory response (Michalski et al., *Am J Physiol Gastrointest Liver Physiol.* 2008;294:G50–G57). To investigate the role of pancreatic PACAP in the development of acute pancreatitis, we employed transgenic mice over-expressing PACAP in pancreatic  $\beta$ -cells (PACAP-Tg). In comparison to wild-type mice, PACAP-Tg mice exhibited more severe pathophysiological signs of the cerulein-induced pancreatitis at 12 h, as evidenced by higher serum amylase and lipase levels accompanied by the exacerbation of pancreatic edema, necrosis, and inflammation. Cerulein treatment increased mRNA expression of several proinflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , and IL-6) at 12 h with similar magnitude both in wild-type and PACAP-Tg mice. In addition, the mRNA and protein levels of regenerating gene III  $\beta$  (RegIII $\beta$ ), a key factor in the pancreatic response to acute pancreatitis, were up-regulated at 24 h in wild-type mice upon cerulein administration, whereas they were attenuated in PACAP-Tg mice. These data indicate that over-expressed PACAP in pancreas enhances the cerulein-induced inflammatory response of both acinar cells, leading to aggravated acute pancreatitis, which was accompanied by a down-regulation of RegIII $\beta$ , an anti-inflammatory factor.

**Keywords:** pituitary adenylate cyclase–activating polypeptide (PACAP), cerulein, acute pancreatitis, regenerating gene III  $\beta$  (RegIII $\beta$ )

**Introduction**

Pituitary adenylate cyclase–activating polypeptide (PACAP) is a neuropeptide that belongs to the vasoactive intestinal polypeptide (VIP) / secretin / glucagon superfamily (1). PACAP regulates a wide range of physiological functions in the central and peripheral

nervous systems (2). In the pancreas, PACAP is found in the nerve fibers and is distributed throughout the organ (3). Similarly, its three receptors, VPAC1, VPAC2, and PAC1, are localized throughout the pancreas (4, 5). With respect to the function of PACAP in the pancreas, several reports have suggested its involvement in the regulation of endocrine functions. It was found that exogenously applied PACAP at an extremely low concentration ( $10^{-14}$  M) stimulates insulin secretion in a glucose-dependent manner (4). In a series of experiments using PACAP-knockout mice (6, 7) and transgenic mice over-expressing PACAP in pancreatic  $\beta$

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cells (PACAP-Tg) (8, 9), we examined the pathophysiological roles of PACAP in pancreatic endocrine function; in PACAP-Tg mice, streptozotocin-mediated destruction of pancreatic  $\beta$  cells was ameliorated (8). In addition, over-expression of PACAP in islets resulted in significant amelioration of hyper-insulinemia and islet hyperplasia in a type II diabetic mouse model (9). These findings suggested that PACAP is involved in regulating the pathophysiology of pancreatic  $\beta$  cells.

With respect to the role of PACAP in pancreatic exocrine function, PACAP stimulates amylase secretion from pancreatic acinar cells by elevating the cellular cAMP level (5, 10–13). Acute pancreatitis is a disease that targets exocrine cells, leading to inflammation, fibrosis, and general tissue disruption of which progression and pathogenesis are mediated by several pro-inflammatory cytokines and anti-inflammatory cytokines that are released from infiltrated monocytes and macrophages. An intravenous infusion of PACAP aggravates the signs of cerulein-induced acute pancreatitis in rats (14). Recently, it has been shown that development of human chronic pancreatitis is associated with intra-pancreatic accumulation of PACAP around nerves and inflammatory infiltrates, with altered patterns of PACAP-mediated cytokine responses (15). Collectively, it is imperative to address the mechanisms underlying the regulatory role of pancreatic PACAP in the development of acute pancreatitis by using pancreas-specific PACAP-Tg mice. The results of the study showed that the cerulein-induced pancreatitis was more aggravated in PACAP-Tg mice than in wild-type mice, indicating that pancreatic PACAP enhanced the inflammatory response of acinar cells during acute pancreatitis.

## Materials and Methods

### *Mice*

The generation of PACAP-Tg mice has been described (8). All mice were kept in a temperature-, humidity-, and light-controlled room with a 12-h light / 12-h dark cycle (lights on from 8:00 AM to 8:00 PM) and allowed free to water and food. All animal experiments were performed in accordance with protocols approved by Institutional Animal Care and Use of Osaka University.

### *Induction of experimental acute pancreatitis in mice by cerulein administration*

After PACAP-Tg and wild-type mice were fasted for 16–18 h with access to water ad libitum, acute pancreatitis was induced in these mice. Seven consecutive hourly intraperitoneal injections of saline solution (0.9% NaCl) containing the pancreatic secretagogue cerulein (50  $\mu$ g/kg body weight) (Sigma, Tokyo) were

administered (10  $\mu$ l/g body weight). The control mice received an equal volume of saline solution. In the following text, the experimental time indicates the time after the first cerulein injection.

### *Measurement of serum amylase and lipase activities*

Blood samples were collected from a tail vein just prior to cerulein administration and at 6, 9, 12, 18, and 24 h after the first cerulein injection. The sample tubes were centrifuged at  $10,000 \times g$  for 10 min, and the plasma samples were stored at  $-80^{\circ}\text{C}$  until use. Amylase and lipase activities were determined with commercially available assays (Roche Diagnostics KK, Tokyo).

### *Tissue preparation and histology*

Pancreases from the saline- or cerulein-treated PACAP-Tg and wild-type mice were dissected for histological examinations. The pancreas was rapidly removed at 12 and 24 h after the first cerulein treatment and fixed overnight in 4% neutral phosphate-buffered paraformaldehyde and embedded in paraffin. The tissues were sectioned using a semi-motorized rotary microtome to generate 5- $\mu$ m sections. These sections were stained with hematoxylin and eosin for visualization of the cellular composition. The stained tissue sections were graded in a blind fashion using the scale system reported previously (16). The sample slides were examined for the presence of edema, inflammation, vacuolization, and necrosis in 5 randomly chosen microscopic fields.

### *Total RNA isolation, reverse transcription, and quantitative real-time PCR*

Total RNA was isolated from pancreases of the saline- or the cerulein-treated PACAP-Tg and wild-type mice by the guanidine thiocyanate method. The concentration and purity of RNA were determined by optical density ratios (value at 260 nm divided by value at 280 nm) and by 1% agarose gel electrophoresis. One microgram of total RNA was used to perform reverse transcription. The RNA samples were mixed with M-MLV reverse transcriptase (Invitrogen, Tokyo), random hexamers, dNTP mix, and first strand buffer. Quantitative real-time PCR was performed using DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) and the following primers for 18S rRNA: 5'-GTA ACC CGT TGA ACC CCA TT-3' (sense) and 5'-CCA TCC AAT CGG TAG TAG CG-3' (antisense), TNF- $\alpha$ : 5'-GGG CCA CCA CGC TCT TCT GTC T-3' (sense) and 5'-GCC ACT CCA GCT GCT CCT CCA C-3' (antisense), IL-1 $\beta$ : 5'-ACA AGT CGG AGG CTT AAT TAC ACA T-3' (sense) and 5'-AAT CAG AAT TGC CAT TGC ACA A-3' (antisense), IL-6: 5'-TCG CTC AGG GTC ACA AGA AA-3'

(sense) and 5'-CCA TCA GAG GCA AGG AGG AA-3' (antisense)

Quantitative real-time PCR was conducted using the DNA Engine Opticon 2 Continuous Fluorescence Detection System (Bio-Rad Laboratories, Tokyo). As an internal control, the results were normalized to 18S ribosomal RNA, and the data were expressed as a fold-change over the wild-type control value, which was assigned the value 1.

#### Western blotting

Pancreases from the saline- or the cerulein-treated PACAP-Tg and wild-type mice were harvested and homogenized in cold extraction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 2  $\mu$ g/ml aprotinin). The homogenized samples were subjected to centrifugation at  $10,000 \times g$  for 20 min at 4°C. The supernatants were recovered, and the protein concentration was determined with the BCA<sup>TM</sup> protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Thirty micrograms of the sample protein was resolved by 15% SDS-PAGE and transferred to immobilon<sup>TM</sup> transfer membranes (Millipore, Tokyo). The blotting was performed as follows: the membrane was incubated in Tris-buffered saline containing 0.1% Tween-20 (TBST) – 5% skim milk for 1 h at room temperature to block non-specific binding. It was then incubated for another 1 h at 37°C with rabbit anti-rat RegIII $\beta$  polyclonal antibody (1:1000) or with anti-mouse  $\beta$ -tubulin antibody

(1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the membrane was washed in TBST and incubated with a secondary goat anti-rabbit-peroxidase antibody (1:1000) (MP Biomedicals, Costa Mesa, CA, USA) for 1 h at 37°C. Subsequently, the membrane was washed in TBST, and the protein detection was performed by chemiluminescence (Western Lightning<sup>TM</sup> Chemiluminescence Reagent Plus; PerkinElmer, Boston, MA, USA).

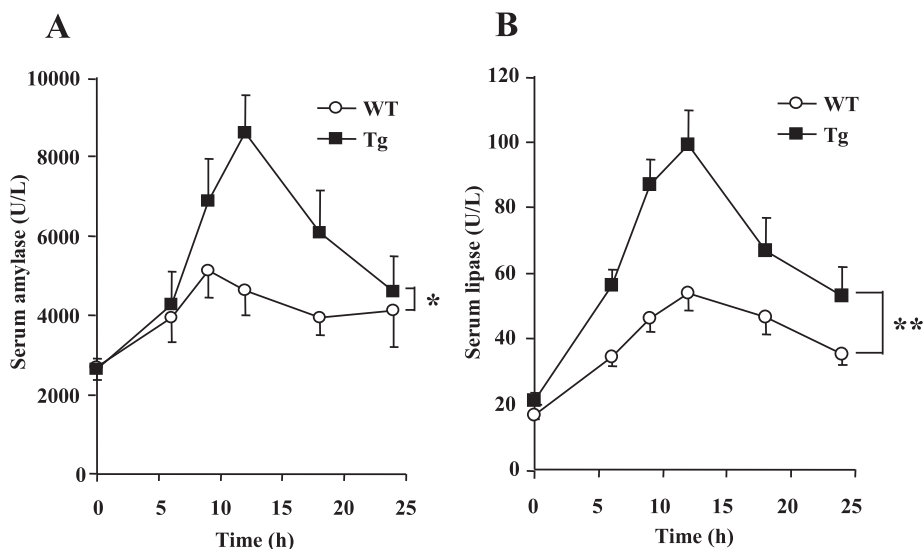
#### Statistical analyses

Data were each expressed as the mean  $\pm$  S.E.M. The differences between the groups were analyzed by a repeated two-way ANOVA, a two-way ANOVA with a post hoc Tukey-Kramer test, or by a Student's *t*-test, using StatView software (SAS Institute, Cary, NC, USA). A *P*-value of <0.05 was considered significant.

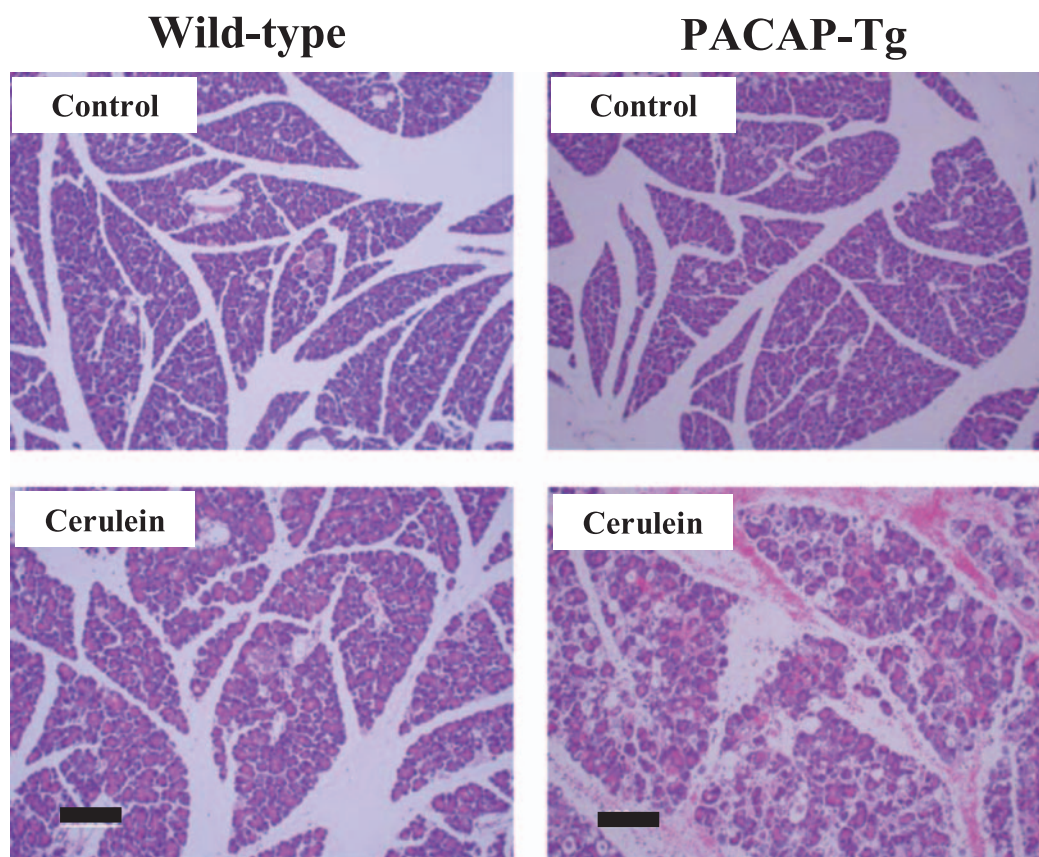
## Results

#### Cerulein-induced secretion of amylase and lipase

Administration of high doses of cholecystokinin (CCK) or its analogue, cerulein, results in the development of pancreatitis that is characterized by increases of serum amylase and lipase activity and an acute necrosis of pancreatic exocrine cells (13). To compare the susceptibility of PACAP-Tg and wild-type mice to the cerulein-induced pancreatitis, the activities of serum amylase and lipase were determined (Fig. 1). The basal activities of serum amylase and lipase were the same in both genotypes of mice. Cerulein induced a time-



**Fig. 1.** Serum amylase and lipase activities in wild-type and PACAP-Tg mice. The activities of serum amylase (A) and lipase (B) were measured for PACAP-Tg and wild-type mice at 0, 6, 9, 12, 18, and 24 h after the cerulein injection. Data are expressed as the mean  $\pm$  S.E.M. ( $n = 6$  per group). \* $P < 0.05$ , \*\* $P < 0.01$ , repeated two-way ANOVA.



**Fig. 2.** Histological examinations of pancreas of cerulein-treated mice. Representative micrographs of hematoxylin and eosin stained pancreatic sections are shown. At 12 h after cerulein (Cerulein) or saline (Control) administration, the pancreas from the wild-type and PACAP-Tg mice were harvested, processed, and stained as described in Materials and Methods. Note the significant increase in edema, infiltration of immune cells, and necrosis in pancreatic acini of the cerulein-treated mice. Arrows represents small blood vessel congestion. Bar = 100  $\mu$ m.

**Table 1.** Histological scores of pancreas after cerulein treatment in PACAP-Tg and wild-type mice

Genotype	Edema	Infiltration	Vacuolization	Necrosis	Total score
At 12 h					
Wild-type	1.6 $\pm$ 0.24	1.8 $\pm$ 0.37	1.4 $\pm$ 0.24	1.0	5.8 $\pm$ 0.86
PACAP-Tg	2.3 $\pm$ 0.21*	3.3 $\pm$ 0.33*	2.0 $\pm$ 0.26	1.8 $\pm$ 0.31*	9.5 $\pm$ 1.11**
At 24 h					
Wild-type	2.0 $\pm$ 0.55	0.8 $\pm$ 0.20	1.2 $\pm$ 0.37	0.8 $\pm$ 0.20	4.8 $\pm$ 1.28
PACAP-Tg	3.0 <sup>#</sup>	1.0 $\pm$ 0.32 <sup>###</sup>	1.6 $\pm$ 0.25	0.8 $\pm$ 0.20 <sup>#</sup>	6.4 $\pm$ 0.51 <sup>##</sup>

Hematoxylin- and eosin-stained sections (see Fig. 2) were evaluated in a blind fashion. Five microscopic fields on each section were scored for edema, vacuolization, infiltration of immune cells, and necrosis. The stained tissue sections were graded from 0 (no change) to 4 (most severe change) in a blind fashion using the scale system reported previously (16). Data were expressed as the mean  $\pm$  S.E.M. (n = 5–6 per group). \* $P$ <0.05, \*\* $P$ <0.01 vs. wild-type mice; <sup>#</sup> $P$ <0.05, <sup>##</sup> $P$ <0.01, <sup>###</sup> $P$ <0.001 vs. PACAP-Tg mice at 12 h, Student's  $t$ -test.

dependent rise in serum amylase and lipase activities with the maximal rises at 8 and 12 h, respectively. In the wild-type mice, the enzyme activities were increased by twofold at 8 h and by threefold at 12 h, whereas the

response was markedly enhanced in PACAP-Tg mice. At the peak time of 12 h, the activities of both enzymes of PACAP-Tg mice were about twofold higher than those of wild-type mice.

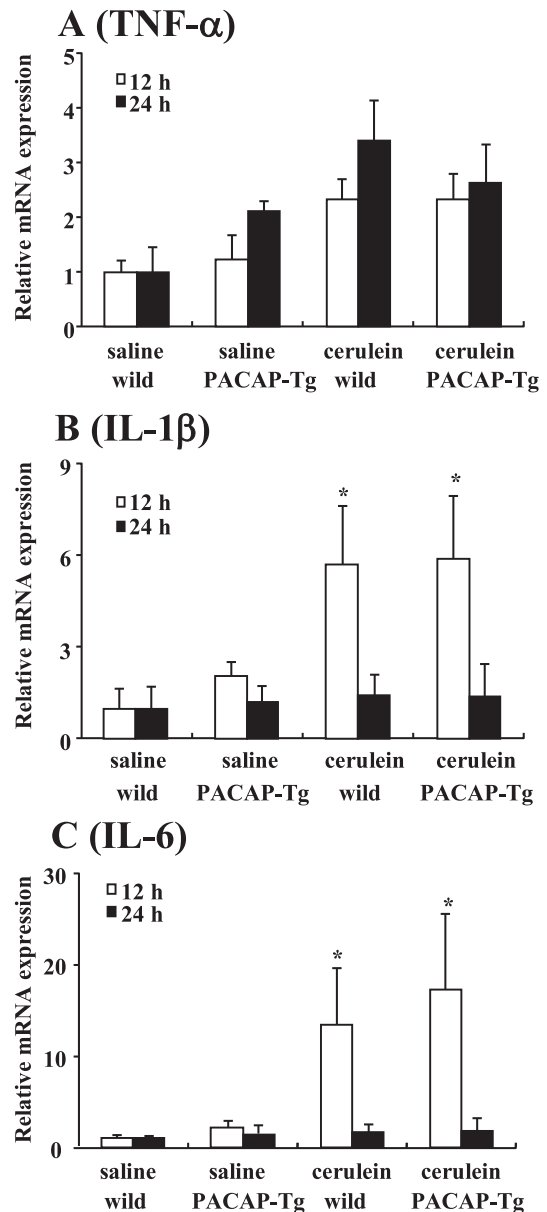
### Histological examination of pancreases from cerulein-treated mice

Next, histological examinations of pancreases from PACAP-Tg and wild-type mice were performed on samples harvested at 12 and 24 h after the cerulein treatment (Fig. 2, Table 1). As shown in Fig. 2, the saline-injected wild-type and PACAP-Tg mice showed no morphological evidence of pancreatic inflammation. In contrast, the systemic injection of cerulein induced pancreatic inflammation in both groups of mice at 12 and 24 h. To assess the magnitude of pancreatic injury, edema, infiltration, vacuolization, and necrosis were graded according to the method reported elsewhere (16), where the extent of damage was given a score from 0 to 4: 0 indicated the absence of injury and 4 indicated the injury being most severe. As shown in Table 1, the cerulein treatment was followed by marked increase in each pancreatitis score at 12 and 24 h. Each score of edema, infiltration of inflammatory cells, and necrosis of pancreas was significantly higher at 12 h in PACAP-Tg mice compared to those in wild-type mice. Accordingly, the total histological score of PACAP-Tg at 12 h was significantly higher than that of wild-type mice ( $P < 0.01$ ). At 24 h in PACAP-Tg mice, the scores of infiltration and necrosis had returned to the levels of wild-type mice, although that of edema was increased further compared to that at 12 h.

### Messenger RNA expression of proinflammatory and anti-inflammatory cytokines

To address whether the increased number of inflammatory cells was responsible for the extensive damage, the presence of pancreatic pro-inflammatory cytokines was determined 12 h after the cerulein treatment (Fig. 3). Development of acute pancreatitis is correlated with an imbalanced production of  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-6}$ , and  $\text{IL-10}$  (17, 18). In the present study, the cerulein treatment resulted in increased mRNA expression of  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  at 12 h both in wild-type and PACAP-Tg mice, although the expression levels of each cytokine were not different between two genotypes. The expressions of  $\text{IL-6}$  and  $\text{IL-1}\beta$  were returned to the control levels at 24 h after the cerulein treatment.

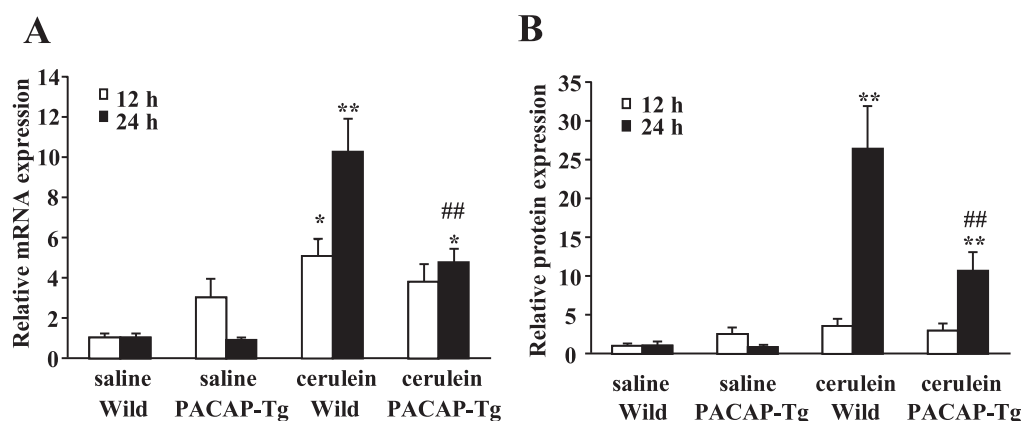
$\text{RegIII}\beta$  (also known as pancreatic associated protein 1, PAP1) is a newly characterized anti-inflammatory factor that was identified in the pancreas (19, 20). Although the expression of pancreatic  $\text{RegIII}\beta$  transcript is quite low in the resting state, it is markedly increased during acute pancreatitis so that its production is much more than any other protein (21). As shown in Fig. 4, the basal levels of  $\text{RegIII}\beta$  mRNA and protein did not significantly differ between the two genotypes. Upon cerulein treatment,  $\text{RegIII}\beta$  mRNA level was increased



**Fig. 3.** Messenger RNA expression of  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  during the cerulein-induced acute pancreatitis. Messenger RNA expression of  $\text{TNF-}\alpha$  (A),  $\text{IL-1}\beta$  (B), and  $\text{IL-6}$  (C) was determined at 12 (open column) and 24 h (closed column) after the cerulein treatment. The levels of mRNA in PACAP-Tg and wild-type mice were determined by real-time PCR and expressed as fold-change relative to the values of saline-treated wild-type mice. Data are expressed as the mean  $\pm$  S.E.M. ( $n = 6-8$  per group). \* $P < 0.05$  vs. saline-treated wild-type mice, two-way ANOVA analysis followed by Tukey Kramer test.

significantly in wild-type mice at 12 and 24 h, but that response was markedly attenuated in PACAP-Tg mice. Similarly, distinct attenuation of the response to cerulein was observed in  $\text{RegIII}\beta$  protein level in PACAP-Tg mice at 24 h.





**Fig. 4.** Expression of RegIII $\beta$  during the cerulein-induced pancreatitis. RegIII $\beta$  mRNA (A) and RegIII $\beta$  protein (B) levels were determined at 12 (open column) and 24 h (closed column) after the cerulein treatment. RegIII $\beta$  mRNA expression was quantitated by real-time PCR, normalized to the 18S rRNA housekeeping gene, and expressed as fold-change relative to the value of saline-treated wild-type mice. RegIII $\beta$  protein level was quantitated by western blotting analysis, normalized to  $\beta$ -tubulin, and expressed as fold-change relative to the value of the saline-treated wild-type mice. Data are expressed as the mean  $\pm$  S.E.M. ( $n = 6 - 8$  per group). \* $P < 0.05$ , \*\* $P < 0.01$  vs. saline-treated wild-type mice at each time, # $P < 0.01$  vs. cerulein-treated wild-type mice at each time, two-way ANOVA analysis followed by Tukey Kramer test.

## Discussion

In the pancreas, PACAP is found in the nerve fibers, distributed throughout the organ (3) and regulates endocrine functions (4–9). Previous findings raise the possible concerns of PACAP in the pathophysiology of pancreatitis (12, 14). Recently, it has been reported that development of human chronic pancreatitis is associated with intrapancreatic accumulation of PACAP (15). Chen et al. (14) demonstrated that an intravenous infusion of PACAP aggravates the signs of cerulein-induced acute pancreatitis in rats. In the same experiment, the PACAP-receptor antagonists also aggravate the cerulein-induced pancreatitis (14). Since PACAP modulates cytokine productions of inflammatory cells such as peritoneal macrophages (22, 23), a systemic application of PACAP possibly causes too complex effects to elucidate the roles of pancreatic PACAP in exocrine function. This is the reason of why the pancreatic-specific PACAP-Tg mice were used in the present study.

The results of our study showed that PACAP-Tg mice exhibit severe pathophysiological signs of cerulein-induced pancreatitis. This was evidenced by the higher serum levels of amylase and lipase (Fig. 1) and by the higher histological scores for edema, infiltration of inflammatory cells, vacuolization, necrosis, and hemorrhage in pancreatic tissue (Fig. 2 and Table 1). PACAP or CCK alone has little effect on amylase secretion from pancreatic acinar cells, while in combination they markedly increase the amylase secretion by sensitizing the acinar cells to CCK-induced zymogen activation (12). Epac/Rap1 signals play an essential role

as a common signal for both CCK- and cAMP-evoked amylase secretions in pancreatic acinar cells (24). In our previous study, we demonstrated that cAMP/Epac/Rap1 may be involved in PACAP-induced gene expression and neurite extension in PC12 cells (25). Collectively, we speculate that over-expressed pancreatic PACAP might sensitize the acinar cells to the cerulein-induced zymogen activation through activation of cAMP/Epac/Rap1 signals.

In experimental pancreatitis, the pathogenesis and progression of acute pancreatitis are accompanied by the enhanced expression of pro-inflammatory cytokines, IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 in serum and pancreas (17, 18). In the present study, pancreatic mRNA levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were increased by the cerulein treatment, although their expression levels did not differ between wild-type and PACAP-Tg mice (Fig. 3). In immune cells, PACAP released after antigenic stimulation suppresses an inflammatory response primarily by inhibiting the production of proinflammatory cytokines (22, 23). On the other hand, PACAP enhances the IL-6 release from macrophages both in the presence and absence of lipopolysaccharide (26). Taken together, it is suggested that pancreatic PACAP does not enhance the cerulein-induced expression of these cytokines in infiltrated inflammatory cells.

RegIII $\beta$ , initially found in the pancreatic juice of rats with acute pancreatitis (19), is a 16-kDa secretory protein structurally related to the C-type lectins (27). The anti-inflammatory function of RegIII $\beta$  in acute pancreatitis was evidenced by various experimental approaches (28–30). RegIII $\beta$  suppresses the expression

of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 (27, 30). The experimentally induced reduction in the expression of pancreatic RegIII $\beta$  is followed by an exacerbation of pancreatitis (29, 30). The expression of RegIII $\beta$  is up-regulated in acinar cells during the acute phase of pancreatitis and paralleled the severity of pancreatitis (21, 28). However, this is not the case in PACAP-Tg mice; while the cerulein-induced pancreatitis is aggravated at 12 h, the expression of RegIII $\beta$  at 24 h was significantly lower than that of wild-type mice (Fig. 4). It is noteworthy that edema was further aggravated at 24 h in PACAP-Tg mice. At present, we have no evidence to explain the reason for why the cerulein-induced expression of RegIII $\beta$  is much lower in PACAP-Tg mice. RegIII $\beta$  expression can be induced by several pro- and anti-inflammatory cytokines and by itself through a Jak/STAT3-dependent pathway (27). PACAP affects several cellular functions including gene expression through cAMP/Epac/Rap1 (25). Based on the functional cross-talk between cAMP signaling and the Jak/STAT signaling pathway in THP-1 cells (31), we assume that possible signal cross-talk between PACAP and Jak/STAT pathways is underlying the reduction of RegIII $\beta$  expression in PACAP-Tg mice.

PACAP is demonstrated in nerve fibers in all compartments of the pancreas and in a subpopulation of intrapancreatic VIP-containing ganglion cells (3). The content of pancreatic PACAP in PACAP-Tg mice is about threefold higher than that of wild-type mice (8). Pancreatic PACAP content is markedly increased during cerulein-induced acute pancreatitis in rats, where exogenously applied PACAP aggravates acute pancreatitis (14). In chronic pancreatitis patients, the intra-pancreatic PACAP mRNA was over-expressed by about 2.5-fold, where altered patterns of PACAP-mediated cytokine responses were observed (15). To address the whole scope of PACAP in pancreatitis, a further study should be carried out on the chronic pancreatitis model in PACAP-Tg mice. Altogether, the present findings provide experimental evidence showing that over-expression of intrapancreatic PACAP enhances progression and pathogenesis of acute pancreatitis.

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