Pituitary adenylate cyclase activating-peptide and its receptor antagonists in development of acute pancreatitis in rats

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AIM: Pituitary adenylate cyclase activating-peptide (PACAP) is a late member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family of brain-gut peptides. It is unknown whether PACAP takes part in the development of acute pancreatitis and whether PACAP or its antagonists can be used to suppress the progression of acute pancreatitis. We investigated the actions of PACAP and its receptor antagonists in acute pancreatitis in rats.

METHODS: Acute pancreatitis was induced in rats with caerulein or 3.5% sodium taurocholate. The rats were intravenously infused for 1 h. Biochemical and histopathological assessments were made at 4 h after infusion. Pancreatic and duodenal PACAP concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Chinese ink-perfused pancreas was fixed, sectioned and cleared for counting the functional capillary density. ELISA revealed that relative concentrations of PACAP in pancreas and duodenum were significantly increased in both sodium taurocholate- and caerulein-induced pancreatitis compared with those in normal controls. Unexpectedly, PACAP6-27 and (4-Cl-D-Phe₆, Leu²)₇ VIP (PACAP receptor antagonists) were intravenously infused for 1 h. Biochemical and histopathological assessments were made at 4 h after infusion. Pancreatic and duodenal PACAP concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Chinese ink-perfused pancreas was fixed, sectioned and cleared for counting the functional capillary density.

RESULTS: PACAP augmented caerulein-induced pancreatitis and failed to ameliorate sodium taurocholate-induced pancreatitis. ELISA revealed that relative concentrations of PACAP in pancreas and duodenum were significantly increased in both sodium taurocholate- and caerulein-induced pancreatitis compared with those in normal controls. Unexpectedly, PACAP6-27 and (4-Cl-D-Phe₆, Leu²)₇ VIP could induce mild acute pancreatitis and aggravate caerulein-induced pancreatitis with characteristic manifestations of acute hemorrhagic/necrotizing pancreatitis. Functional capillary density of pancreas was interpreted in the context of pancreatic edema, and calibrated functional capillary density (calibrated FCD), which combined measurement of functional capillary density with dry weight/wet weight ratio, was introduced. Hyperemia or congestion, rather than ischemia, characterized pancreatic microcirculatory changes in acute pancreatitis.

CONCLUSION: PACAP may take part in the pathogenesis of acute pancreatitis in rats. The two PACAP receptor antagonists might act as partial agonists. Calibrated functional capillary density can reflect pancreatic microcirculatory changes in acute pancreatitis.

INTRODUCTION

The pathogenesis of acute pancreatitis has not been well understood until now. Its therapies are primarily supportive measures, and the morbidity and mortality of acute hemorrhagic/necrotizing pancreatitis are still high[11]. Various factors have been tested in experimental acute pancreatitis, among them brain-gut peptides, including cholecystokinin (CCK), glucagon, secretin and somatostatin, are the subjects of intensive study[2-4]. Pituitary adenylate cyclase-activating peptide (PACAP), which was characterized by the end of last century, is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family of brain-gut peptides[5,6,7]. PACAP exists in nerve fibres in all compartments of the pancreas. Its three characterized receptors are distributed in the pancreas[6,7] suggesting its possible actions on this organ. PACAP can stimulate pancreatic exocrine and endocrine secretions[5,6,14]. It is a highly potent vasorelaxant peptide, which can dilate blood vessels and increase pancreatic blood flow, notably in the exocrine part of pancreas[15,16]. However, it is unknown whether PACAP takes part in the development of acute pancreatitis and whether PACAP or its antagonists can suppress the progression of acute pancreatitis.

Microcirculatory impairment has been considered as an important pathogenic factor of acute pancreatitis[21-25]. Edematous pancreatitis is associated with hyperemia of pancreas. But in acute hemorrhagic/necrotizing pancreatitis, intravital microscopy often shows reduced functional capillary densities of pancreata[26,27]. This change is termed ischemia by some authors. However, acute pancreatitis is always accompanied with pancreatic edema, i.e., widening of interlobular septa and separation of acinar cells, and this change was previously ignored when the functional capillary density was calculated.

MATERIALS AND METHODS

Materials

PACAP-27, PACAP6-27, (4-Cl-D-Phe₆, Leu²)₇ VIP, caerulein and sodium taurocholate were purchased from Sigma Company (St. Louis, USA). Goat polyclonal antibody against PACAP C-19 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA).

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Experimental design

Male Wistar rats, weighing 190-360 g, were obtained from the Laboratory Animal Center of Sichuan University. Experiments were performed in accordance with Chinese regulations concerning the use of laboratory animals. After the rats were fasted overnight but with free access to water, they were anesthetized with intraperitoneal administration of 50 mg/kg sodium pentobarbital, and their left jugular veins were catheterized. They were observed for 4 h, then re-anesthetized and laparotomized. Blood was collected via inferior vena cava for the measurement of serum amylase, and the duodenal portions of pancreas were rapidly excised. One part of the pancreas was blotted dry and weighed, then desiccated at 80 °C for 24 h and re-weighed. The dry weight/wet weight ratio was thus calculated, representing the presence and extent of pancreatic edema. Another part of the pancreas was fixed with 100 mL/L formaldehyde, embedded in paraffin, and sectioned for hematoxylin-eosin staining.

Five-and-half µg/kg and 7.5 µg/kg caerulein were subcutaneously injected into the nape at 0 and 1 h, respectively, to induce mild acute pancreatitis. Five mL/(kg·h) normal saline was intravenously infused to maintain water and electrolyte balance.

Immediately after the catheterization of jugular vein, an upper midline abdominal incision was made and a small cannula was retrogradely placed into biliopancreatic duct transduodenally. A small clip was applied at the proximal part of the biliopancreatic duct. One milliliter per kilogram 3.5% sodium taurocholate was infused via the cannula. The infusion was begun after a five-minute stabilization period and the infusion pressure was kept below 30 mmHg. As venous catheterization plus laparotomy would pose a greater challenge to homeostasis, and ascites would produce in this group, the animals were readily dehydrated and 7.5 mL/(kg·h) normal saline was administered intravenously.

Rats were intravenously administered 5 (n = 11), 10 (n = 11), 15 (n = 11) and 30 µg/kg (n = 11) of PACAP diluted in normal saline in the first 90 min, followed by infusion of 5 mL/(kg·h) normal saline.

The rats with caerulein-induced pancreatitis were administered 15 (n = 11) or 30 µg/kg (n = 11) of PACAP intravenously in the first 90 min, followed by infusion of 5 mL/(kg·h) normal saline.

The rats with sodium taurocholate-induced pancreatitis were intravenously administered 5 (n = 6) or 10 µg/kg (n = 11) of PACAP in the first 90 min, followed by infusion of 7.5 mL/(kg·h) normal saline.

Normal rats were intravenously administered 10 (n = 6) or 100 µg/kg (n = 6) of PACAP6-27 diluted in normal saline within the first 60 min, followed by infusion of 5 mL/(kg·h) normal saline.

The rats with caerulein-induced pancreatitis were intravenously infused 10 (n = 6) or 100 µg/kg (n = 6) of PACAP6-27 within the first 60 min.

The rats with sodium taurocholate-induced pancreatitis were intravenously administered 10 (n = 6) or 100 µg/kg (n = 6) of PACAP6-27 within the first 60 min.

The experimental designs for (4-Cl-D-Phe6, Leu7)VIP were essentially the same as that for PACAP6-27, i.e., normal rats or rats with caerulein-induced pancreatitis or rats with sodium taurocholate-induced pancreatitis were intravenously infused 10 (n = 6) or 100 µg/kg (n = 6) of (4-Cl-D-Phe6, Leu7)VIP within the first 60 min.

The jugular veins of 15 normal rats were catheterized and normal saline was infused at 5 mL/(kg·h) for 4 h.

Functional capillary density

At the end of experiment, at a site distal to the origins of renal arteries and proximal to the aortic bifurcation, a small catheter was retrogradely placed into aorta, then 100 IU of heparin was injected via the catheter. The thoracic segment of inferior vena cava was cut, and thoracic aorta was ligated, then warm heparinized-normal saline was infused through the catheter with an infusion pressure not exceeding 120 mmHg. When the outflow was clear, undiluted Chinese ink was injected. One to two hours were needed for the hardening of Chinese ink in the vessels. Finally the pancreatic head was removed, fixed with 100 mL/L formaldehyde, and embedded in paraffin. Each specimen was cut into three 20 µm thick sections, and cleared with dimethylbenzene for assessment of functional capillary density.

Functional capillary density was defined as the capillary length of all RBC-perfused capillaries (here Chinese ink-perfused ones) per observation area. Quantification of functional capillary density was made possible by connecting Olympus Provis AX70 microscope with Panasonic BT-H1450Y color monitor through Olympus U-PMTVC. According to the method described by Schmidt-Schönbein et al, a grid was printed on a transparency and fixed on the screen of the monitor. Five fields in each section were randomly superimposed on the grid, intersections between the grid and capillaries were counted twice, and the functional capillary density was calculated according to the following equation:[29]

\[ Lc = \frac{\Pi L}{2NcPd} \]

where \( Lc \) = perfused capillary length (dimension /cm), \( Nc \) = mean for numbers of intersections, \( P \) = number of squares in the grid, and \( d \) = length of the edge of the grid. The mean calculated from 10 fields in each section represented the functional capillary density of that section.

ELISA for PACAP

Pancreatitis was induced by caerulein and sodium taurocholate as described above. At the end of experiment, the pancreas and a segment of duodenum were rapidly excised and weighed. The specimens were then immersed in 10-fold (volume/pancreatic weight) 0.2 mol/L Tris-HCl buffer (pH 7.3) containing 20 mmol/L EDTA, immediately boiled (100 °C) for 10 min to denature residual protease activity, and homogenized for 30 s. The homogenates were centrifuged 2 000 g for 10 min at 4 °C. The supernatant was stored at -70 °C for later assay.

Since commercial PACAP ELISA kit was not available, the following procedure was adopted. In brief, 10 µL supernatant of duodenal tissue or 50 µL pancreatic supernatant was mixed with 1 mL 0.05 mol/L carbonate buffer (pH 9.6), and the mixture was incubated in a 96-well plate overnight at 4 °C. After blocking with 10 mL/L bovine serum albumin at 37 °C for 2 h, anti-PACAP antibody at a dilution of 1:1 000 was incubated overnight at 4 °C. Then biotinylated rabbit anti-goat IgG at a dilution of 1:200 was incubated at room temperature for 1.5 h. Horseradish peroxidase streptavidin at a dilution of 1:200 was incubated overnight at 4 °C. Then biotinylated rabbit anti-goat IgG at a dilution of 1:1 000 was incubated at room temperature for 1 h. Horseradish peroxidase streptavidin at a dilution of 1:200 was incubated for 1 h, and then for another 30 min with substrate solution (TMB and H2O2 dissolved in phosphate-citric acid buffer). The reaction was terminated with 2 mol/L H2SO4, and the plate was read at 450 nm on a Bio-Rad Model 550 microplate reader.

A supernatant was randomly chosen as a standard, serially diluted, by the same procedure of assay as described above. A standard curve consisting of optical density and relative concentration could thus be plotted. Relative concentrations of PACAP could be calculated from optical densities read by the microplate reader.

Light microscopy

Each paraffin-embedded specimen was cut into three 5 µm thick sections, which were stained with hematoxylin and eosin. The severities of histopathological changes (i.e., pancreatic hemorrhage, necrosis, vacuolization of acinar cells and leukocyte infiltrate) were graded as follows: 0 for normal; 1 for changes found on one section; 2 for changes detected on two sections; and 3 for changes observed on three sections. Although this scoring
system was not satisfactory, it matched the severities of histopathological changes.

**Assays**

Blood for amylase assay was collected from inferior vena cava, and sent to the Medical Assay Center of our hospital and assayed for serum amylase with Beckman coulter CX7 Super Clinical System Synchron. Peritoneal fluid/ascites was also assayed for amylase and nucleated cells.

**Statistical analysis**

All data was expressed as mean±SE. The differences between the means of all studies were analyzed by using analysis of variance. *P*<0.05 was considered statistically significant.

### RESULTS

**Animal models**

The pancreas of control group was macroscopically and microscopically normal. Caerulein-induced pancreatitis was characterized by gross swelling of pancreas, histological evidence of edematous pancreatitis (acinar cell vacuolization, leukocyte sequestration and edema) and significant increase in serum amylase. Sodium taurocholate-induced pancreatitis was characterized by marked enlargement of pancreas, gross hemorrhage, saponification of pancreas and peripancreatic tissues, ascites formation, histological evidence of acute hemorrhagic/necrotizing pancreatitis (bleeding, parenchymal necrosis, acinar cell vacuolization, marked leukocyte infiltration and severe edema) and significant increase in serum amylase.

**Effect of PACAP on normal pancreas**

The pancreas of PACAP-treated rats was grossly normal. Pancreatic dry weight/wet weight ratio was insignificantly decreased by PACAP treatment. Serum amylase was mildly increased. Acinar cell vacuolization, leukocyte sequestration and parenchymal necrosis were histologically observed in some cases (Tables 1-3).

**Effect of PACAP on caerulein-induced pancreatitis**

PACAP treatment aggravated the pancreatic edema. There were gross hemorrhage and pancreatic/peripancreatic saponification in some cases. Ascites occurred in 3 and 8 cases of caerulein plus 15 µg/kg PACAP group and caerulein plus 30 µg/kg PACAP group, ranging from 1.5 to 2.5 mL and from 0.5 to 2.5 mL, respectively. Amylase level in ascites varied widely. Microscopically, PACAP treatment led to bleeding and parenchymal necrosis in caerulein-induced pancreatitis (Tables 1-3).

**Effect of PACAP on sodium taurocholate-induced pancreatitis**

PACAP treatment insignificantly improved pancreatic edema. Serum amylase was significantly reduced by 10 µg/kg PACAP in sodium taurocholate-induced pancreatitis (*P*<0.05). Hemorrhage was aggravated, and occasionally 2-7 foci of hemorrhage were found on a single section (Tables 1-3).

**PACAP levels in pancreas and duodenum**

In both sodium taurocholate- and caerulein-induced pancreatitis, relative concentrations of PACAP in pancreas and duodenum were found to be significantly increased compared with the normal control (Table 4).

**Effect of PACAP6-27 on normal pancreas**

No gross change in pancreas was found after PACAP6-27 treatment. Serum amylase was significantly elevated compared with normal control. Pancreatic dry weight/wet weight ratio slightly decreased. Microscopically, leukocyte sequestration was evident (Tables 5-7).

**Effect of PACAP6-27 on caerulein-induced pancreatitis**

PACAP6-27 treatment aggravated caerulein-induced pancreatitis. Severe vacuolization of acinar cells, pancreatic bleeding and parenchymal necrosis, characteristics of acute hemorrhagic/necrotizing pancreatitis, were evident (Tables 5-7).

**Effect of PACAP6-27 on sodium taurocholate-induced pancreatitis**

PACAP6-27 treatment augmented serum amylase elevation induced by intraductal injection of sodium taurocholate. The infusion of 100 µg/kg PACAP6-27 led to extensive pancreatic hemorrhage in one case of sodium taurocholate-induced pancreatitis (Tables 5).

**Effect of (4-Cl-D-Phe¹, Leu¹⁷)VIP on normal pancreas, caerulein-induced pancreatitis and sodium taurocholate-induced pancreatitis**

(4-Cl-D-Phe¹, Leu¹⁷)VIP showed similar effects as PACAP6-27 (Tables 5).

### Functional capillary density of pancreas

Pancreatic functional capillary density was slightly but significantly increased in caerulein-induced pancreatitis, whereas other experimental groups showed reduced functional capillary densities compared with controls (Table 8). These findings are consistent with other researches²⁶,²⁷. When examined closely, functional capillary density might not reflect the real scene and could be misleading. Since each group other than the control had pancreatic edema to a greater or less extent, interlobular and intercellular distances were increased, and this

### Table 1 Ascites/peritoneal fluid and their gross appearance (action of PACAP) (mean±SE)

| Group                                      | Ascites | Amylase | Nucleated cells | Saponification | Hemorrhage |
|--------------------------------------------|---------|---------|-----------------|----------------|------------|
| Control                                   | 2134±7  | 1.71±0.02 | 9               | 2              |
| Caerulein-induced pancreatitis            | 39 075±50 341/3  | 7.54±2.85 | 4               | 7              |
| Sodium taurocholate-induced pancreatitis  | 3.55±1.86/12 | 11 463±5 486/10⁴ | 2.9±3.06/8 | 2              |
| PACAP 5 µg/kg                             |         | 3.26±1.89 | 6               | 4              |
| PACAP 10 µg/kg                            |         | 2.3±0.98  | 6               | 4              |
| PACAP 15 µg/kg                            |         | 1.76±1.02 | 11              | 3              |
| Sodium taurocholate+PACAP 5 µg            | 2.33±0.82 | 26 917±11 145/6⁴ | 2.3±0.98  | 2/6           |
| Sodium taurocholate+PACAP 10 µg           | 4.77±2.44 | 5 956±3 546/8⁴ | 1.76±1.02 | 11             |
| Caerulein+PACAP 15 µg                     | 0.55±0.96 | 926/1     | 1               | 1              |
| Caerulein+PACAP 30 µg                     | 0.91±0.83 | 63 918±8 884/8 | 1.73±1.23 | 3              |

*P<0.05 vs normal control.
Table 2: Histological scoring (action of PACAP) (mean±SE)

| Group                                    | Hemorrhage | Parenchymal necrosis | Vacuolization | Inflammation |
|------------------------------------------|------------|----------------------|---------------|--------------|
| Caerulein-induced pancreatitis          | 1.67±0.60  | 2.5±0.49             |               | 1.67±0.60    |
| Sodium taurocholate-induced pancreatitis | 1.5±0.67   | 2.5±0.49             | 0.5±0.49      | 3            |
| PACAP 5 µg                               | 0.33±0.33  | 0.17±0.17            | 0.17±0.17     |              |
| PACAP 10 µg                              | 0.17±0.17  | 0.67±0.49            | 1.33±0.61     |              |
| PACAP 15 µg                              | 0.33±0.33  | 0.17±0.17            | 1.5±0.67      |              |
| PACAP 30 µg                              | 0.17±0.17  | 0.83±0.54            | 1.5±0.5       |              |
| Sodium taurocholate+PACAP 5 µg           | 1.67±0.49  | 2.67±0.33            | 2±0.63        | 3            |
| Sodium taurocholate+PACAP 10 µg          | 2±0.63     | 2.5±0.56             | 1.5±0.56      | 3            |
| Caerulein+PACAP 15 µg                    | 1.5±0.5    | 2±0.63               | 2.5±0.22      | 1.67±0.60    |
| Caerulein+PACAP 30 µg                    | 1.83±0.60  | 2.17±0.31            | 2.33±0.49     | 2.17±0.48    |

Table 3: Dry weight/wet weight ratio of pancreas and serum amylase (action of PACAP) (mean±SE)

| Group                                    | Pancreatic dry weight/wet weight ratio (%) | Serum amylase (IU/L) |
|------------------------------------------|-------------------------------------------|----------------------|
| Normal control                           | 29.21±5.65                                | 520.8±163.27         |
| Caerulein-induced pancreatitis           | 21.83±3.013a                              | 3 699.33±3 826.56    |
| Sodium taurocholate-induced pancreatitis | 17.52±1.505a                             | 3 690.87±2 277.99    |
| PACAP 5 µg                               | 25.86±1.974a                             | 818.09±404.76        |
| PACAP 10 µg                              | 24.81±1.312a                             | 675.55±217.14        |
| PACAP 15 µg                              | 23.88±2.532a                             | 671.36±151.98        |
| PACAP 30 µg                              | 25.17±0.897a                             | 899.09±474.49        |
| Sodium taurocholate+PACAP 5 µg           | 19.18±2.102a                             | 2 944.33±1 182.47    |
| Sodium taurocholate+PACAP 10 µg          | 20.87±5.597a                             | 1 986.91±710.97      |
| Caerulein+PACAP 15 µg                    | 17.66±4.652a                             | 4 053.55±2 164.07    |
| Caerulein+PACAP 30 µg                    | 13.45±2.045a                             | 5 243.46±3 769.73    |

Table 4: ELISA for PACAP in pancreas and duodenum (mean±SE)

| Group                                    | In pancreas | In duodenum |
|------------------------------------------|-------------|-------------|
| Caerulein-induced AP                     | 0.02215±0.01123 (n = 4) | 0.000335±0.000074 (n = 5) |
| Sodium taurocholate-induced AP           | 12.0712±8.40536 (n = 4) | 0.003797±0.002301 (n = 5) |
| Sodium taurocholate+PACAP 5 µg           | 0.2799±0.22932 (n = 4) | 0.001564±0.000529 (n = 4) |

Table 5: Ascites and its gross appearance (effect of PACAP receptor antagonists) (mean±SE)

| Group                                    | Volume of ascites (mL) | Ascites amylase (IU/L) | Nucleated cell count of ascites (×10⁸/L) | Saponification (n) | Hemorrhage (n) |
|------------------------------------------|------------------------|------------------------|----------------------------------------|-------------------|----------------|
| Normal control                           | 213±44/7               | 1.71±1.02/9            | 7.5±2.85/4                             | 2/12              | 3/12           |
| Caerulein-induced pancreatitis           | 3.55±1.86/12           | 11.46±3.15/8           | 2.9±3.06/8                             | 2/12              | 3/12           |
| Sodium taurocholate-induced pancreatitis | 3.42±1.99/6            | 3.44±1.7/6             | 4/6                                    | 2/6               |
| Sodium taurocholate+10 µg PACAP6-27      | 3.52±1.86/6            | 49.46±45.52/6          | 2.35±1.81/6                            | 1/6               | 3/6            |
| Sodium taurocholate+100 µg PACAP6-27     | 3.42±1.99/6            | 64.08±40.65/6          | 3.44±1.7/6                             | 4/6               | 2/6            |
| Sodium taurocholate+10 µg (4-Cl-D-Phe⁶, Leu¹⁷) VIP | 1.200/1               | 5.52±2.38/4            | 3.4/1                                  | 1/1               | 3/12           |
| Sodium taurocholate+100 µg (4-Cl-D-Phe⁶, Leu¹⁷) VIP | 17.200/1              | 1.06±0.51/3            | 3/6                                    | 2/6               |

*P<0.05 vs normal control.
change also occurred in pancreatic microvasculature. It was reasonable to take pancreatic edema into account when calculating functional capillary density. Thus calibrated FCD was introduced. Calibrated FCD = FCD × R/R', where R is the dry weight/wet weight ratio of normal controls and R' is the dry weight/wet weight ratio of experimental group. Here it was assumed that the specific weight of pancreatic tissue was 1, and the pancreatic tissue mass or dry weight experienced no change.

**Table 6** Pancreatic dry weight/wet weight ratio and serum amylase (effect of PACAP receptor antagonists) (mean±SE)

| Group                              | Dry weight/wet weight ratio (%) | Serum amylase (IU/L) |
|------------------------------------|---------------------------------|---------------------|
| Normal control                     |                                 |                     |
| Caerulein-induced pancreatitis     |                                 |                     |
| Sodium taurocholate-induced pancreatitis |                      |                     |
| 10 µg PACAP6-27                    | 17.52±1.05                      | 464.33±2.65         |
| 100 µg PACAP6-27                   | 26.21±1.57                      | 692.17±3.12         |
| Caerulein+10 µg PACAP6-27          | 22.75±3.523                     | 484.33±1.59         |
| Caerulein+100 µg PACAP6-27         | 21.4±1.152                      | 485.5±3.52         |
| Sodium taurocholate+10 µg PACAP6-27| 20.81±3.94*                     | 502.8±3.63         |
| Sodium taurocholate+100 µg PACAP6-27| 17.99±3.594                     | 766.67±4.20         |
| 10 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 25.61±2.389<sup>a</sup> | 337.17±3.34          |
| 100 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 26.77±1.37                      | 781±5.27            |
| Caerulein+10 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 24.33±0.939<sup>a</sup> | 875.33±5.82          |
| Caerulein+100 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 22.36±2.13<sup>a</sup> | 807.83±2.03         |
| Sodium taurocholate+10 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 16.89±1.18<sup>a</sup> | 684.33±993.35       |
| Sodium taurocholate+100 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 18.71±4.048<sup>a</sup> | 264.67±2.83          |

<sup>a</sup>P<0.05 vs normal control.

**Table 7** Histological scoring (effect of PACAP receptor antagonists) (mean±SE)

| Group                              | Hemorrhage | Parenchymal necrosis | Vacuolization | Inflammatory infiltration |
|------------------------------------|------------|----------------------|---------------|--------------------------|
| Caerulein-induced pancreatitis     | 1.5±0.67   | 2.5±0.49             | 0.5±0.49      | 2.5±0.22                 |
| Sodium taurocholate-induced pancreatitis | 1.5±0.5   | 3.5±0.5              | 0.5±0.5       | 2.5±0.5                 |
| 10 µg PACAP6-27                    | 0.83±0.54  | 0.17±0.17            | 1.83±0.60     | 2.5±0.5                 |
| 100 µg PACAP6-27                   | 1.67±0.49  | 1.0±0.52             | 2.17±0.31     | 2.5±0.22                |
| Caerulein+10 µg PACAP6-27          | 1.5±0.67   | 3.5±0.6              | 1.3±0.61      | 2.5±0.22                |
| Sodium taurocholate+100 µg PACAP6-27| 1.5±0.5   | 2.83±0.17            | 2±0.52        | 3.5±0.5                 |
| 10 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 0.33±0.33  | 3.0±0.33             | 2.5±0.33      | 2.0±0.6                 |
| 100 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 1.0±0.63  | 1.67±0.60            | 2.0±0.60      | 2.5±0.22                |
| Caerulein+10 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 0.17±0.17  | 0.17±0.17            | 0.6±0.40      | 2.0±0.5                 |
| Sodium taurocholate+100 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 0.33±0.33  | 1.67±0.49            | 2.17±0.48     | 2.1±0.48                |
| Sodium taurocholate+10 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 1.5±0.56  | 2.5±0.49             | 1.17±0.60     | 3.5±0.5                 |
| Sodium taurocholate+100 µg (4-Cl-D-Phe<sub>6</sub>, Leu<sub>17</sub>) VIP | 1.5±0.67  | 2.33±0.33            | 1.3±0.60      | 3.5±0.5                 |

<sup>a</sup>P<0.05 vs normal control.

When functional capillary density was converted into calibrated functional density, most of the experimental groups showed increased pancreatic calibrated functional capillary densities compared with the normal controls. In caerulein-induced pancreatitis and sodium taurocholate-induced pancreatitis treated with PACAP, calibrated functional capillary densities of pancreas were greatly increased. The most prominent increase in calibrated functional capillary density of pancreas was associated with sodium taurocholate-induced pancreatitis, in which functional capillary density change was usually interpreted as ischaemia.

**DISCUSSION**

Pituitary adenylate cyclase activating peptide (PACAP) is a brain-gut peptide<sup>5</sup>. It stimulates secretion of pancreatic enzyme, bicarbonate and fluid, and produces additive effects on CCK- or carbachol-induced enzyme secretion<sup>8,10</sup>. PACAP is a highly potent vasorelaxant peptide, which can induce vasodilation and increase pancreatic blood flow, notably in the exocrine part of pancreas<sup>11,16</sup>. However, it is unknown whether PACAP takes part in the development of acute pancreatitis and whether PACAP or its antagonists can suppress the progression of acute pancreatitis.

This experiment was carried out to investigate the action of...
PACAP on the development of acute pancreatitis in rats. Treatment with PACAP alone induced mild pancreatitis in some rats. Concurrent administration of PACAP aggravated caerulein-induced pancreatitis. PACAP failed to ameliorate sodium taurocholate-induced pancreatitis. It was reported that PACAP-27 dose-dependently stimulated pancreatic secretion of fluid, bicarbonate, and protein in rats. This effect is mediated by release of both secretin and CCK, and a combination of CCK-A receptor antagonists and anti-secretin serum eliminates the PACAP-stimulated pancreatic secretion[17]. We speculate that PACAP administration increases CCK level, and a higher level of CCK greatly inhibits exocytosis of zymogen granules from pancreatic acinar cells, consequently leading to enhanced intrapancreatic zymogen activation.

We further determined whether PACAP concentration in pancreas was increased with the induction of caerulein- and sodium taurocholate-induced pancreatitis. Since CCK is predominantly released from the intestinal mucosal CCK cells when stimulated by CCK releasing factors[18], it is of interest to know whether duodenal PACAP concentration experiences any change. Moreover, PACAP is found to be present in intestinofugal neurons projecting to pancreatic ganglia. Ganglia in pancreas contains a rich network of PACAP-immunoreactive fibers but no PACAP-positive neurons. It is suggested that some of the PACAP-containing axons in the pancreatic ganglia originate from PACAP-containing myenteric neurons in the duodenum[19]. Thus it is reasonable to measure duodenal PACAP levels as well. Although enzyme-linked immunosorbent assay employed in this study was not an accurate assay, the examination clearly showed that relative concentrations of PACAP in pancreas and duodenum significantly increased in both sodium taurocholate- and caerulein-induced pancreatitis compared with the normal controls, suggesting that PACAP might be involved in the pathogenesis of acute pancreatitis in rats.

Our study showed that PACAP infusion aggravated pancreatitis and its duodenal and pancreatic concentrations were significantly elevated. Thus it is hypothesized that PACAP antagonists might ameliorate acute pancreatitis in rats. PACAP-6-27 is a N-terminal truncated derivative of PACAP-27, its affinity to receptors and ability to activate adenylyl cyclase are greatly reduced compared with PACAP-27. (4-Cl-D-Phe6, Leu17)VIP is found to competitively bind to VPAC1 and VPAC2 receptors (receptors with the similar affinity for VIP and PACAP). Contrary to our expectation, PACAP-6-27 failed to reverse acute pancreatitis in rats. PACAP-6-27 alone could induce mild acute pancreatitis, aggravate caerulein-induced pancreatitis with characteristic manifestations of acute hemorrhagic/necrotizing pancreatitis, and could not improve histopathological changes in sodium taurocholate-induced pancreatitis. (4-Cl-D-Phe6, Leu17)VIP exhibits similar effects as PACAP-6-27. It was reported that [4Cl-D-Phe6, Leu17]VIP at higher concentrations (100 and 300 pmol/L) caused slight increase in amylase release, which might result from slight agonist activity of [4Cl-D-Phe6, Leu17]VIP for the VPAC1 and VPAC2 receptors at high concentrations. Amylase release stimulated by cabachol and CCK is not inhibited by [4Cl-D-Phe6, Leu17]VIP. In fact, [4-Cl-D-Phe6, Leu17]VIP augments release caused by these agents[29]. We conjecture that PACAP-6-27 and [4-Cl-D-Phe6, Leu17]VIP partially agonized the PACAP receptors in this study, and that the PACAP-binding capacity was not saturated and these PACAP receptor antagonists could not displace PACAP from its receptors.

Microcirculatory impairment has been considered an important pathogenic factor in acute pancreatitis[31-35]. Edematous pancreatitis is associated with hyperemia of pancreas. But in acute hemorrhagic/necrotizing pancreatitis (as induced with bile and trypsin[26] and ischemia-reperfusion[27]), intravital microscopy often shows reduced functional capillary densities of pancreas. These findings are supported by the observations with hydrogen clearance technique, electromagnetic flow probe and radioactive isotopes. This kind of change is termed ischemia by some authors. This interpretation has provoked researchers to test the efficacy of various vasodilators in the treatment of acute pancreatitis. However, experiments with vasodilators often yield conflicting results. The dispute over the pancreatic perfusion status in acute hemorrhagic/necrotizing pancreatitis is unsettled.

When functional capillary density of pancreas is discussed in the context of pancreatic edema, this misunderstanding concerning pancreatic perfusion status has been put to an end. Assume that the number of capillaries of the whole organ does not reduce or increase significantly and that the dry weight of the organ remains the same, the length of capillaries in a given area is decided by the change in water content of the organ and the opening or closing of capillaries, calibrated functional capillary density, which combines measurement of functional capillary density with dry weight/wet weight ratio is introduced. The introduction of calibrated FCD by us provides an insight of the pancreatic microcirculatory change. It is obvious that the FCD of pancreas in experimental acute pancreatitis, especially in acute hemorrhagic/necrotizing pancreatitis, was underestimated.

Based on our study, hyperemia or congestion, rather than ischemia, characterizes the pancreatic microcirculatory changes in acute pancreatitis. The reduction of pancreatic blood flow in acute hemorrhagic/necrotizing pancreatitis could be attributed to the great reduction in blood velocity. Mechanisms, including hemodynamic change[26,27,29,30], extensive venular leukocyte adherence[27,31], increase in interstitial pressure[32-34] and intrapancreatic blood flow redistribution[35] other than arteriolar constriction, possibly contribute to pancreatic microcirculatory changes.

The therapeutic value of vasodilators is questionable, whereas vasoconstrictors may possibly be helpful in the treatment of acute hemorrhagic/necrotizing pancreatitis. Vasopressin can ameliorate experimental pancreatitis and reduce mortality rate while maintaining blood supply for vital organs[36]. The finding that endothelin-1 could provide protection against caerulein-induced pancreatitis seems reliable[27]. It should also be pointed out that the action of somatostatin derivatives on acute pancreatitis could be partly attributed to its vasoconstrictor effect[38]. In short, this study demonstrated that intravenous administration of PACAP aggravated acute pancreatitis in rats, and that its antagonists failed to arrest the progression of acute pancreatitis. On the contrary, its antagonist acted very much the same way as itself, indicating that PACAP receptor antagonists at the doses used here might be, in fact, partial agonists. The PACAP concentrations in pancreas and duodenum were elevated significantly in caerulein- and sodium taurocholate-induced pancreatitis, suggesting that PACAP might participate in the pathogenesis of acute pancreatitis. It should be emphasized that functional capillary density of pancreas should be interpreted in the context of pancreatic edema. The introduction of calibrated functional capillary density provides an insight of the real nature of pancreatic microcirculation during acute pancreatitis. Acute hemorrhagic/necrotizing pancreatitis is associated with congestion and capillary stasis rather than ischemia. So different strategies should be adopted to improve pancreatic microcirculatory changes in acute pancreatitis.

REFERENCES
1 Zhou ZG, Chen YD. Influencing factors of pancreatic microcircu-
latory impairment in acute pancreatitis. World J Gastroenterol 2002; 8: 4064-4066
2 Hofbauer B, Saluja AK, Lerch MM, Bhagat L, Bhattacharjee M, Lee HS, Frossard JL, Adler G, Steer ML. Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. Am J Physiol 1998; 275: G352-G362
3 Leach SD, Modlin IM, Scheele GA, Gorelick FS. Intracellular activation of digestivezymogens in rat pancreatic acini. Stimulation by high doses of cholecystokinin. J Clin Invest 1991; 87: 362-366
4 Parain H, Mayo A, Parain D, Neufeld D, Shwartz I, Zissin R, Singer P, Kaplan O, Skornik Y, Freund U. Octreotide treatment in patients with severe acute pancreatitis. Dig Dis Sci 2000; 45: 2247-2251
5 Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev 2000; 52: 269-324
6 Sandler F, Ekblad E, Absood A, Hakanson R, Kovas K, Arimura A. Pituitary adenylate cyclase activating peptide: a novel vasoactive intestinal peptide-like neuropeptide in the gut. Neuroscience 1992; 46: 439-454
7 Hannibal J, Fahrenkrug J. Pituitary adenylate cyclase-activating polypeptide in intrinsinc and extrinsic nerves of the rat pancreas. Cell Tissue Res 2000; 299: 99-70
8 Schmidt WE, Seebeck J, Hocker M, Schwarzroff R, Schafer H, Fornefeld H, Morys-Wortmann C, Folsch UR, Creutzfeldt W. PACAP and VIP stimulate enzyme secretion in rat pancreatic acini via interaction with VIP/PACAP-2 receptors: additive augmentation of CCK/carbachol-induced enzyme release. Pancreas 1993; 8: 476-487
9 Raufman JP, Malhotra R, Singh L. PACAP-38, a novel peptide from ovine hypothalamus, is a potent modulator of amylase release from dispersed acini from rat pancreas. Regul Pept 1991; 36: 121-129
10 Onaga T, Okamoto K, Harada Y, Mineo H, Kato S. PACAP stimulates pancreatic exocrine secretion via the vagal cholinergic nerves in sheep. Regul Pept 1997; 72: 147-153
11 Borboni P, Porzio O, Pierucci D, Cicconi S, Magnaterra R, Federici M, Sesti G, Lauro D, D'Agata V, Cavallaro S, Marlier LN. Molecular and functional characterization of pituitary adenylate cyclase-activating polypeptide (PACAP-38)/vasoactive intestinal polypeptide receptors in pancreatic beta-cells and effects of PACAP-38 on components of the insulin secretion system. Endocrinology 1999; 140: 5530-5537
12 Davalli AM, Bertuzzi F, Meoni C, Scaglia L, Socci C, Pozza G, Pontioli AE. Insulin and intracellular calcium responsiveness to glucagon-like peptide-1 and pituitary adenylate cyclase-activating peptide by dispersed adult porcine islet cells. Transplantation 1999; 67: 174-176
13 Yada T, Sakurada M, Ishihara H, Nakata M, Shioda S, Yaeura K, Hamakawa N, Yanagida K, Kikuchi M, Oka Y. Pituitary adenylate cyclase-activating polypeptide (PACAP) is an islet substance serving as an intra-islet amplifier of glucose-induced insulin secretion in rats. J Physiol 1997; 505(Pt 2): 319-328
14 Carlsson PO, Ostenson CG, Efendic S, Langel U, Jansson L. Pituitary adenylate cyclase activating peptide (PACAP) redistributes the blood within the pancreas of anesthetized rats. Regul Pept 1996; 63: 123-129
15 Ito O, Naruse S, Kitagawa M, Ishiguro H, Ko S, Nakajima M, Hayakawa T. The effect of VIP/PACAP family of peptides on pancreatic blood flow and secretion in conscious dogs. Regul Pept 1998; 78: 105-112
16 Filipsson K, Tornoe K, Holst J, Ahren B. Pituitary adenylate cyclase-activating polypeptide stimulates insulin and glucagon secretion in humans. J Clin Endocrinol Metab 1997; 82: 3093-3098
17 Lee ST, Lee KY, Li P, Coy D, Chang TM, Chey WY. Pituitary adenylate cyclase-activating peptide stimulates rat pancreatic secretion via secretin and cholecystokinin receptors. Gastroenterology 1998; 114: 1054-1060
18 Liddle RA, Misukonis MA, Pacy L, Balber AE. Cholecystokinin cells purified by fluorescence-activated cell sorting respond to monitor peptide with an increase in intracellular calcium. Proc Natl Acad Sci U S A 1998; 95: 5147-5151
19 Kirchgesner AL, Liu MT. Pituitary adenylate cyclase activating peptide (PACAP) in the enteropancreatic innervation. Anat Rec 2001; 262: 91-100
20 Pandol SJ, Dharmathorn K, Schoeffield MS, Vale W, Rivier J. Vasoreactive intestinal peptide receptor antagonist [4Cl-D-Phe6, Leu11] VIP. Am J Physiol 1986; 250: G553-G557
21 Pluszcz T, Borsari S, Westermann S, Menger M, Feifel G. ET-1 induces pancreatitis-like microvascular deterioration and acinar cell injury. J Surg Res 1999; 85: 301-310
22 von Doschuetz E, Hoffmann T, Messmer K. Inhibition of neutrophil proteinases by recombinant serpin Lex032 reduces capillary no-reflow in ischemia/reperfusion-induced acute pancreatitis. J Pharmacol Exp Ther 1999; 290: 782-788
23 Pluszcz T, Westermann S, Rathgeb D, Feifel G. Acute pancreatitis in rats: effects of sodium taurocholate, CCK-8, and secretin on pancreatic microcirculation. Am J Physiol 1997; 272: G310-G320
24 Kerner T, Vollmar B, Menger MD, Waldner H, Messmer K. Determinants of pancreatic microcirculation in acute pancreatitis in rats. J Surg Res 1996; 62: 165-171
25 Hoffmann TF, Leiderer R, Waldner H, Arboagst S, Messmer K. Ischemia reperfusion of the pancreas: a new in vivo model for acute pancreatitis in rats. Res Exp Med (Berl) 1995; 195: 125-144
26 Klar E, Schratt W, Foytik T, Buhr H, Herfarth C, Messmer K. Impact of microcirculatory flow pattern changes on the development of acute edematous and necrotizing pancreatitis in rabbit pancreas. Dig Dis Sci 1994; 39: 2639-2644
27 Menger MD, Bonhoff K, Vollmar B. Ischemia-reperfusion-induced pancreatic microvascular injury. An intravital fluorescence microscope study in rats. Dig Dis Sci 1996; 41: 823-830
28 Schmid-Schoenbein GW, Zweifach BW, Kovalcheck S. The application of stereological principles to morphometry of the microcirculation in different tissues. Microvasc Res 1977; 14: 303-317
29 Horton JW, Burnweit CA. Hemodynamic function in acute pancreatitis. Surgery 1988; 103: 538-546
30 Ais G, Lopez-Farre A, Gomez-Garre DN, Cacho R, Romeo JM, Braquet P, Lopez-Novoa JM. Role of platelet-activating factor in hemodynamic derangements in an acute rodent pancreatic model. Gastroenterology 1992; 102: 181-187
31 Bloechle C, Kusterer K, Kuhn RM, Schneider C, Knoefel WT, Izbicki JR. Inhibition of bradykinin B2 receptor preserves microcirculation in experimental pancreatitis in rats. Am J Physiol 1998; 274: G42-G51
32 Widdison AL, Alvarez C, Schwartz M, Reber HA. The influence of ethanol on pancreatic blood flow in cats with chronic pancreatitis. Surgery 1992; 112: 202-208, discussion 208-210
33 Reber HA, Karanja ND, Alvarez C, Widdison AL, Leung FW, Ashley SW, Lutrinf FJ. Pancreatic blood flow in cats with chronic pancreatitis. Gastroenterology 1992; 103: 652-659
34 Karanja ND, Singh SM, Widdison AL, Lutrinf FJ, Reber HA. Pancreatic ductal and interstitial pressures in cats with chronic pancreatitis. Dig Dis Sci 1997; 37: 268-273
35 Schmunn J, Waldner H, Vollmar B, Goetz A, Conzen P, Schweiberer L, Brendel W. Pancreatic blood flow at micro- and macro-scale in experimental pancreatitis. Br J Surg 1988; 75: 1254-1255
36 Andreous P, Kiriakou K, Tountas C. Vasopressin in the treatment of acute experimental pancreatitis. Ann Surg 1967; 166: 913-918
37 Kogire M, Inoue K, Higashide S, Takaori K, Echigo Y, Gu YJ, Sumi S, Uchida K, Imamura M. Protective effects of endothelin-1 on acute pancreatitis in rats. Dig Dis Sci 1995; 40: 1207-1212
38 Chen CC, Wang SS, Lee FY, Tsay SH, Wu SL, Lu RH, Chang FY, Lee SD. Prophylactic octreotide reduces the severity of histopathological changes and hemorrhagic shock in early taurodeoxycholate-induced experimental pancreatitis. Proc Natl Sci Counc Repub China B 1999; 23: 1-6