INTRODUCTION

Apelin was discovered and isolated from the bovine stomach in 1998 by Tatemoto and co-workers (1) as an endogenous ligand for the G protein-coupled orphan receptor (APJ). Several molecular isoforms of apelin, consisting 36, 17, 13 and 12 amino acids, were described (1, 2). All those isoforms are produced during posttranslational processing of a 77 amino acid pre-prohormone. Apelin is expressed in the gastrointestinal (GI) tract, heart, brain, lung, kidney, skeletal muscle, pregnant and lactating breast what was confirmed by real-time PCR studies (2-4). Northern blotting analysis showed 4-fold higher apelin mRNA expression in the stomach as compared to the duodenum, jejunum and colon, and 2-fold higher expression in comparison to the ileum (4). Immunohistochemistry studies revealed apelin peptide stores in gastric epithelial cells in vesicle-like structures adjacent to the lumen of the gastric glands, and failed to identify apelin-containing cells in the duodenum (4).

Apelin is also expressed in adipose tissue, suggesting adipokine functions. Accordingly, plasma apelin positively correlates in humans with body mass index (BMI), being ca. 4-fold higher in obese than in non-obese individuals (5). Apelin was shown to control pituitary hormone release and body fluid equilibrium, lower blood pressure, stimulate drinking behaviour in rats, and inhibit proinflammatory cytokine production (2, 6-9). The role of apelin in controlling the GI system remains still unclear.

In mice, concentrations of circulating apelin depend upon the nutritional status. Apelin levels are low in the fasting state and restored to normal by re-feeding (10). In streptozotocin-treated mice, a dramatic fall in insulin production along with a strong decrease in apelin expression were observed (10). These data suggest that insulin may regulate apelin gene expression and secretion.

The apelin receptor (APJ) is expressed in pancreatic β-cells (11), suggesting a direct regulation of insulin secretion and/or release. Indeed, apelin-36 was shown to inhibit glucose-stimulated insulin secretion in mice in vivo and in vitro studies (12). Apelin also inhibited the insulin response to intravenous glucose in mice fed high-fat diet (10). Boucher et al. (10) showed that apelin acts as a negative feedback signal to inhibit insulin secretion in situations of high insulin levels. Since endocrine pancreatic hormones like insulin, control the function of the exocrine pancreas (insulo-acinar axis), apelin could potentially affect the exocrine pancreas though it has not been studied so far.

THE EFFECT OF EXOGENOUS APELIN ON THE SECRETION OF PANCREATIC JUICE IN ANAESTHETIZED RATS

Apelin is known to stimulate cholecystokinin (CCK) and inhibit insulin release, however the mechanisms on pancreatic secretion remain unclear. The present study aimed to determine the expression of apelin and apelin receptor in the pancreas by immunofluorescence studies and the effect of exogenous apelin on the secretion of pancreatic juice in anesthetized rats. Pancreatic-biliary juice (P-BJ) was collected from Wistar rats treated with apelin (10, 20 and 50 nmol/kg b.w., boluses given every 30 min intravenously or intraduodenally). The same apelin doses were administered to rats subjected to intraduodenal tarazapide, capsaicin or vagotomy. Pancreatic blood flow was measured by a laser doppler flowmeter. Direct effects of apelin were tested on dispersed acinar cells. Apelin receptor was expressed on acinar cells, pancreatic duct and islets cells, whereas apelin in pancreatic acini, but not in the islets. Intravenous apelin decreased P-BJ volume, protein and trypsin outputs in a dose-dependent manner. In contrast, intraduodenal apelin stimulated P-BJ secretion. Pharmacological block of mucosal CCK1 receptor by tarazepide, vagotomy and capsaicin pretreatment abolished the effects of intravenous and intraduodenal apelin on P-BJ volume, protein and trypsin outputs. Apelin decreased the pancreatic blood flow. Apelin at 10^{-6} M increased the release of amylase from non-stimulated and CCK-8-stimulated acinar cells. In conclusion, apelin can affect the exocrine pancreas through a complex mechanism involving local blood flow regulation and is driven by vagal nerves.

Key words: apelin, capsaicin, cholecystokinin, pancreas, regulation, vagotomy, acinar cells, pancreatic blood flow
Apelin is produced in both exocrine and endocrine gastric cells (4, 13). Strong expression of apelin was reported in the rat stomach-oxyntic epithelium and in the neck region of the stomach mucosa but not in the muscular layer. Susaki et al. (13) detected apelin peptide in mucous neck cells, parietal cells and chief cells. The localization of apelin in gastric exocrine cells suggests that apelin is secreted into the gastric lumen in response to secretagogues that activate gastric exocrine cells (4). Tatemoto et al. (14) have already demonstrated that apelin may stimulate cholecystokinin (CCK) secretion. Their findings were confirmed by Wang et al. (4), who demonstrated involvement of the mitogen-activated protein kinases (MAPK) signal induction pathway in CCK release from murine small-intestinal cell line STC-1 cells by apelin-13 in vitro. Thus gastric apelin, like leptin, when secreted into the gastric lumen, may reach the intestinal lumen and stimulate CCK release (15, 16). Previous studies showed that exogenous apelin in controlling the secretion of pancreatic juice and to determine the mechanisms involved.

MATERIAL AND METHODS

Animals

The animal studies were approved by the Local Ethics Committee. A total of 92 Wistar male rats (200±15 g of body weight) were used. Animals were housed in a light- and temperature-controlled room with free access to standard food and water. The rats were fasted during the night before the experiment.

Immunofluorescence studies

Apelin and APJ receptor expression were examined using immunofluorescence and confocal microscopy. Rats (n=6) were slaughtered and tissue samples of pancreas, stomach (corpus, fundus, pylorus), duodenum and jejunum were embedded in OCT embedding matrix (CellPath), immediately frozen in liquid nitrogen and stored at –80°C. Tissues were cut into 15 µm slices in cryostat and were placed on silanized microscope slides. Slides were incubated in 1% bovine serum albumin in PBS for 1 hour to block nonspecific reaction. For visualization of apelin and APJ receptor in pancreas, sections were incubated with rabbit anti-apelin or anti-APJ receptor antibody (dilution 1:100) (Abcam, UK) for 1 hour at room temperature. After incubation sections were rinsed twice in PBS and labelled with secondary antibodies DyLight 488-goat anti-rabbit antibody (dilution 1:500) (Jackson Immuno Research, USA) for 1 hour at room temperature. Counterstaining of nuclei was performed using 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA) for 1 minute at room temperature. The slides were analysed using an FV 500 laser scanning confocal microscope (Olympus Optical Co., Hamburg, Germany).

Animal preparation for pancreatic-biliary secretion studies

Under mixed xylazine (12 mg/kg body weight, i.m., Rometar, SPOFA, Praha, Czech) and ketamine (35 mg/kg b.w., i.m., Bioketan, Biowet, Gorzow, Poland) anaesthesia 60 rats were surgically prepared for pancreatic-biliary juice (P-BJ) collection. Body temperature was continuously controlled and maintained by heating lamps. The right external jugular vein was catheterized with silicone tube and fixed with ligatures. Continuous intravenous infusion of saline (0.9% NaCl, peristaltic pump speed 2 ml/h) started immediately after cannulation and was continued until the end of the experiment. The infusion was stopped during intravenous apelin injections. Following midline laparotomy, polyethylene tube was inserted into the common pancreatico-biliary duct for collection of pancreatic-biliary juice. A second polyethylene tube was inserted into the duodenum through the pylorus, and secured by silk ligature for the reintroduction of P-BJ and apelin administration. The P-BJ was infused into the duodenum at the rate of secretion, and apelin boluses were administered as described further below. The anaesthesia was maintained during the experiment by intraperitoneal administration of anaesthetics.

The involvement of vagal nerves was examined following subdiaphragmatic vagotomy and capsaicin differentiation as previously described (21, 17). Involvement of the duodenal mucosal CCK1 receptor was studied (20) following intraduodenal administration of a selective CCK1 receptor antagonist, tarazepide (generously supplied by Solvay Pharmaceuticals GmbH, Hannover, Germany).

Experimental protocol

Collection of P-BJ started immediately after surgery. P-BJ was collected in 15 min intervals into 1.5 ml polyethylene tubes held on ice. Vehicle (0.9% NaCl) and three apelin-13 boluses (10, 20 and 50 nmol/kg b.w.; Apelin-13, Phoenix Pharmaceutical Inc., Belmont, USA) were given intravenously every 30 min. A total of 10 series of experiments, each consisting of 6 rats, was performed to test the effect of apelin-13 on P-BJ secretion. In the first series, the effect of apelin-13 administered alone (i.v.) was studied. In the second series, the effect of apelin-13 given i.v. on P-BJ secretion was determined in vagotomized rats. In the third series, the effect of apelin-13 (i.v.) on P-BJ secretion was determined in capsaicin-pretreated rats. In the fourth series, the effect of apelin-13 i.v. following tarazepide on P-BJ secretion in rats was determined. The same protocol (4 series) was applied for apelin-13 administration into the duodenum. The in vivo study was resumed with respective control collections with intravenous or intraduodenal vehicle instead of apelin-13 administrations, and following subdiaphragmatic vagotomy, capsaicin deafferentation, and duodenal administration of tarazepide (5 control series).

The P-BJ samples were weighed and 0.1 ml P-BJ samples were stored at –20°C for further analyses. Samples were analysed for total protein using the Lowry method, performed on 96-well microwell plates with bovine serum albumin (Sigma, USA) as the standard. Intra- and inter-assay variations for protein determination were 3.1 and 3.6%, respectively. Trypsin (EC 3.4.21.4) activities were estimated after enterokinase (Sigma-Aldrich) estimation using N-alpha-benzoyl-DL-arginine-p-nitroanilide (Sigma) as the substrate (17). Intra- and inter-assay variations for the trypsin determination were 2.8 and 3.2%, respectively.

Intragastric apelin administration

A total of 14 rats were used to examine the effect of intragastric administration of apelin on plasma CCK
concentration. **Ad libitum** fed rats received either vehicle (saline) or apelin-13 (50 nmol/kg b.w. daily) by stomach tube for 2 weeks. Twelve hours after the last administration of apelin-13, venous blood samples were harvested, centrifuged and plasma CCK was measured using a commercially available RIA-kit for CCK (cat. no. RK-069-04, Phoenix Pharmaceutical Inc.). A non-sulfated CCK (26-33) octapeptide fragment was used as the standard. The antibody shows 100% cross reactivity with CCK (26-33) non-sulfated fragment, porcine CCK-33, human gastrin-1, and human big gastrin-1, 78% cross reactivity with CCK octapeptide sulfated fragment, 63% cross reactivity with CCK 27-33 fragment, and 14% with CCK 30-33 fragment, and no cross reactivity with human pancreatic polypeptide (PP), and human, porcine or rat vasoactive intestinal peptide (VIP). The detection limit was 60.3 pg/ml.

**Preparation of dispersed acinar cells**

Dispersed acinar cells were obtained from rat pancreas by collagenase digestion (21), CCK-8-stimulated (10⁻⁸ M), and incubated with apelin (10⁻⁹–10⁻⁶ M) in vitro. Pancreatic tissue was incubated in 10 mM HEPES buffered Ringer-solution (HR) containing 100 U/ml collagenase, 6 mM glucose, 0.5% BSA, 0.1% SBTI, and Eagle’s minimum amino acid supplement. After 30 min incubation at 37°C under O₂ saturation in a shaking water bath, acini were dispersed using mild shearing forces, passed through double filter gauze and purified by sedimentation in 4% BSA. Pancreas acini were washed three times in HR and added in aliquots to prepared vials containing apelin-13 with or without CCK8. The vials were incubated under O₂ saturation at 37°C in a shaking water bath for 30 min. The reaction was stopped by placing the vials on ice and cell were separated by centrifugation. Amylase released into the medium and total acinar amylase content were measured by modified Bernfeld method using starch as a substrate. All experiments were performed in triplicates. Amylase released was expressed as a percentage of initial total amylase content.

**Determination of pancreatic blood flow**

Rats were anesthetized with ketamine (50 mg/kg i.p., Bioketal, Vetoquinol Biowet, Gorzow Wielkopolski, Poland). The abdominal cavity was opened and pancreatic blood flow was measured by a laser Doppler flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Jarfalla, Sweden), as described previously (23). Apelin boluses were administered intravenously in doses of 20 nmol/kg b.w.

**Statistical analysis**

Results were calculated as means (±SEM) for 15 min P-BJ collection intervals. The data pertaining to the effect of apelin-13 on P-BJ were expressed in absolute values. One-way analysis of variance for repeated measures was followed by Tukey’s post-test and linear trend analysis (GraphPad Prism v. 3, Graph Pad Software, San Diego, CA, USA) to investigate the relationships between the dose of apelin and P-BJ secretory response. The t-test was used to analyse the differences between control and apelin experiments. A value of P<0.05 was considered statistically significant.

**RESULTS**

**Immunofluorescence studies**

Immunofluorescence analysis of pancreas slides showed apelin immunoreactivity in pancreatic acini, but not in the...
Fig. 2. Representative microphotographs of: (A) APJ receptor localization (red fluorescence) in fundus with visualization of neurofilaments (green fluorescence); (B) apelin localization (red fluorescence) in fundus with visualization of neurofilaments (green fluorescence); (C) APJ receptor localization (red fluorescence) in mucosal membrane of jejunum with visualization of neurofilaments (green fluorescence); (D) apelin localization (red fluorescence) in mucosal membrane of jejunum with visualization of neurofilaments (green fluorescence). Bar=100 µm.

Fig. 3. Effect of intravenous (i.v.) bolus infusions of apelin-13 (0, 10, 20 and 50 nmol/kg body weight) on pancreatico-biliary juice (P-BJ) volume (top) and protein output (bottom) in anaesthetized rats. From the left in each group of bars, the open bar represents control secretion after i.v. vehicle, the black bar - secretion after i.v. apelin-13 alone, and the next three, respectively, secretion after i.v. apelin-13 in combination with capsaicin, tarazepide, and vagotomy pretreatment. Each bar represents a 15 min P-BJ sample after i.v. infusion of vehicle alone or apelin-13. Mean and S.E.M. (n=6). The mean value is significantly different from the respective vehicle infusion (one-way ANOVA for repeated measurements followed by Tukey's test, * P<0.05, ** P<0.01, *** P<0.001).
islets (Fig. 1A). The expression of APJ receptor was observed in the acinar cells, pancreatic duct and islets cells (Fig. 1B). In the duodenum, apelin expression was expressed on the villi, mainly on their top, but not in the crypts (Fig. 1C). The APJ expression was detected in the tunica mucosa, in particular in the duodenal epithelium (Fig. 1D). No apelin peptide and no APJ expression was found in the cardiac region of the stomach. In the fundic region, apelin peptide and APJ expression were abundant (Fig. 2A and 2B). APJ expression was much higher in the fundic glands than in the submucosal layer. In the pyloric region, weak apelin immunofluorescence and no APJ expression was detected. In the jejunum, apelin and APJ expression were located mostly in the upper half part of the villi. No apelin, was found in jejunum crypts, which weakly expressed APJ. In the jejunum, a colocalization of apelin peptide and APJ with neurofilaments was observed (Fig. 2C and 2D).

Pancreato-biliary juice secretion in anaesthetized rats

Intravenous administration of apelin-13 (20 and 50 nmol/kg b.w.) significantly reduced P-BJ volume and pancreatic output. The effect was dose-dependent as indicated by linear trend analysis (P<0.001). Capsaicin and tarazepide pretreatment abolished the effects of intravenous apelin on P-BJ (Fig. 3). In contrast to intravenous administration, intraduodenal application of apelin-13 (20 and 50 nmol/kg b. w.) stimulated P-BJ volume and protein output. The effect was dose-dependent (linear trend analysis, P<0.001). Capsaicin and tarazepide treatment as well as vagotomy abolished intraduodenal stimulation by exogenous apelin-13 (Fig. 4). The effects of intravenous and intraduodenal apelin-13 boluses on trypsin activity were similar to those on respective protein outputs (data not shown).

Effect of intragastric administration of apelin on cholecystokinin release

Intragastric administration of apelin-13 for 2 weeks resulted in a significant increase in basal plasma immunoreactive-CCK concentration as compared to vehicle-treated controls (control 348±31 pg/ml vs. 436±23 pg/ml; unpaired t-test, P=0.04).

Effect of apelin-13 on amylase release from acinar cells

The highest dose of apelin-13 (10^{-6} M) stimulated basal and potentiated CCK-8-stimulated amylase release from acinar cells (Fig. 5), whereas lower doses (10^{-7}-10^{-8} M) of apelin-13 were ineffective.

Effect of apelin-13 on pancreatic blood flow

Intravenous administration of apelin-13 boluses (20 nmol/kg b.w.) decreased pancreatic blood flow. The effect was immediate (started within a minute) and lasted for up to 8 min (Fig. 6).
DISCUSSION

Our immunofluorescence study confirmed that apelin is produced in both gastric exocrine and endocrine cells (13). Apelin peptide was detected in mucous neck cells, parietal cells and chief cells but not in parietal cells in the neck region. Parietal cells localized at the very top of gastric glands (i.e. at the upper part of the isthmus), destined for apoptosis, showed apelin staining. Apelin-positive cells were not identified in the stomach muscle. Localization of apelin in gastric exocrine cells suggests that apelin may be secreted into the gastric lumen in response to secretagogues. This makes sense to physiological regulation of pancreatic secretion by some apelin-related luminal mechanisms. Immunohistochemical examination of

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**Fig. 5.** The effect of apelin-13 (10^{-10}-10^{-6} M) on amylase release from non-stimulated (top) and stimulated with 10^{-10} M CCK-8 (bottom) rat dispersed pancreatic acinar cells in vitro. The amylase release results were calculated as the concentration of amylase per mg of total protein. The results expressed are means and S.E.M. taken from 6–8 separate experiments (one-way ANOVA *P<0.05).

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**Fig. 6.** Effect of intravenous (i.v.) bolus infusions of apelin-13 (20 nmol/kg body weight) on pancreatic blood flow in anaesthetized rats. Mean and S.E.M. (n=6). Arrow indicates apelin injection. The mean value is significantly different from the respective vehicle infusion (one-way ANOVA for repeated measurements followed by Tukey’s test, * P<0.05, ** P<0.01, *** P<0.001).
adult rat stomach showed abundant apelin peptide in the cytoplasm of epithelial cells in the oxyntic mucosa (4). A high-power magnification indicates that apelin peptide is stored in vesicle-like structures and that apelin staining is localized adjacent to the lumen of the gastric glands. Immunohistochemical examination of the adult human stomach showed apelin staining in the gastric epithelium, in rats duodenum failed to identify apelin-containing cells. In the stomach epithelium, APJ immunostaining was associated with the cell membrane and cytoplasm (27).

The APJ receptor is expressed in rat and human intestine (8, 26, 28). A strong APJ immunostaining was also present in the smooth muscle layer of the rat stomach, duodenum and colon. In contrast to the surface epithelium, APJ immunostaining the smooth muscle layer was robust at all ages examined, APJ immunostaining was associated primarily with the cell membrane and some APJ immunostaining was observed in the cytoplasm. APJ immunostaining was also observed in the venules of intestinal villi and in endothelial cells.

To our knowledge this is the first report showing that exogenous apelin-13 may control exocrine pancreas function in anaesthetized rat. Importantly, depending on the route of administration of apelin, intravenous vs. intraduodenal, we observed opposite effects on P-BJ secretion, suggesting different mechanisms involved in the regulation by circulating and luminal apelin. Although APJ expression was found on the acinar cells and apelin expression was found in the pancreatic islets like in other species (29), the direct mechanism on pancreatic acini is rather doubtful, since only the highest (apparently pharmacological) dose of apelin was effective in vitro. Our results corroborate with study by Flemstrom et al. (24) who found that apelin do not induce release of amylase from the pancreatic acini bioassay system. Low number of apelin IR islet cells in rodents as compared to human islets may explain our results.

An apparent increase in circulating apelin following intravenous application led to significant dose-dependent inhibition of pancreatic secretion (Fig. 3). This is in agreement with earlier studies by Sorhede Winzell et al. (12) who showed inhibition of insulin secretion in mice treated with apelin-36. In humans, plasma apelin levels correlate positively with BMI (5). It seems, therefore, that circulating apelin may control the secretion of pancreatic juice by insulo-acinar axis. If human apelin operates through a similar mechanism to that found in mice and rats, apelin may be important for reducing pancreatic exocrine secretion and thereby food digestibility.

Another mechanism involving circulating apelin is reduction of local blood flow observed in our study. The rate of pancreatic secretion positively correlates with pancreatic blood flow, thus flow reduction would result in the inhibition of the secretion of pancreatic juice.

Tatemoto et al. (25) demonstrated that apelin is located within the endothelia of small arteries in the liver, spleen, lung, pancreas, intestine, kidney, and adipose tissues. The presence of apelin receptors and apelin in the heart and blood vessels suggest that this peptide may have a cardiovascular role. Apelin peptides were found to significantly lower arterial blood pressure (25, 26) as well as to reduce blood flow in the present study. The depressor response of apelin in anaesthetized rats was accompanied by an increase of the plasma concentrations of nitrite/nitrate (NOx) and was inhibited by pretreatment with the nitric oxide synthase inhibitor (25).

In contrast to intravenous administration, the intraduodenal boluses of apelin led to dose-dependent stimulation of pancreatic juice volume and protein (trypsin) output. The effect was dependent on an intact mucosal CCK1-receptor mechanism that operates in the duodenum (20). Our conclusion is supported by results obtained by Tatemoto et al. (14) who showed that luminal apelin can stimulate the release of endogenous CCK as well as by our observation in rats receiving CCK by gastric gavage. Participation of duodenal CCK1-receptor mechanism was confirmed by abolishing intraduodenal apelin effects during pharmacological blockade of the duodenal mucosal CCK, receptor, degeneration of vagal afferent fibers, or vagotomy (Fig. 2). Our pilot studies showed no change in circulating apelin after intraduodenal administration of apelin (unpublished data), thus suggesting presence of two distinct pools of apelin, circulating and luminal, which may be released concomitantly but play divergent roles in controlling exocrine pancreas.

In conclusion, circulating and luminal apelin may control the secretion of rat pancreatic juice through distinct, indirect mechanisms. The direct effect on pancreatic acini is apparently pharmacological.

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