CCK-, secretin-, and cholinergic-independent pancreatic fluid hypersecretion in protease inhibitor-treated rats

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IT IS WELL KNOWN THAT the presence of trypsin and chymotrypsin in the proximal small intestine exerts a negative feedback regulation on pancreatic exocrine secretion in rats (5, 8, 16, 20, 40), guinea pigs (15), pigs (11), and humans (13, 19, 22, 32, 33). Inhibition of luminal proteolytic activities by intraduodenal infusion of soybean or synthetic trypsin inhibitors (6, 8, 19, 30, 31, 40) or intact protein (17, 18) or by diversion of biliary-pancreatic secretions away from the small intestine (5, 8, 20) has been shown to increase plasma concentrations of cholecystokinin (CCK) (6, 16, 30, 40) and secretin (31, 40) and to strongly stimulate pancreatic exocrine secretion (6, 16, 30, 40). In our previous studies (29, 30), we have demonstrated that oral administration of the synthetic trypsin inhibitor camostat mesylate has two different stimulatory effects, immediate and delayed, on pancreatic fluid secretion. Oral or intraduodenal administration of camostat mesylate stimulates an increase in plasma levels of endogenous CCK (6, 30, 31, 40), irrespective of whether rats were fed or fasted, causing an immediate pancreatic exocrine secretion. The immediate pancreatic fluid hypersecretion occurred within 30 min, whereas the delayed hypersecretion was observed only in fed rats at 6–24 h (peak increase at 12 h) after camostat feeding (29, 30). Moreover, the early effects of camostat on pancreatic exocrine secretion were abolished in in vitro isolated perfused pancreas, in which humoral and neural influences on the pancreas are eliminated, whereas the late effects were still observed in the absence of humoral and neural influences (29, 30).

The present experiments were designed therefore to further clarify the underlying mechanism of the delayed pancreatic fluid hypersecretion after camostat treatment. In the present study, we used a newly synthesized protease inhibitor (PI), ONO-3403, which is orally active and, in comparison to camostat, is 5–10 times more potent in inhibiting protease activities and has a longer duration of action (35).

MATERIALS AND METHODS

Materials

Synthetic PI (ethyl N-allyl-N-(E)-2-methyl-3-[4-(4-amidino-phenoxycarbonyl)phenyl]propanoylamino acetate methanesulfonate; ONO-3403) was a generous gift from Ono Pharmaceutical (Osaka, Japan). A potent and specific CCK-A receptor antagonist, loxiglumide, was obtained from Kaken Pharmaceutical (Tokyo, Japan).

We purchased the following materials: cholinergic receptor antagonist atropine sulfate from Nacalai Tesque (Kyoto, Japan), antisecretin rabbit serum from Peptide Research Institute (Osaka, Japan), 5-bromo-2-deoxyuridine (BrdU) labeling and detection kit II from Boehringer Mannheim Biochemica (Mannheim, Germany), and DST800 multitracer system from Radiometer (Copenhagen, Denmark).

Animals and Treatments

Male Wistar rats weighing 220–250 g were used in all experiments. The animals were kept at 22°C on a 12:12-h light-dark cycle with free access to water and a standard laboratory diet containing 53% carbohydrates, 25% protein, and 6% fat (Oriental Yeast, Tokyo, Japan). PI dissolved in 0.15 M NaCl (20 mg·2 ml–1·kg body wt–1) or the same volume of 0.15 M NaCl solution (2 ml/kg body wt) was given via an orogastric tube directly into the stomach.

Secretory Studies

PI was given to awake rats by an orogastric tube, and rats were kept with free access to water and a standard laboratory rat diet until surgery. Pancreatic exocrine function was studied at specified time points after oral administration of PI by the method described in detail previously (38). Briefly, rats were laparotomized under urethane anesthesia (1.6 g/kg body wt sc). The proximal end of the bile duct was ligated near the liver, and the bile was drained into the duodenum through a
Experimental Protocol

Basal pancreatic secretion. The basal pancreatic fluid secretion was collected for 60 min, and fluid volume, protein output, bicarbonate and chloride concentrations were determined before and 3, 6, and 12 h after oral PI administration. To examine the underlying mechanism of the fluid hypersecretion in PI-treated rats, the following experiments were performed in rats at 12 h after oral PI administration. In these experiments, PI was given at 12:00 AM to awake rats by an orogastric tube, and rats were kept with free access to water and a standard laboratory rat diet. Surgery was started at 10:30 AM, and pancreatic fluid secretion was collected for 120 min, from 11:00 AM to 1:00 PM. First, a potent and specific CCK-A receptor antagonist, loxiglumide, at a dose of 50 mg·kg body wt⁻¹·h⁻¹, or the cholinergic receptor antagonist atropine sulfate at a dose of 100 µg·kg body wt⁻¹·h⁻¹ was intravenously infused at a rate of 1 ml/h for 60 min, from 12:00 to 1:00 PM. Pancreatic fluid was collected from 60 min before to 60 min after the commencement of the antagonist infusion. This dose of loxiglumide (26) or atropine (23) can effectively suppress basal or stimulated pancreatic fluid secretion. In addition, antisecretin rabbit serum was administered by intravenous bolus injection at a dose of 100 µl at 12:00 PM to immunoneutralize the endogenous secretin, and pancreatic fluid was collected from 60 min before to 60 min after the injection. This dose completely abolishes the pancreatic exocrine secretion stimulated by intravenous infusion of 1.0 U·kg body wt⁻¹·h⁻¹ secretin (39). Any cross-reactions against human gastrin-releasing peptide, human gastrin, human glucagon, and somatostatin are not observed at much higher doses (1-10 nmol/ml). This antibody showed 100% cross-reactivity with human and porcine secretin (according to communication with the manufacturer). Second, the CCK-A receptor antagonist loxiglumide at a dose of 50 mg/kg body wt sc was given together with oral administration of PI. Third, the same dose of loxiglumide was subcutaneously given at 3 h after oral PI administration.

In another set of experiments, the same dose of PI was orally given to rats that were fasted from 12 h before and fasted another 12 h after the dose. The basal pancreatic fluid secretion, protein output, and chloride and bicarbonate concentrations were determined at 12 h after PI feeding.

Cellular source of fluid hypersecretion. We then investigated the cellular source of the fluid hypersecretion in the PI-treated rats. Because pancreatic acinar cells produce chloride-rich fluid whereas pancreatic ductal cells produce bicarbonate-rich fluid (37), chloride and bicarbonate concentrations in the pancreatic fluid were determined. In addition, in vivo labeling of proliferating cells with BrdU was determined. One hour before the rats were killed, 10 mmol/l BrdU labeling reagent (1 ml/100 g body wt) was intravenously injected into both control and PI-treated rats to label proliferating cells (7).

Assays

Protein and bicarbonate concentrations in the pancreatic fluid were determined by the method of Lowry et al. (21) and by the DST800 multititration system (34), respectively. Chloride concentration in pancreatic fluid was determined according to the method of Schales and Schales (34). CCK concentrations in plasma were measured by a sensitive and specific radioimmunoassay, using antisera OAL-656 with CCK-8 as a standard (36).

Data Analysis

Results were expressed as means ± SE. Statistical analysis was performed by using the Mann-Whitney U test. Paired data were analyzed using a paired t-test. Differences with a P value of <0.05 were considered statistically significant.

RESULTS

Plasma CCK Levels after PI treatment

In fed rats, plasma CCK concentrations increased significantly from the basal value of 2.59 ± 0.13 pmol/l to the peak of 27.6 ± 4.1 pmol/l 60 min after an oral administration of 20 mg/kg body wt PI, but then decreased to 15.8 ± 2.6 pmol/l after 2 h and returned to the preload levels after 12 h (2.93 ± 0.24 pmol/l) (Fig. 1).

PI also caused a significant increase in plasma CCK levels in fasted rats from the basal levels of 1.48 ± 0.12 pmol/l to the peak of 10.91 ± 1.56 pmol/l after 30 min and returned to the preload levels after 4 h (1.77 ± 0.24 pmol/l) (Fig. 2).

Histological Examination

A portion of the pancreatic tissue was fixed for 24 h in a 10% formaldehyde solution for hematoxylin and eosin staining for light microscopy examination. To identify proliferating cells, BrdU incorporated into replicating DNA was stained using a specific monoclonal antibody. At least 1,000 acinar cells and 300 ductal cells were counted. The labeling index was considered to be the number of BrdU-positive cells per 100 counted cells.

Fig. 1. Time course of plasma CCK response to a single oral dose of protease inhibitor (PI) at a dose of 20 mg/kg body wt in rats fed ad libitum (●) and in those fasted from 12 h before to the end of the experiments (○). Values are means ± SE of 5-8 rats. aSignificant difference vs. fed rats at the corresponding time. bSignificant difference vs. preloading value.
However, plasma CCK levels in fasted rats were significantly lower than those in fed rats at all corresponding time points (Fig. 1).

**Basal Pancreatic Fluid and Protein Output**

Oral administration of PI had no influence on basal fluid secretion at 3 h after, but it caused a significant increase in pancreatic fluid secretion at 6 h after (61.0 ± 9.6 vs. 27.3 ± 2.3 µl/30 min in control rats, P < 0.01) and further increased pancreatic fluid secretion at 12 h after (Table 1, Fig. 2). PI also increased protein output at 12 h after (Table 1, Fig. 2). The pancreatic fluid was rich in chloride but poor in bicarbonate concentration (Table 1). In contrast to fed rats, basal pancreatic fluid, protein output, and bicarbonate and chloride concentrations in fasted rats were similar to those in the control rats (Table 1).

**Mechanism of Pancreatic Fluid Hypersecretion**

Because the basal fluid secretion was elevated in PI-treated fed rats, we examined the underlying mechanism in rats at 12 h postfeeding. Intravenous infusion of the CCK-A receptor antagonist loxiglumide at 12 h post-PI treatment at a dose of 50 mg·kg body wt⁻¹·h⁻¹ for 60 min, which could eliminate the possible effect of endogenous CCK on the fluid secretion, had no significant influences on the fluid and bicarbonate secretion but significantly suppressed protein output (Table 2). Subcutaneous injection of loxiglumide at 3 h after PI treatment could not modify the pancreatic fluid hypersecretion at 12 h after. Loxiglumide, however, when subcutaneously given together with PI administration, completely abolished PI-induced changes in the exocrine pancreatic secretion to the control levels, although plasma CCK concentration at this time point was significantly higher than that after PI alone (Table 1).

Intravenous infusion of the cholinergic receptor antagonist atropine at a dose of 100 µg·kg body wt⁻¹·h⁻¹ was also unable to modify the fluid and bicarbonate secretion, but it significantly suppressed protein output (Table 2). Similarly, antisecretin serum had no significant influence on fluid hypersecretion, although it further decreased bicarbonate concentration from 41.9 ± 3.0 to 22.0 ± 3.0 mmol/l (Table 2).

**Cellular Source of Fluid Hypersecretion**

Chloride and bicarbonate concentrations in pancreatic fluid. PI treatment increased chloride concentration, whereas it reciprocally decreased bicarbonate concentration with time (Table 1, Fig. 3). These electrolyte changes in the pancreatic fluid were observed

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**Table 1. Plasma [CCK], and pancreatic fluid volume, protein output, [Cl⁻], and [HCO₃⁻] in pancreatic fluid at 12 h after PI treatment in fed and fasted rats**

<table>
<thead>
<tr>
<th></th>
<th>Plasma [CCK], pmol/l</th>
<th>Fluid Volume, µl/30 min</th>
<th>Protein Output, µg/30 min</th>
<th>[Cl⁻], meq/l</th>
<th>[HCO₃⁻], mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fed rats</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>2.59 ± 0.13</td>
<td>27.3 ± 2.3</td>
<td>581 ± 52</td>
<td>92.4 ± 2.3</td>
<td>66.3 ± 3.0</td>
</tr>
<tr>
<td>PI (20 mg/kg body wt)</td>
<td>2.93 ± 0.24</td>
<td>100.9 ± 8.2*</td>
<td>916 ± 58*</td>
<td>124.3 ± 3.7*</td>
<td>45.8 ± 2.5*</td>
</tr>
<tr>
<td>With loxiglumide</td>
<td>4.04 ± 0.87†</td>
<td>23.3 ± 3.2†</td>
<td>539 ± 82†</td>
<td>95.7 ± 7.9†</td>
<td>60.0 ± 1.6†</td>
</tr>
<tr>
<td>With loxiglumide 3 h after PI</td>
<td>4.15 ± 0.62*†</td>
<td>78.1 ± 13.6*</td>
<td>1162 ± 104*</td>
<td>110.3 ± 3.5*</td>
<td>55.9 ± 2.0†</td>
</tr>
<tr>
<td><strong>Fasted rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>1.48 ± 0.12</td>
<td>28.0 ± 2.5</td>
<td>333 ± 24</td>
<td>90.4 ± 2.3</td>
<td>67.3 ± 3.6</td>
</tr>
<tr>
<td>PI (20 mg/kg body wt)</td>
<td>1.73 ± 0.20</td>
<td>33.6 ± 2.4</td>
<td>299 ± 15</td>
<td>93.2 ± 8.3</td>
<td>64.1 ± 2.9</td>
</tr>
<tr>
<td>With loxiglumide 3 h after PI</td>
<td>4.04 ± 0.87†</td>
<td>23.3 ± 3.2†</td>
<td>539 ± 82†</td>
<td>95.7 ± 7.9†</td>
<td>60.0 ± 1.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5–8 rats. Loxiglumide at a dose of 50 mg/kg body wt sc was given together with or 3 h after oral administration of protease inhibitor (PI). [CCK], CCK concentration. *Significant difference vs. respective control; †significant difference vs. PI alone.

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**Fig. 2.** Pancreatic fluid volume and protein output at 3, 6, and 12 h after an oral administration of PI at a dose of 20 mg/kg body wt. Values are means ± SE of 5–8 rats. aSignificant difference vs. control value.
The proliferating cells labeled with BrdU were seen mainly in acinar cells. The labeling index of acinar cells showed a 7.7-, 2.7-, and 1.3-fold increase above control value at 3, 6, and 12 h, respectively, after PI treatment (Table 3, Fig. 4). In contrast, the labeling indexes of acinar and duct epithelial cells in rats given loxiglumide together with PI were slightly lower, although statistically not significant, than those in the controls (Table 3).

In fasted rats, the labeling index of acinar cells at 3 h after PI treatment was significantly lower than that in the controls (0.20 ± 0.09% vs. 0.46 ± 0.08%, P < 0.01).

### DISCUSSION

The results of the present study have confirmed our earlier studies (29, 30) demonstrating that a single oral PI administration causes pancreatic fluid hypersecretion only in fed rats at 6–12 h postfeeding and have extended our previous understanding by revealing that the fluid hypersecretion is not under the regulation of CCK-, secretin-, or cholinergic-dependent mechanisms.

Previous studies have demonstrated that a single oral dose of PI causes an increase in plasma CCK concentrations and pancreatic exocrine secretion for more than 2 h (14, 29, 31). However, the expected increase in basal pancreatic exocrine secretion in rats that had been given PI 3 h before was not seen, but the basal pancreatic fluid secretion was increased 6–12 h postfeeding. These results, together with previous observations (29, 30), suggest different mechanisms between the early and the late response after PI feeding.

There are several possible explanations for pancreatic fluid hypersecretion in rats late after oral administration of PI. It is commonly accepted that the exocrine secretion of the pancreas is regulated by a CCK-mediated negative-feedback mechanism, which is triggered by lowering of the duodenal concentration of proteolytic enzymes either by diversion of biliary-pancreatic secretions away from the small intestine (5, 8, 20) or by infusion of trypsin inhibitors (6, 8, 30, 31, 40) or intact protein into the small intestine (5, 8). This feedback mechanism is thought to be mediated by endogenous CCK-releasing peptides in the lumen. One such peptide, monitor peptide (MP), is secreted by the pancreas into pancreatic juice (12). The others appear to be secreted by the intestine into the lumen (22, 24) and have been identified as “luminal CCK-releasing factor,” or LCRF (36), and “diazepam-binding inhibitor,” or DBI (37).

### Table 3. Labeling index of BrdU-positive cells in rats at 3 h after oral administration of PI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acinar Cells, %</th>
<th>Ductal Cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed control rats</td>
<td>0.46 ± 0.08</td>
<td>0.53 ± 0.17</td>
</tr>
<tr>
<td>PI treatment (20 mg/kg body wt)</td>
<td></td>
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<tr>
<td>In fed rats</td>
<td>3.56 ± 0.29*</td>
<td>1.40 ± 0.13*</td>
</tr>
<tr>
<td>Together with loxiglumide</td>
<td>0.25 ± 0.07</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>In fasted rats</td>
<td>0.20 ± 0.09*</td>
<td>0.59 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5–8 rats. Loxiglumide at a dose of 50 mg/kg body wt sc was given together with oral administration of PI. Fasted rats were deprived of food 12 h before PI administration until the end of the experiment. BrdU, 5-bromo-2′-deoxyuridine. *Significant difference vs. fed control rat.
or DBI (10). These peptides are all trypsin sensitive and exert CCK-releasing action by avoiding trypsin degradation in the presence of intraluminal trypsin inhibitors or food that transiently binds trypsin. One possible explanation for the delayed increase in pancreatic exocrine secretion after an oral dose of PI is that PI reduces intraduodenal activities of proteolytic enzymes (6, 30, 40) or acts directly on CCK-producing cells in the small intestinal mucosa, resulting in a significant increase in CCK (2, 17), which stimulates pancreatic enzyme secretion including MP as well as trypsin and chymotrypsin into the duodenum. In a fasted state, these proteolytic enzymes presumably hydrolyze and inactivate MP, LCRF, or DBI and thus inhibit further release of CCK, whereas these CCK-releasing peptides can survive intact in fed rats because dietary protein in the duodenum competes for the trypsin and chymotrypsin and thus continuously stimulates the release of CCK. If, however, the prolonged increase of endogenous CCK were related to the delayed hypersecretion, the potent and highly specific CCK-A receptor antagonist loxiglumide (50 mg/kg body wt) (26, 27) might inhibit pancreatic fluid hypersecretion seen at 12 h postfeeding. Moreover, if endogenous CCK were related to the delayed hypersecretion, fluid secretion might be increased in rats given loxiglumide together with oral PI, because plasma CCK levels at 12 h after PI treatment in these rats were significantly higher than those in rats given PI alone. Because the plasma CCK levels at 12 h after PI treatment were similar to the preloading basal levels and since intravenous infusion of loxiglumide was unable to suppress the fluid hypersecretion, it is unlikely that the pancreatic fluid hypersecretion is caused by the direct effect of endogenous CCK.

The possible effect of cholinergic tone on the fluid hypersecretion is also refuted by the ineffectiveness of atropine in inhibiting the fluid hypersecretion. On the other hand, immunoneutralization of circulating secretin slightly but not significantly suppressed the pancreatic fluid hypersecretion and greatly decreased bicarbonate concentration (~47%). This provides clear evidence that endogenous secretin does not play an important role in fluid hypersecretion in PI-treated rats. In support of this view, the camostat-induced basal pancreatic fluid hypersecretion is observed even in in vitro perfused pancreas, in which humoral and neural influences are eliminated, while the circulatory system is maintained via intact arteries and veins (29, 30). Taken together, it is unlikely that any humoral and neural factors are responsible for the basal fluid hypersecretion in rats at 12 h after PI feeding.

Subcutaneous administration of loxiglumide together with oral PI completely abolished fluid hypersecretion, but it had no inhibitory effects when given 3 h after PI feeding. On the other hand, intravenous infusion of atropine at a dose of 100 μg·kg body wt·h⁻¹ for 60 min starting immediately after an oral administration of PI had no influence on fluid hypersecretion at 12 h postfeeding (unpublished observations). These results suggest that the fluid hypersecretion is related to the early changes in the exocrine pancreas induced by endogenous CCK release at a very early time point after PI feeding (within 3 h). It is conceivable therefore that some functional changes in the exocrine pancreas, which can be prevented by cotreatment but cannot be suppressed by posttreatment with loxiglumide, might have occurred in the pancreas after oral administration of PI, leading to a persistent fluid hypersecretion.

The time course of the fluid hypersecretion appears to agree with the proliferating process of acinar cells. This process is probably triggered by complex mechanisms involving gastrointestinal hormones such as CCK and secretin. Among them, CCK is thought to be the most important (25). The proliferative process might be triggered by the initial increase in endogenous CCK. Thus the subsequent blockade of CCK might have no effect, because the increased proliferation of acinar cells is already underway and does not require CCK for maintenance, as we have shown in our recent studies that maintenance of hyperplasia requires less CCK than induction of hyperplasia (25, 28). Indeed, the labeling index of acinar cells with BrdU showed a 7.7-fold increase above the control value at 3 h after, and subcutaneous injection of loxiglumide at this time point could not inhibit the increase. Moreover, the labeling index of acinar cells in rats given loxiglu-
mide together with oral PI was lower than that at control levels, and the pancreatic fluid hypersecretion was completely abolished in this group of rats.

An increased mitotic activity after PI treatment was seen as early as 3 h in both the acinar and ductal cells. In accordance with this proliferative process, chloride concentration in pancreatic fluid was significantly increased, whereas bicarbonate concentration was markedly decreased, indicating an acinar source of the fluid. The increases in protein output as well as fluid volume and chloride concentration in the PI-treated rats further suggest the acinar source of the fluid hypersecretion. However, because a simultaneous increase in acinar and ductular proliferation is seen, it is possible that not only acinar but also ductal cells contribute to fluid hypersecretion in the PI-treated rats. Although secretin is known to cause pancreatic fluid secretion mainly from the duct system and to increase bicarbonate concentration with a reciprocal fall in chloride concentrations (1), PI feeding decreased bicarbonate concentration and increased chloride concentration with an increase in fluid secretion. Acinar cell proliferation appears to be the source of the increase in CCK-, secretin-, and cholinergic-independent pancreatic fluid hypersecretion in the PI-treated rats.

Similar to PI-pretreated rats, we found in our recent study that basal pancreatic fluid secretion, rich in chloride but poor in bicarbonate, is greatly increased at 24 h after the commencement of caerulein-induced pancreatitis (fluid vol. 105.1 ± 4.6 µl/min) (3). The labeling index with BrdU of acinar cells in these pancreaticitic rats showed a 12.1-fold increase above the control levels at 6 h but decreased to the preloading level at 24 h after. In the present study, the labeling index of acinar cells peaked at 3 h, whereas fluid hypersecretion was observed later than 6 h after PI treatment. Taken together, these results suggest that acinar cells that have progressed beyond the synthesis period cause pancreatic fluid hypersecretion, because BrdU is specifically incorporated into the cells in the synthesis period of the cell cycle (7).

In contrast to fed rats, in fasted rats PI did not cause any significant increase in the basal pancreatic fluid secretion, as we have demonstrated in our previous study (30). However, oral administration of PI in fasted rats caused an eightfold increase in plasma CCK levels, as was the case in the fed rats (Fig. 1). Because the rats were fasted from 12 h before to 12 h after PI administration, the absence of fluid hypersecretion can be explained by a deficit of substrates (amino acids) or energy (calories) for protein synthesis in the pancreas. Cell proliferation requires energy and substrates for protein synthesis, and depletion of either can slow or prevent replication. Indeed, recent studies have demonstrated that a low-protein diet or the absence of food in the gastrointestinal tract decreases the trophic effects of both exogenous and endogenous CCK on the rat pancreas (4, 9). Thus oral intake of adequate protein is important to induce the full effect of PI.

In conclusion, the present study demonstrated that the pancreatic fluid hypersecretion seen after PI treatment is not under the regulation of a CCK-, secretin-, or cholinergic-dependent mechanism. Acinar cell proliferation is the most probable source of the agonist-independent fluid hypersecretion.

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Received 20 June 1997; accepted in final form 13 November 1997.

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