Discrepancy between the Potency of Various Trypsin Inhibitors to Inhibit Trypsin Activity and the Potency to Release Biologically Active Cholecystokinin-pancreozymin

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Abstract Injection of various trypsin inhibitors into the lumen of the isolated perfused rat duodenum increased the amount of biologically active cholecystokinin-pancreozymin (CCK-BA) in the vascular perfusate. The potency to induce CCK-BA release of the various trypsin inhibitors differed. Injection of ethyl p-(6-guanidinohexanoyloxy) benzoate methanesulfonate (FOY-007; 100 μmol), p-ethoxy-carbamoyl-thio-6-guanidino caproate phosphate (FOY-129; 110 μmol), 4-(4-guanidino-benzoyloxy) phenylacetic acid (FOY-251; 128 μmol), N,N-dimethylcarbamoylmethyl-4-(4-guanidinobenzoyloxy) phenylacetate methanesulfonate (FOY-305; 80 μmol), and p-aminobenzamidine dihydrochloride (p-ABA, Sigma; 300 μmol) caused release of CCK-BA amounting to 1,042, 247, 252, 682, and 302 pm, respectively. The potency to induce CCK-BA release was not correlated with the potency to inhibit trypsin activity. The present results do not support the hypothesis that a negative feed-back regulation of pancreatic enzyme secretion is exerted by intraluminal trypsin in the rat.

Key Words: trypsin inhibitors, cholecystokinin-pancreozymin secretion, isolated perfused pancreas, isolated perfused duodenum.

Chronic ingestion of a natural trypsin inhibitor extracted from bovine lung (IHSE et al., 1975), soybean (FÖLSCH et al., 1974; FÖLSCH and WORMSLEY, 1974; YANATORI and FUJITA, 1976), or egg white (ROTHMAN and WELLS, 1969), as well as a synthetic trypsin inhibitor FOY-305, induced hypertrophy and hyperpyknia in the rat pancreas (YONEZAWA, 1983). The hypertrophy and hyperpyknia induced by these trypsin inhibitors are thought to be mediated by accelerating the release of endogeneous cholecystokinin-pancreozymin (CCK). Evidence to support this view is provided by KHAYAMBASHI and LYMAN (1969), who demonstrated that feeding soybean trypsin inhibitor caused the release of CCK-like substances
into the bloodstream in rats. This view is also supported by the recent finding that the intra-duodenal injection of 100 mg/kg body weight FOY-305 (about 40 \( \mu \text{mol} \)) caused release of CCK-BA into the portal vein in the isolated perfused rat duodenum (KANNO et al., 1979).

The mechanism by which these trypsin inhibitors induce CCK-BA release has not yet been fully defined. However, GREEN and LYMAN (1972) and SCHNEEMAN and LYMAN (1975) have proposed that trypsin in the upper small intestine prevents the release of CCK-BA into the blood. Thus by binding the trypsin, soybean trypsin inhibitor would effectively remove the enzyme from the intestine, thereby releasing CCK-BA and increasing pancreatic enzyme output. Their hypothesis would be supported if the trypsin inhibitory potency of trypsin inhibitors is directly correlated with the potency of inducing CCK-BA release. Therefore, in the present study the trypsin inhibitory potencies of five synthetic trypsin inhibitors (FOY-007, FOY-129, FOY-251, FOY-305, and \( p \)-ABA) were compared with their potencies to induce CCK-BA release.

**METHODS**

*Preparation of isolated perfused pancreas and duodenum.* Male Sprague-Dawley strain rats weighing about 200 g were fasted for 18 hr before each experiment. The isolated perfused rat pancreas with attached duodenum was prepared by the method of KANNO et al. (1976). In brief, under ether anesthesia, the mesenteric and the coeliac arteries as inlets and the portal vein as outlet were cannulated and perfused at 2.0/min with the aid of a roller pump. After ligating the hepatic end of the common bile duct, the duodenal end of the pancreatic duct was cannulated with a stainless steel tube. The blood supply to stomach, spleen, and liver was stopped by tying the arteries. The mesentery with its embedded whole pancreas and the attached duodenum was then removed and placed in a Lucite chamber containing about 20 ml of a modified Krebs-Henseleit solution and maintained at 37°C.

*Solution and drugs.* The perfusing solution was a modified Krebs-Henseleit solution to which 5 vol. \% Dextran T-70 (Pharmacia Uppsala) had been added, and through which 5% \( \text{CO}_2 \) in \( \text{O}_2 \) was continuously bubbled. Additionally, 8 vol. \% dog erythrocytes were added to the perfusing solution because to obtain CCK-BA release in sufficient quantity, there must be an adequate supply of oxygen (KANNO and IMAI, 1976). The composition of the standard Krebs-Henseleit solution used to perfused and bathe the preparation was as follows (final concentration, mm): \( \text{NaCl}, 131; \text{KCl}, 5.6; \text{CaCl}_2, 2.5; \text{NaHCO}_3, 25; \text{MgCl}_2, 1.0; \text{NaH}_2\text{PO}_4, 1.0; \) glucose, 5.0.

Synthetic trypsin inhibitors, FOY-007, FOY-129, FOY-251, and FOY-305 (ONO Pharmaceutical Co., Osaka, Japan), and \( p \)-ABA (Sigma, St. Louis) were dissolved in distilled water and injected into the lumen of the isolated duodenum.
to stimulate the CCK-BA releasing cells in the mucosa of the duodenum. Soybean trypsin inhibitor (SBTI, [Sigma Type II-S]), egg white trypsin inhibitor (EWTI, [Sigma Type II-O]) and arginine hydrochloride (Sigma, St. Louis) were also injected into the duodenum. Pure natural cholecystokinin-pancreozymin (CCK; 3,500 Ivy dog units/mg, [GIH Research Unit, Karolinska Institute, Stockholm, Sweden]) was infused through a cannula into the inlets of the vascular perfusion system. Concentration CCK was expressed in pm.

Estimation of protein output and flow of pancreatic juice. Estimation of the flow of pancreatic juice was made as follows. A calibrated silicon-rubber tube (about 0.5 mm OD) was attached to the free end of the pancreatic duct cannula. At specified intervals the tube was replaced, and the volume of pancreatic juice in the tube was measured. The collected juice was then diluted with 0.9% saline, and the total protein in the juice was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Anti-trypsin activities of various trypsin inhibitors. Anti-trypsin activity of FOY-007, FOY-129, FOY-251, or FOY-305 was quoted from the data of ONO Pharmaceutical Co., Ltd. measured as follows: 0.4 ml of 1.5 µg/ml trypsin was added to 0.5 ml of 20 mM p-N-tosyl-arginine methyl ester (TAME) and 0.1 ml of 0.1 M Tris buffer solution containing 100 mM CaCl₂, and hydrolysis of TAME by trypsin was estimated by the method of Hesterin (1949). By replacing the Tris buffer solution with various concentrations of a trypsin inhibitor dissolved in 0.1 M Tris buffer solution, the concentration required for 50% inhibition of hydrolysis of TAME by trypsin (ID₅₀) was measured. Anti-trypsin activities of SBTI and EWTI were quoted from Sigma's catalogue. Anti-trypsin activity was expressed as the amount of trypsin with approx. 10,000 BAEE units per mg protein inhibited by 1 mg of a trypsin inhibitor. One BAEE unit was defined as the change of 0.001 per minute in absorbance at 253 µm with N,a-benzoyl-L-arginine ethyl ester as substrate at pH 7.6 at 25°C by trypsin.

RESULTS

CCK-BA bioassay procedure

The isolated rat pancreas was perfused with the modified Krebs-Henseleit solution for 20 min, and then with the solution containing 730 pM CCK for 5 min. This perfusion sequence was then repeated three times. Initial perfusion with 730 pM CCK resulted in a large increase in pancreatic juice flow and protein output. The initial response was followed by a slight and gradual diminution in the subsequent responses. In the other experiments, 146 or 365 pM CCK rather than 730 pM CCK was used for the second stimulation (Fig. 1). When the protein output in response to the second stimulation with 730 pM CCK was taken as 100% biological effect, a linear relation was obtained between the dose of CCK given at the second stimulation (X) and the relative value of protein output induced by
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The regression line for the relation was $Y = 0.13X + 5$ and the linear correlation coefficient was 0.96 (Fig. 2). The linear relation was used to calculate CCK-BA contained in the portal perfusate as follows: Each trypsin inhibitor or arginine was injected and retained in the lumen of the isolated

Fig. 1. Secretory responses of the isolated perfused rat pancreas induced by successive 5 min (solid bars) perfusion with various concentrations of CCK at regular intervals of 25 min. Each value represents the mean (±S.E.) of five experiments.

Fig. 2. The relation between the CCK concentration ($X$) and the protein output ($Y$). The regression line calculated from the relation was $Y = 0.13X + 5$ and the correlation coefficient was 0.96. Each value is obtained from the results depicted in Fig. 1.

Fig. 3. The linear relation between the dose of FOY-305 ($X$) and the concentration of CCK-BA release ($Y$). The regression line calculated from the relation was $Y = 7.5X + 130$ and the correlation coefficient was 0.79. Each value represents the mean (±S.E.) of five experiments.

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duodenum, the vascular system of which was continuously perfused with the modified Krebs-Henseleit solution, and the vascular perfusate was collected from the portal vein for the following 30 min. The perfusate was oxygenated and its biological activity on the isolated perfused rat pancreas was examined between the preceding and the following perfusion with solution contained 730 pm CCK.

Dose response relation for FOY-305 induced release of CCK-BA

In a previous study (Kanno et al., 1980), the intraduodenal injection of 100 mg/kg body weight FOY-305 (about 40 µmol) caused the release of CCK-BA amounting to 7 mU/ml CCK (510 pm). In the following experiments, we injected and retained different doses of FOY-305 into the lumen of the isolated duodenum. Increase in the dose of FOY-305 injected into the duodenal lumen induced release of perfusate containing higher CCK-BA concentration. As shown in Fig. 3, a linear relation was obtained between the intraduodenal dose of FOY-305 (X) in the range of 0 to 80 µmol per rat and the concentration of CCK-BA in the perfusate (Y in pm). The regression line calculated from the relation was Y = 7.5X ± 130 and the correlation coefficient was 0.79.

Correlation between the trypsin inhibitory potency of various trypsin inhibitors and their potency to induce CCK-BA release

The potency to induce CCK-BA release of five synthetic trypsin inhibitors, FOY-007, FOY-129, FOY-251, FOY-305, and p-ABA, was compared with their potencies to inhibit trypsin activity. There was no correlation between these two potencies (Table 1). For example, FOY-305 had the strongest potency to inhibit trypsin activity among the synthetic trypsin inhibitors tested but its po-

<p>| Table 1. Comparison of the potency of various trypsin inhibitors to inhibit trypsin activity and to release CCK-BA. |
|---------------------------------|----------------|-----------------|-----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Compounds</th>
<th>ID50 for anti-trypsin activity (m)</th>
<th>Dose (µmol)</th>
<th>Amount of CCK-BA released in the perfusate (pm)</th>
<th>Potency to release CCK-BA (pm/µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ABA</td>
<td>10^-2-10^-3</td>
<td>300</td>
<td>302</td>
<td>1.0</td>
</tr>
<tr>
<td>FOY-305</td>
<td>8.5 x 10^-9</td>
<td>80</td>
<td>682</td>
<td>8.5</td>
</tr>
<tr>
<td>FOY-251</td>
<td>2.9 x 10^-8</td>
<td>128</td>
<td>252</td>
<td>2.0</td>
</tr>
<tr>
<td>FOY-007</td>
<td>2.0 x 10^-5</td>
<td>100</td>
<td>1,042</td>
<td>10.4</td>
</tr>
<tr>
<td>FOY-129</td>
<td>1.1 x 10^-4</td>
<td>110</td>
<td>247</td>
<td>2.2</td>
</tr>
<tr>
<td>SBTI</td>
<td>0.6 mg*</td>
<td>200**</td>
<td>267</td>
<td>—</td>
</tr>
<tr>
<td>EWTI</td>
<td>1.0 mg*</td>
<td>200**</td>
<td>153</td>
<td>—</td>
</tr>
<tr>
<td>Arginine</td>
<td>—</td>
<td>300</td>
<td>443</td>
<td>(1.5)</td>
</tr>
</tbody>
</table>

The potency of antitrypsin activity was measured by the use of tosyl-arginine methyl ester as a substrate. * Anti-trypsin activity was expressed as the amount of trypsin with activity of approx. 10,000 BAEE units per mg protein inhibited by 1 mg of a trypsin inhibitor. The data were quoted from Sigma's catalogue. ** mg/kg body weight.
tency to induce release of CCK-BA was about 80% of that of FOY-007. FOY-007 had the third weakest potency to inhibit trypsin activity, whereas it induced the release of the highest concentration of CCK-BA. The order of potencies to inhibit trypsin activity among these five synthetic trypsin inhibitors was FOY-305 > FOY-251 > FOY-007 > FOY-129 > p-ABA, whereas the order of potencies to release biologically active CCK was FOY-007 > FOY-305 > FOY-129 > FOY-251 > p-ABA.

The discrepancy between the potency to release CCK-BA and the potency to inhibit trypsin activity was further confirmed with the natural trypsin inhibitors, SBTI and EWTI. The potency to inhibit trypsin activity was EWTI > SBTI whereas the potency to release CCK-BA was SBTI > EWTI.

Arginine (300 μmol), the most potent amino acid known to induce CCK-BA release in the anesthetised rat (Fujita and Yanatori, 1976), induced the release of 443 pm CCK-BA, only 1/7 the potency of FOY-007 to release CCK-BA.

DISCUSSION

Green and Lyman (1972) and Schneeman and Lyman (1975) have proposed that intraluminal trypsin has a negative feed-back regulation of pancreatic enzyme secretion. This hypothesis is supported by the following: 1) Trypsin or bile-pancreatic juice, infused intestinally, suppressed the secretion of pancreatic enzymes. 2) Diversion of bile-pancreatic juice from the intestine as well as intraintestinal infusion of SBTI or protein stimulated the pancreatic enzyme output several-fold. 3) Suppression of enzyme secretion by trypsin as well as its stimulation by SBTI occurs only in the upper one-third of the small intestine, where CCK is known to be released. 4) Trypsin, whose active center is blocked by diisopropyl-fluorophosphate, does not suppress pancreatic enzyme secretion. Furthermore, Fujita and Yanatori (1976) have proposed that proteolytic enzymes from the pancreas are adsorbed to the plasma membrane of the microvilli in the CCK producing cell and represent the receptors of the cell. The enzyme molecules when combined with the molecules of substrate proteins, certain amino acids, or specific inhibitors, undergo allosteric deformation which may cause electrochemical changes in the cell membrane and trigger emiocytotic granule release. In the rat, trypsin represents the main receptors of the CCK producing cell. This hypothesis is supported by the following: 1) The effect of SBTI and amino acids upon pancreatic enzyme secretion is prevented by washing the rat intestine with a saline solution. 2) SBTI stimulation of pancreatic enzyme secretion occurs effectively only when pancreatic juice or trypsin itself is returned to the intestine during the cannulation experiments in the rat. On the other hand, their hypothesis is not supported by the present results since: 1) Although pancreatic juice or trypsin itself was not returned to the intestine, a considerable amount of CCK-BA was released by intraduodenal injection of trypsin inhibitors. 2) If blocking the active...
center of trypsin plays a major role in the action of trypsin inhibitors to induce CCK-BA release, there should be a close correlation between the potency to induce CCK-BA release and the potency to inhibit trypsin activity. In the present experiments such correlation was not obtained. Furthermore, it is difficult to conceive that trypsin in the intestinal mucosa can easily be washed out by flushing the intestine with a saline solution.

The difference in the qualitative results obtained by GREEN and LYMAN (1972), SCHNEEMAN and LYMAN (1975), and FUJITA and YANATORI (1976), and the present results may in part be due to the difference in preparations, especially since their preparation involved the administration of an anesthetic. Thus the fact that washing with saline prevents SBTI-induced pancreatic enzyme secretion could be explained by the possibility of indirect influences of humoral, hormonal, and neuronal factors. Since the present quantitative study used the isolated perfused rat duodenum, in which possible influences of humoral, hormonal, and neuronal factors were excluded, we conclude that there is no correlation between the potency to induce CCK-BA release and the potency to inhibit trypsin activity of various trypsin inhibitors.

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REFERENCES


