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Degradation of Insulin and Calcitonin and Their Protection by Various Protease Inhibitors in Rat Caecal Contents: Implications in Peptide Delivery to the Colon

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Abstract

The objective of this study was to examine the metabolism of insulin and calcitonin, and their protection by various protease inhibitors, in the large intestine.

Fresh caecal contents were prepared from non-fasted rats and the degradation of insulin and calcitonin was studied in a suspension of rat caecal contents, as a model of the content of the large intestine. Both insulin and calcitonin were metabolized in suspensions of rat caecal contents, but the degradation of calcitonin was much faster than that of insulin. The degradation of insulin was fastest at pH 6·8. Protease inhibitors such as camostat and aprotinin inhibited the degradation of insulin and calcitonin in rat caecal contents, which was consistent with the high chymotrypsin activity of these contents.

These findings suggest that care should be taken when administering peptide drugs to the large intestine for colon-specific drug delivery because they can be degraded in rat caecal contents. Protease inhibitors might be useful for increasing the stability of these peptides in the large intestine, thereby improving their large-intestinal absorption to the systemic circulation.

Peptides and proteins such as insulin and calcitonin are usually administered parenterally because when administered orally they are degraded by proteolytic enzymes in the gastrointestinal tract. Because it is known that the activities of various proteases responsible for peptide degradation in the large intestine are generally lower than those in the small intestine (Lee et al 1991; Yamamoto et al 1990, 1994), many studies have investigated the possibility of delivering peptide and protein drugs to the colon (Saffran et al 1986; Kopeckova et al 1994).

Various microorganisms are, on the other hand, distributed throughout the gastrointestinal tract, and most of these microorganisms are found in the large intestine where they mediate hydrolytic digestive functions using carbohydrate and proteins as substrates. It has recently been demonstrated that these microorganisms can metabolize some drugs and other foreign compounds (Shamat 1993). Peppercorn & Goldman (1972) reported that sulphasalazine, which consists of sulphapyridine and 5-aminosalicylic acid, is primarily metabolized by intestinal bacteria in conventional and germ-free rats. In addition, it was shown that isosorbide dinitrate can be metabolized in-vitro by the aerobic intestinal microorganisms of both rat and man (Shamat 1993). Thus, the intestinal microflora represent a distinct and a potentially important role for metabolic transformation of drugs, because all drugs released in the large intestine will undoubtedly come into contact with microorganisms. Few studies have, however, examined whether peptide and protein drugs were metabolized in the contents of the large intestine.

We have reported that thyrotropin-releasing hormone and its analogue, azetirelin, were metabolized in rat caecal contents (Sasaki et al 1994). No study of the metabolism of peptide drugs with higher molecular weights, such as insulin and calcitonin, in the contents of the large intestine has, however, been performed, despite the devotion of much attention to the colon-specific delivery of these drugs. In this study, therefore, insulin and calcitonin were chosen as model peptides and their degradation characteristics were examined in rat caecal contents as a model of the contents of the large intestine. We also examined the effects of various protease inhibitors on the degradation and proteolytic activities of the peptides in rat caecal contents.

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Materials and Methods

Materials

Insulin, sodium glycocholate, aprotinin, 7-amino-4-methyl coumarin, succinyl (Suc)-Ala-Pro-Ala-4-methylcoumaryl-7-amide (MCA) and *t*-butyloxycarbonyl (Boc)-Gln-Ala-Arg-MCA were purchased from Sigma (St Louis, MO, USA). Bacitracin, soybean trypsin inhibitor, trifluoroacetic acid and glucose B test Wako were obtained from Wako Pure Chemical Industries (Osaka, Japan). Arg-MCA, Leu-MCA and Ala-MCA were obtained from Peptide Institute (Osaka, Japan). Camostat mesilate was a gift from Ono Pharmaceutical (Osaka, Japan) and human calcitonin was a gift from Suntory (Osaka, Japan). All other chemicals and solvents were of reagent grade.

Preparation of drug solutions

Bovine insulin and human calcitonin were dissolved in isotonic phosphate buffer at pH 7·4 to give a final concentration of 0·1 mM.

Preparation of suspension of rat caecal contents

Experiments were performed on male Wistar rats, 200–250 g; water and food were freely available to the animals before

experiments. The rats were anaesthetized with pentobarbital (32 mg kg^{-1}) given by intraperitoneal injection and the caecum was removed after the rats had been killed by exsanguination from the aorta. Fresh caecal contents were suspended in two volumes of bicarbonate buffer ($NaHCO_3$, 110 mM; $Na_2HPO_4 \cdot 12H_2O$, 20 mM; NaCl, 8·0 mM; KCl, 6·0 mM; $CaCl_2 \cdot 2H_2O$, 0·5 mM; $MgCl_2 \cdot 6H_2O$, 0·4 mM). The pH of the buffer was adjusted to 7·0 by bubbling CO_2 gas through it before use. The pH of the buffer was adjusted by addition of HCl or NaOH when the studies were performed at different pHs. The suspension was filtered through four layers of gauze. The boiled suspension was obtained by boiling the caecal suspension for 1 min.

In-vitro stability experiments

The degradation of insulin and human calcitonin in the suspension of rat caecal contents was studied under CO_2 conditions by incubating a 33% suspension of rat caecal contents (200 μL) with insulin or calcitonin solution (0·1 mM; 400 μL) in the presence or absence of 200 μL of camostat mesilate (20 mM), sodium glycocholate (20 mM), aprotinin (2 mg mL^{-1}) and soybean trypsin inhibitor (10 mg mL^{-1}). In the control experiments, 200 μL of phosphate-buffered saline (PBS) used for the various protease inhibitors was added. The concentrations of various protease inhibitors used in this experiment were adjusted to our results of insulin absorption and stability experiments reported previously (Yamamoto et al 1994). Samples were withdrawn from the incubation mixture (37°C) and 50% acetic acid was added to terminate the reaction. The resulting mixture was then centrifuged for 5 min to remove the precipitated protein and caecal contents. The supernatant (25 μL) was purified by Millipore filtration (pore size 0·45 μM) and analysed by HPLC (Tozaki et al 1995). The apparent inhibition constant (Ki_{app}) was calculated by non-linear least-squares regression analysis (Yamaoka et al 1981) from the equation:

$$a = 100/[1 + ([I]/Ki_{app})]$$

where a is the residual activity (%) of camostat on the degradation of insulin in the rat caecal contents, [I] is the concentration of camostat and Ki_{app} is the inhibition constant.

HPLC conditions

The stability of insulin and calcitonin was assayed by reversed-phase HPLC on a Vydac protein and peptide 5C18-AR-300 column (4·6 × 250 mm). The HPLC system consisted of Hitachi L-6200 and L-6000 pumps, a Hitachi L-4000 variable wavelength absorption monitor, a Hitachi AS-4000 sample injector and a Hitachi D-2500 data processor. The mobile phase was a mixture of distilled water, acetonitrile and 0·1% trifluoroacetic acid. The proportion of acetonitrile in the mobile phase was increased linearly from 15 to 60% during the first 35 min, and then maintained at 60% for the next 10 min. The flow rate was 1·0 mL min^{-1}. Insulin and calcitonin eluting from the column were detected by UV absorption at 210 nm.

Determination of the activities of various enzymes in rat caecal contents

Enzyme activity was assayed as described by Ekstrom & Westrom (1991). The principle of the assay of the activities of various proteases is based on the fluorimetric measurement of 7-amino-4-methylcoumarin liberated from the fluorogenic substrate, peptidyl-MCA. The incubation mixture consisted of phosphate-buffered saline (pH 7·4), substrate and the 33% suspension of rat caecal contents. The activity of aminopeptidase B, leucine aminopeptidase, aminopeptidase N, elastase, chymotrypsin and trypsin were examined using Arg-MCA as a substrate with KCl as activator, Leu-MCA, Ala-MCA with $CaCl_2$, Suc-Ala-Pro-Ala-MCA with $CaCl_2$, Suc-Ala-Ala-Pro-Phe-MCA with $CaCl_2$ and t-Boc-Gln-Ala-Arg-MCA, respectively. Trichloroacetic acid (50%) was added to terminate the reaction and the resulting mixture was then centrifuged for 5 min to remove the precipitated protein and caecal contents. The released 7-amino-4-methylcoumarin in the supernatant was determined by use of a Hitachi F-2000 spectrofluorimeter using excitation and emission wavelengths of 380 nm and 440 nm, respectively. The protease activity was calculated from specific protease enzyme activity (nmol min^{-1} mg $protein^{-1}$) × protein concentration in the 33% suspension (mg mL^{-1}) × volume of the 33% suspension (mL).

Statistical analysis

Results are expressed as the mean ± s.d. and statistical analysis was performed by use of the Student's *t*-test.

Results

Degradation of insulin and calcitonin in the rat caecal contents

The concentration-time profiles for the degradation of insulin and calcitonin by the rat caecal contents are shown in Fig. 1. Both insulin and calcitonin were metabolized in suspensions of rat caecal contents, but the degradation of calcitonin was significantly faster than that of insulin ($P < 0.01$). The degradation of these peptides followed first-order kinetics. The half-life for the proteolysis of calcitonin (2·5 min) in the suspension of rat caecal contents was 13-fold smaller than that for insulin (34 min).

Effect of pH on the degradation of insulin in the rat caecal contents

The pH-rate profile for the degradation rate constant (k) of insulin in the suspension of rat caecal contents is shown in Fig.

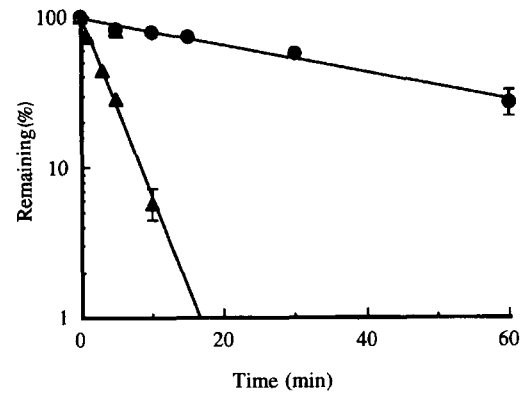

FIG. 1. Time course of insulin and calcitonin degradation in rat caecal contents. Results are expressed as the mean ± s.d. of three experiments. ●, insulin, suspension; ▲, calcitonin, suspension.

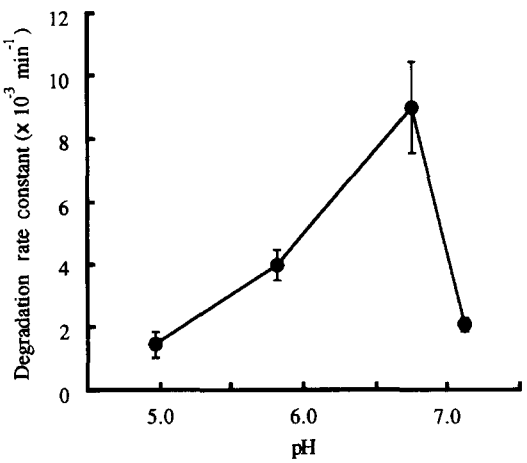

FIG. 2. pH-Dependence of rate constant for insulin degradation in suspension of rat caecal contents. Results are expressed as the mean ± s.d. of three experiments.

2. The degradation of insulin in the suspension of rat caecal contents was the fastest when the pH of the buffer was adjusted at 6·8. The degradation rate slowed as the pH deviated from 6·8.

Effects of various protease inhibitors on the degradation of insulin and calcitonin in the rat caecal contents

The concentration-time profiles for the degradation of insulin and calcitonin in rat caecal contents in the presence of various protease inhibitors are shown in Fig. 3 and Table 1. No insulin degradation was found in the boiled suspension. The degradation of insulin in the unboiled suspension was inhibited by various protease inhibitors. In particular, 20 mM camostat and 2 mg mL^{-1} aprotinin significantly inhibited its degradation; the degradation half-lives were prolonged 5·5 and 5·6-fold, respectively, compared with the control (no additive). Sodium glycocholate (20 mM) had no effect on the inhibition of insulin degradation, however.

The degradation of calcitonin in the presence of various protease inhibitors was also investigated in rat caecal contents. The effects of 20 mM camostat and 2 mg mL^{-1} aprotinin were significant, but 20 mM sodium glycocholate had no effect on the inhibition of calcitonin degradation. Soybean trypsin inhibitor (10 mg mL^{-1}) did not, however, inhibit calcitonin degradation, although it did inhibit the degradation of insulin in the suspension.

Fig. 4 shows the degradation rate constants of insulin and its degradation half-lives with different concentrations of camostat in the suspension of rats caecal contents. The degradation of insulin in the suspension was inhibited by camostat in a dose-dependent manner. The apparent inhibition constant (Ki_{app}) of camostat for the inhibition of insulin degradation was calculated to be approximately 1·8 mM.

Activities of various proteases in the suspension of rat caecal contents

The activities of various proteases in the suspension are shown in Table 2. The results indicate high activity of chymotrypsin

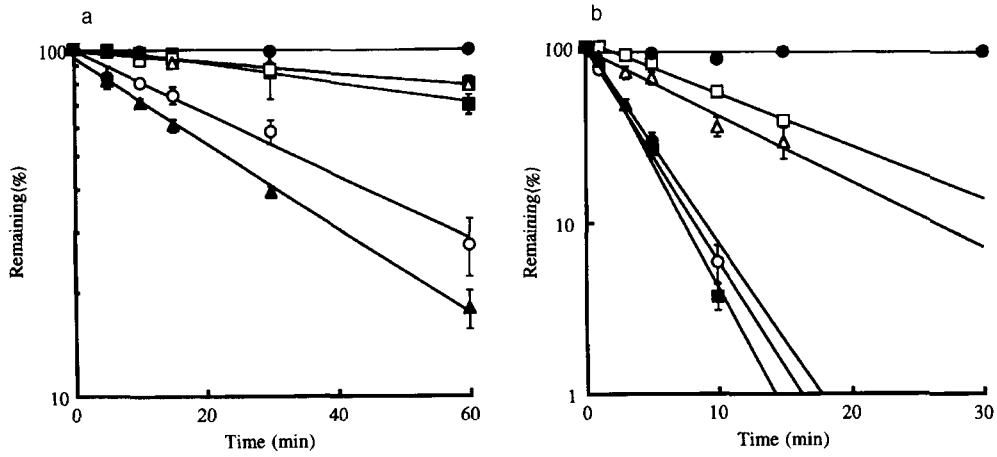

FIG. 3. Effects of various protease inhibitors on the degradation of a, 0·1 mM insulin and b, calcitonin in suspensions of rat caecal contents. Results are expressed as the mean ± s.d. of three experiments. ○, control; ●, boiled suspension; □, camostat, 20 mM; ■, soybean trypsin inhibitor, 10 mg mL^{-1}; △, aprotinin, 2 mg mL^{-1}; ▲, sodium glycocholate, 20 mM.

Table 1. Degradation half-lives of 0·1 mM insulin and calcitonin in suspensions of rat caecal contents.

	Concn	Insulin $t_{\frac{1}{2}}$ (min)	Ratio	Calcitonin $t_{\frac{1}{2}}$ (min)	Ratio
Control	–	34 ± 5.8	–	2.5 ± 0.3	–
Camostat	20 mM	190 ± 14**	5.5	16 ± 0.7**	6.4
Sodium glycocholate	20 mM	25 ± 2.6	0.7	26 ± 0.2	1.0
Aprotinin	2 mg mL^{-1}	190 ± 24**	5.6	8.0 ± 1.3*	3.2
Soybean trypsin inhibitor	10 mg mL^{-1}	110 ± 27*	3.2	2.1 ± 0.1	0.8
Boiled 33% suspension	–	not degraded	–	not degraded	–

The $t_{\frac{1}{2}}$ values are the means ± s.d. of three experiments. *$P < 0.01$, **$P < 0.001$, compared with control.

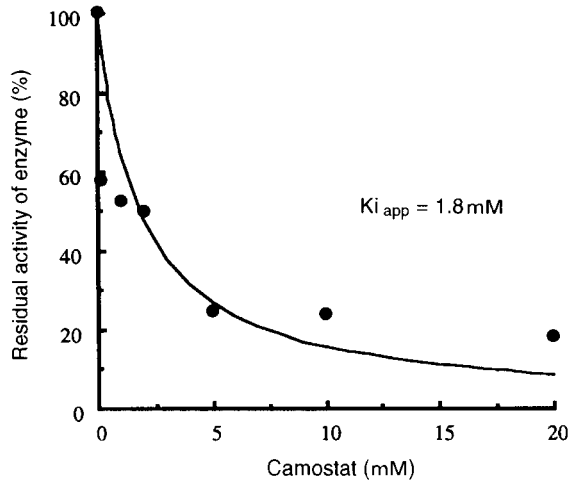

FIG. 4. Degradation rate constants of insulin, and its degradation half-lives, in the presence of different concentrations of camostat in suspensions of rat caecal contents. Results are expressed as the mean of three experiments.

Table 2. Activities of various proteases in suspensions of rat caecal contents.

	Protease activity (nmol min^{-1})
Aminopeptidase B	0·00 ± 9·72
Leu-aminopeptidase	0·00 ± 13·3
Aminopeptidase N	9·33 ± 11·2
Elastase	0·00 ± 9·03
Chymotrypsin	116·0 ± 35·0
Trypsin	2·56 ± 13·4

Results are expressed as the mean ± s.d. of three experiments.

in the rat caecal contents. Although small amounts of aminopeptidase N and trypsin activity were also observed in the rat caecal contents, no aminopeptidase B, leucine aminopeptidase and elastase activity were observed.

Discussion

These findings indicate that insulin and calcitonin were degraded by some proteolytic enzymes in rat caecal contents and in the small- or large-intestinal mucosal homogenates and small intestinal fluid of rats (Hayakawa et al 1989; Yamamoto et al 1990, 1994; Okagawa et al 1994) and that calcitonin was more susceptible to proteolysis in rat caecal contents than was insulin. We demonstrated that the degradation rate constant (k) for insulin hydrolysis was the fastest at pH 6·8 (Fig. 2). It was also observed that insulin and calcitonin were not metabolized in the boiled suspension (Fig. 3, Table 1), in which all the enzymes had been inactivated by the boiling. These results suggested that the degradation of peptides in rat caecal contents was related to enzyme reactions and pH 6·8 is optimum for insulin degradation in rat caecal contents.

In this study, we examined the effect of various protease inhibitors on the degradation of insulin and calcitonin in rat caecal contents and found that aprotinin and camostat reduced the degradation of these peptides more effectively than did soybean trypsin inhibitor or sodium glycocholate in such contents. Schilling & Mitra (1991) reported that insulin was degraded by trypsin and α-chymotrypsin in solution. These results suggest that these enzymes play an important role in the degradation of these peptides in rat caecal contents. This speculation was also supported by our present enzyme activity studies which indicate high activity of chymotrypsin in rat caecal contents. Soybean trypsin inhibitor inhibited the degradation of insulin, however, whereas the degradation of calcitonin was not inhibited. This finding suggests that the enzymes responsible for the breakdown of insulin might be different from those of calcitonin.

Hirai et al (1981) reported that sodium glycocholate inhibited the activity of Leu-aminopeptidases, thereby reducing the degradation of insulin in rat nasal mucosa. In our present study, however, we found almost no Leu-aminopeptidase activity in rat caecal contents. Thus, the negative effect of sodium glycocholate in reducing insulin degradation might be because of the low enzymatic activity of Leu-aminopeptidase in rat caecal contents.

In this study, the rank order of the effectiveness of these protease inhibitors for insulin proteolysis was aprotinin = camostat > soybean trypsin inhibitor > sodium glycocholate, whereas that for calcitonin proteolysis was camostat > aprotinin > sodium glycocholate = soybean trypsin inhibitor. These rank orders are inconsistent with our previous results of the effects of protease inhibitors (Yamamoto et al 1994); then we reported that sodium glycocholate was more effective than camostat and aprotinin at reducing insulin degradation in large intestinal mucosal homogenates. These findings suggest, therefore, that the types of proteases in rat caecal contents might be considerably different from those in large-intestinal mucosal homogenates.

The apparent inhibition constant (Ki_{app}) indicates the concentration (IC50) at which camostat inhibits 50% of the degradation of insulin in rat caecal contents (Fig. 4). This result therefore suggested that the degradation of insulin in caecal contents was inhibited by the use of camostat at concentrations greater than 1·8 mM. Camostat mesilate inhibits the activities of aminopeptidase and proteases such as trypsin, plasmin and kallikrein. Morimoto et al (1991) reported that this compound enhanced nasal absorption of vasopressin and was slightly absorbed through the nasal mucosae. Our present result suggests, therefore, that camostat mesilate might have a dose-dependent inhibitory effect on the activities of proteolytic enzymes.

In conclusion, this study indicated that peptide drugs such as insulin and calcitonin were degraded in rat caecal contents. We, therefore, must take note of the degradation of peptide drugs in the large intestine, especially when colon-specific peptide drug delivery systems are designed. Some protease inhibitors could, furthermore, inhibit the degradation of peptides in rat caecal contents, suggesting that these protease inhibitors might be useful for improving the large-intestinal absorption of peptides by the systemic circulation.

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