Oral Absorption of Peptides: Influence of pH and Inhibitors on the Intestinal Hydrolysis of Leu-Enkephalin and Analogues

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Leu-enkephalin (YGGFL) and several analogues were chosen as model peptides for the study of peptide absorption and hydrolysis in the rat jejunum. An HPLC assay was adapted to detect YGGFL or the analogues and metabolites. Peptide hydrolysis was studied in the rat jejunum using a single-pass perfusion method. Extensive hydrolysis of YGGFL was observed in the rat jejunum and approaches to reduce its metabolism were studied. The brush border enzymes are a major site of enkephalin hydrolysis. Lumenal peptidases were secondary to the brush border enzymes in hydrolyzing the enkephalins in this system. In the *in situ* perfusion system, YGGFL is hydrolyzed primarily to Tyr and GGFL by the brush border aminopeptidase and to YGG and FL by brush border endopeptidase. Lowering the jejunal pH below 5.0 significantly reduces aminopeptidase activity and, to a lesser extent, endopeptidase activity. An aminopeptidase inhibitor, amastatin, produced more pronounced inhibitory effects at higher pH and the endopeptidase inhibitors, tripeptides YGG and GGF, are effective even below pH 5.0. Coperfusion of YGGFL with a combination of aminopeptidase and endopeptidase inhibitors, e.g., amastatin and YGG, is more effective in inhibiting hydrolysis since both metabolic pathways are inhibited. Leu-D(Ala)^2-enkephalin, while showing enhanced stability against aminopeptidase hydrolysis, is hydrolyzed at the Gly–Phe bond by the endopeptidase. Its hydrolysis is not affected by pH changes or amastatin but is decreased by YGG. The YGGFL wall permeability was estimated and is not a limiting factor for oral absorption.

KEY WORDS: aminopeptidase; brush border enzymes; endopeptidase; enkephalins; enzyme inhibitors; opioids peptides; peptide absorption.

INTRODUCTION

A large number of biologically active peptides have been evaluated recently for their therapeutic activity and are proposed as candidates for drugs (1,2). However, oral administration of peptides often results in very low bioavailability as a result of extensive hydrolysis of the peptides by digestive enzymes of the gastrointestinal (GI) tract. Nevertheless, transport of intact oligopeptides across adult mammalian jejunum has been demonstrated *in vitro* (3–6), *in vivo* (7–9), and in combination with peptidase inhibitors (10). A better understanding of the fate of the peptides in the GI tract is needed in order to overcome the hydrolysis and to design an oral dosage form for peptide delivery. A model peptide, leu-enkephalin (YGGFL), and several of its analogues were chosen for this study.

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YGGFL has a physiological role in many organs. In the brain, YGGFL serves as a neural mediator where a fast degradation is essential to establish an immediate control (6,11–13). YGGFL hydrolysis has been described for different organs and a similar pattern of hydrolysis has been reported (14–19). Moreover, it has been shown recently that the enzymes hydrolyzing the YGGFL in the different tissues (kidney, jejunum, brain) are related immunologically (14,18). The previously defined "enkephalinase" is now considered to be a general endopeptidase capable of hydrolyzing YGGFL as well as insulin and tachykinins, angiotensin, cholecystokinin, and others (11,14).

Substitution of the second amino acid of YGGFL with a (D) amino acid and methylation of YGGFL at different locations have been shown to increase biological response and to improve oral bioavailability (20,21).

However, replacement in the sequence of amino acids comprising a peptide, which leads to a hydrolysis resistant peptide, may not always be successful since the introduction of a (D) amino acid, in many cases, reduces the pharmacological intrinsic activity. Since the pharmacological response a peptide drug elicits is a result of its metabolic stability and intrinsic activity, both of these processes must be studied in order to optimize drug efficacy. This study was aimed at describing the gastrointestinal hydrolysis of YGGFL and examining the potential for successful absorption of the intact peptide from the jejunum.

MATERIALS AND METHODS

Materials

^{125}I-YGGFL (Peninsula Laboratories, Belmont, CA), ^{14}C-polyethylene-glycol (NEN, Boston, MA), YGGFL, GGFL, YGG, GGF, FL, Leu-D(Ala)^{2}enkephalin, Leu-D(Ala)^{2}enkephalinamide, YGGFM, Met-D(Ala)^2-enkephalin, and polyethylene-glycol 4000 (Sigma Chemical Co., St. Louis, MO) were used.

All buffer and mobile-phase components were analytical or HPLC grade and used as received.

Perfusate Solution

The perfusate solution consisted of Sorensen phosphate and citrate buffers of pH 3.5–7.0, 0.01% PEG 4000 with a trace amount of its ^{14}C isotope, YGGFL or one of its analogues, and NaCl to adjust the final solution osmolality to 290 (±5)mOsm/Kg.

Rat Perfusion

An *in situ* rat perfusion method was used without modification as described previously (22–25). Briefly, a jejunal segment of 6 to 8 cm of a previously fasted, anaesthesized rat is exposed and cannulated. Precleaning of the perfused jejunal segment is performed by passing 20 ml of plain buffer through the segment until the effluent is clear. The studied compound in the buffer solution is then perfused at a flow rate of 0.2 ml/min. Throughout the inhibition experiments two adjacent segments of the jejunum of the same rat were perfused, one segment with the test compound and the other

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with the test compound and the inhibitor so that each animal served as its own control.

In Vitro Stability

A fraction of perfused buffer was collected and YGGFL (or one of its analogues) was spiked into the perfused luminal washouts. Stability was measured in the nonperfused buffers as a control. Samples were collected every 5 min in order to define the initial hydrolysis rate.

Assay Method

Radioactivity. Dual-label counting, ^{125}I with ^{14}C, was performed by means of dual-channel dpm calculations and quench curves, Beckman LS counter 3801 (Beckman Instruments, Inc., Fullerton, CA). Water absorption or desorption was determined by measurement of ^{14}C-PEG 4000, a nonabsorbed, nonhydrolized marker. A 0.5-ml sample was mixed with 10 ml of scintillation cocktail (Bio-Safe, RPI, Mount Prospect, IL) and counted using a Beckman LS 9000 counter (Beckman Instruments Inc., Fullerton, CA).

HPLC. The instrumentation consisted of two pumps (Model 510), a WISP automatic sampler (Model 712), and a UV detector (Model 481) from Waters (Milford, MA). Pump and gradient control, as well as data acquisition and integration, was provided by a Waters Baseline 810 software package (Dynamic Solution, Ventura, CA). All peptides were separated on a reverse-phase column (Ultrasphere ODS 51, Beckman, San Ramon, CA), with mobile phase A–acetonitrile and B–0.05 *M* phosphate buffer acidified with phosphoric acid to pH 3.5; detection wavelength was 210 nm. A gradient elution was applied with 8% A from 0 to 4 min, increased gradually to 40% from 4 to 16 min. Retention times for YGGFL and derivatives were as follows: YGGFL, 15.8 min; GGFL, 14.9 min; FL, 14.5 min; and YGG, 4.5 min. Retention times for other peptides were as follows: YG, 3.9 min; GFL, 9.4 min; and GF, 8.0 min.

Estimation of Intestinal Wall Permeability

The physiological maximum wall permeability reflecting the maximum absorption rate can be estimated with the knowledge of the stagnant or aqueous diffusion layer (22–25). The following set of equations was used to estimate the wall permeability (P^*wall) of YGGFL and analogues (22–25).

$$P^*\text{wall} = P^*\text{eff}/[1 - (P^*\text{eff}/P^*\text{aq})] \quad (1)$$

$$P^*\text{eff} = Q[1 - (C_m/C_o)]/2\,p\,R\,L \quad (2)$$

$$P^*\text{aq} = 1/(A^*\text{Gz}^{1/3}) \quad (3)$$

$$\text{Gz} = P\,DL/2Q \quad (4)$$

where P^*wall is the dimensionless wall permeability, P^*eff is the dimensionless effective permeability, P^*aq is the dimensionless aqueous permeability, Q is the flow rate, C_m/C_o is the experimental output/input ratio, R is the jejunal radius, L is the length of the perfused jejunal segment, A is a predetermined constant, Gz is the Graetz number, and D is the compound aqueous diffusivity.

RESULTS AND DISCUSSION

Jejunal Hydrolysis of YGGFL at pH 7.0

In a preliminary study only 10 ± 6% of intact YGGFL was recovered after perfusion through the rat jejunum, as detected by HPLC. When ^{125}I-YGGFL was perfused, about 85% of the radioactivity was detected in the perfusate, indicating that substantial metabolism was occurring during perfusion. Metabolism by brush border amino peptidase giving free ^{125}I-tyrosine which in turn is only partially absorbed would account for these results. Luminal hydrolysis as measured *in vitro* in collected perfusate accounts for, at most, up to 10% of the total hydrolysis measured (Fig. 1). The relative stability of YGGFL in perfusate, even in an initial fraction that was collected at the beginning of the precleaning procedure, suggests that the hydrolysis is predominantly caused by the brush border enzymes.

pH Influence on YGGFL Hydrolysis

It has been reported that below pH 3.5 undesirable hystological changes develop in the rat jejunum (23). Therefore, pH 3.5 was the lowest pH employed in this study. The hydrolysis of YGGFL is reduced as the pH of the perfusion buffer is lowered from pH 7.0 to pH 4.5 (Fig. 2). YGGFL at pH 7.0 is hydrolyzed at the first and at the third peptide bonds from the amino terminus, to Tyr + GGFL and YGG + FL. On a molar basis, about 20% of YGGFL is absorbed and 80% metabolized, and of that, 80% is metabolized to GGFL, 20% is converted to YGG and FL (Fig. 2).

Aminopeptidase Versus Endopeptidase Inhibition

YGGFL hydrolysis is inhibited by amastatin and by the tripeptides GGF and YGG. Higher YGGFL levels were detected when coperfused with these inhibitors (Fig. 3). Amastatin is a very potent inhibitor of aminopeptidase. It inhibits GGFL production at concentrations of 20 μ*M*, whereas higher concentrations of the tripeptides are needed (10 m*M*) in order to inhibit significantly YGGFL hydrolysis. Amastatin is more effective in inhibiting the aminopeptidase, whereas the tripeptides are more effective in inhibiting the

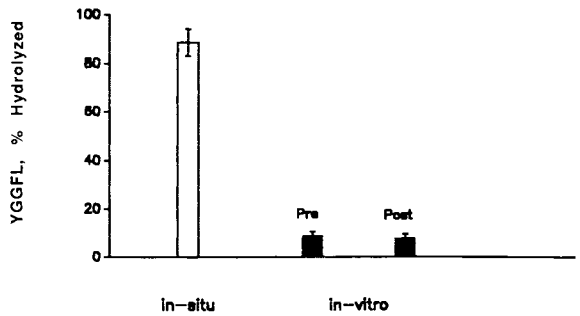

Fig. 1. Comparison of *in situ* vs. *in vitro* YGGFL hydrolysis. Amount of YGGFL hydrolyzed *in vitro* is calculated for average residence time of the perfusion solution in the jejunum, corresponding to the *in situ* experiment. Pre, *in vitro* hydrolysis of YGGFL in perfusate samples collected before the precleaning of the jejunal segment, post, after the precleaning. Mean ± SD for triplicate determinations.

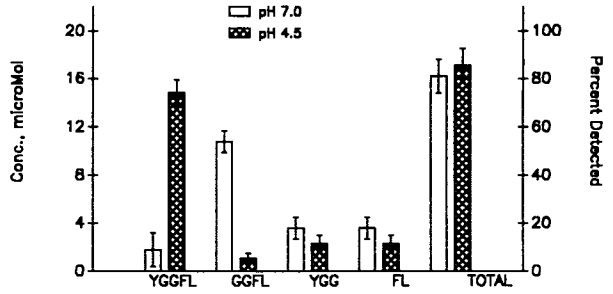

Fig. 2. YGGFL, GGFL, YGG, and FL levels as detected by HPLC after perfusion at pH 4.5 and 7.0. Mean of four rats ± SD in each group.

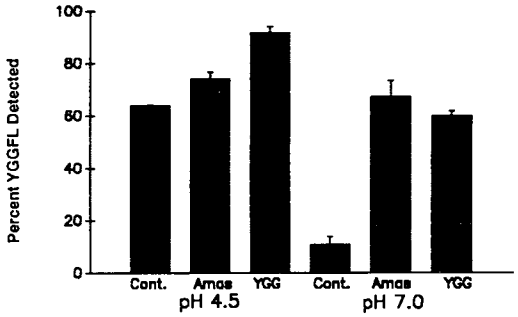

Fig. 4. Influence of peptidase inhibitors, amastatin, YGG, and amastatin with YGG on YGGFL hydrolysis at pH 4.5 and 7.0 as detected by HPLC. Mean of four rats ± SD.

endopeptidase. From Fig. 4 it can be seen that simultaneous reduction of pH and coperfusion with YGG results in the highest reduction of enzymatic hydrolysis. In this condition 94 ± 4% of YGGFL is recovered after jejunal perfusion, probably due to almost-complete inhibition of the brush-border enzyme hydrolytic activity.

YGGFL Analogues

Replacing the second amino acid of YGGFL, Gly, with D-Ala significantly decreased the hydrolysis, most notably at high pH, where YGGFL hydrolysis was most significant (Fig. 5). Amidation of the carboxy peptide terminus does not contribute to peptide stability against enzymatic hydrolysis. This can be seen from the hydrolysis profile of Leu-D(Ala)^2-enkephalin*amide* versus Leu-D(Ala)^2-enkephalin (Fig. 5). No significant difference in the hydrolysis profile of YGGFM and YGGFL (Met and Leu-enkephalin) can be seen. Similarity in brush border hydrolysis between YGGFM and YGGFL is also demonstrated by the similar hydrolysis pattern while coperfused with amastatin or YGG. Met-D(Ala)^2-enkephalin*amide* was also tested and no difference in its hydrolysis profile compared to Leu-D(Ala)^2-enkephalin or Leu-D(Ala)^2-enkephalin*amide* was observed. The influence of amastatin and YGG on the hydrolysis of Leu-D(Ala)^2-enkephalin in comparison to YGGFL is shown in Fig. 6. Amastatin does not significantly improve Leu-D(Ala)^2-enkephalin stability, while the YGG levels increase by about 20%. This suggests that the analogue is resistant to aminopeptidase hydrolysis and sensitive to hydrolysis by endopeptidase. Moreover, the extent of endopeptidase hydrolysis of Leu-D(Ala)^2-enkephalin is similar to that of YGGFL, as observed from the YGG or FL levels appearing after its hydrolysis at pH 7.0 (Fig. 2). Apparently, replacing Gly^2 with D-Ala protects against aminopeptidase hydrolysis of the first peptide bond but does not alter the hydrolysis of the third peptide bond, Gly–Phe, by the endopeptidase.

Estimation of YGGFL Wall Permeability

The molecular weight of YGGFL (MW 562.5) is not a limiting factor in its absorption since many drugs with molecular weights in this range are orally absorbed. The estimation of YGGFL wall permeability is based on the experimental *in situ* perfusion output/input ratio while hydrolysis is inhibited. Equivalent levels of YGGFL and Leu-D(Ala)^2-enkephalin are detected, 94%, when hydrolysis is inhibited. YGGFL output at low pH and coperfused with YGG is 93 ± 3% (Fig. 4) and Leu-D(Ala)^2-enkephalin also yields 94 ± 4% when coperfused with YGG (Fig. 6). Moreover, considering luminal hydrolysis of 10% (Fig. 1) and the total amount of YGGFL and the degradation products (GGFL and YGG or FL) on a molar basis add up to 93 ± 5% (Fig. 3). These results taken together suggest that 6% of YGGFL was absorbed under the perfusion conditions.

The wall permeability of YGGFL can be estimated according to the model published elsewhere (24,25) considering the experimental conditions of 6–8 cm rat jejunum perfused at a flow rate of 0.2 ml/min and an aqueous diffusivity of 0.000323 cm^2/min. YGGFL wall permeability, P*wall, is

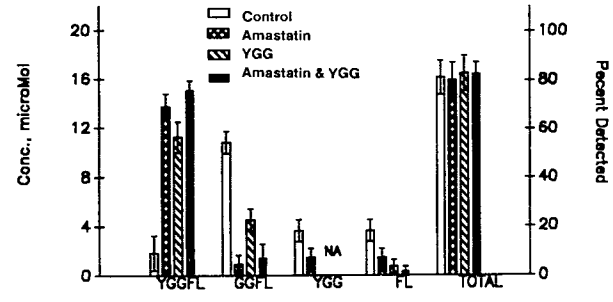

Fig. 3. Averages ± SD (four rats in each condition) of intact YGGFL, GGFL, YGG, and FL and cumulative value of YGGFL and its metabolites as detected by HPLC after perfusion through rat jejunum at pH 7.0. Influence of peptidase inhibitors, amastatin, YGG, or amastatin with YGG.

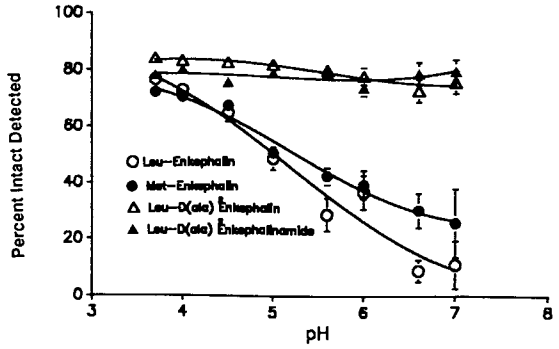

Fig. 5. Intact YGGFL and analogue levels as detected by HPLC after perfusion through rat jejunum at pH 3.7 to 7.0. Mean of four to eight rats in each condition ± SD.

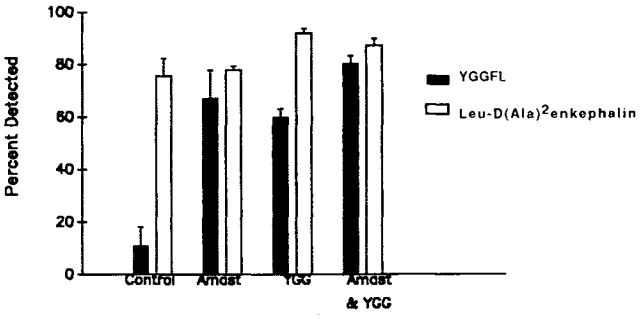

Fig. 6. Influence of aminopeptidase inhibitors, amastatin, and YGG, on levels of intact YGGFL and Leu-D(Ala)^2-enkephalin perfused through rat jejunum at pH 7.0. Mean of four rats ± SD for each condition.

then calculated to be 1.83 ± 0.9%. Since YGGFL and Leu-D(Ala)^2-enkephalin do not differ significantly in their aqueous diffusivity, identical output values result in very similar wall permeabilities. Drugs with P^*wall above 1.00 have been found to be well absorbed (22), whereas the absorption of those with P^*wall below 0.5 is generally more limited. The calculated wall permeabilities indicate that YGGFL is transported through the intestinal microvillar membrane and that membrane permeability factors do not limit absorption of these pentapeptides.

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