Aminopeptidase Activity in the Jejunal and Ileal Peyer's Patches of the Albino Rabbit

Eiji Hayakawa[1] and Vincent H. L. Lee[2,3]

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The objectives of this study were (a) to compare the aminopeptidase activity in the Peyer's patches of the jejunum and ileum of the albino rabbit against that in the adjacent patch-free segments and (b) to determine the relative sensitivities of the aminopeptidase activity in the Peyer's and non-Peyer's patches to aminopeptidase inhibitors and penetration enhancers. The results indicated that the Peyer's patches were about equal in aminopeptidase activity in the jejunum and in the ileum but were only 20–30% as rich in aminopeptidase activity as their neighboring patch-free areas. Compared to non-Peyer's patches, the aminopeptidase activity in the Peyer's patches was not as sensitive to the inhibitory effect of amastatin. It was, however, much more sensitive to the inhibitory effect of puromycin and *p*-chloromercuribenzoate and was somewhat more sensitive to the inhibitory effect of Na deoxycholate, Na glycocholate, and polyoxyethylene-9-lauryl ether. Therefore, based on substrate preferences and on the relative sensitivity of aminopeptidase activity to inhibition by aminopeptidase inhibitors and penetration enhancers, the relative proportions of various aminopeptidases in the Peyer's patches and in the non-Peyer's patches are likely different.

KEY WORDS: aminopeptidases; peptide bioavailability; Peyer's patches; aminopeptidase inhibitors; penetration enhancers.

INTRODUCTION

Peyer's patches, groups of subepithelial lymphoid follicles located in increasing frequency toward the distal end of the gastrointestinal tract (1), have received considerable attention as possible sites for the oral administration of peptide and protein drugs (2). Such an interest in Peyer's patches is apparently stimulated by the observation that M cells, which overlie the lymphoid follicles, are more active than the enterocytes in endocytosing macromolecules, microbes, and particulates from the intestinal milieu (1,3–8). Keljo and Hamilton (9), for instance, demonstrated that horseradish peroxidase, MW 40,000, penetrated the piglet jejunal segment with Peyer's patches about three times better than the segment without Peyer's patches. Ho *et al.* (10) also reported that poly(D-lysine), MW 55,000, penetrated the rabbit jejunal Peyer's patches better than the non-Peyer's patches. Endocytosis was involved in the transport of both proteins, as indicated by its inhibition by metabolic inhibitors.

Although the alkaline phosphatase and esterase activities in the Peyer's patches are known (11), there has been no direct determination of the proteolytic activity that could limit the fraction of peptide and protein drugs absorbed from the Peyer's patches. The objectives of this study were (a) to compare the aminopeptidase activity in the Peyer's patches of the jejunum and ileum of the albino rabbit against that in the adjacent patch-free segments and (b) to determine the relative sensitivities of the aminopeptidase activity in the Peyer's patches and in the non-Peyer's patches to aminopeptidase inhibitors and penetration enhancers.

Aminopeptidases are a family of exopeptidases which cleave peptides and proteins at their N termini. These proteases possess broad substrate specificities (12), are widely distributed in the body (13), and play a principal role in terminating the activity of several neuropeptides (14–16). In this study, aminopeptidase activity was determined by monitoring the initial formation rate of 4-methoxy-2-naphthylamine from one of four aminopeptidase substrates, namely, 4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-glutamic acid, and L-arginine. Within limits, these substrates are preferentially hydrolyzed by leucine aminopeptidase (EC 3.4.11.1), aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), and aminopeptidase B (EC 3.4.11.6), respectively. None of these substrates is hydrolyzed by serine or thiol proteinases (17).

MATERIALS AND METHODS

Materials

4-Methoxy-2-naphthylamides of leucine, alanine, glutamic acid, and arginine; amastatin; bestatin; puromycin; Na *p*-chloromercuribenzoate (PCMB); Na deoxycholate (DC); Na glycocholate (GC); and polyoxyethylene-9-lauryl ether (PLE) were obtained from Sigma Chemical Company (St. Louis, MO) and were used as received.

Collection of Mucosal Tissues

Two groups of six female albino rabbits (ABC Rabbitry, Pomona, CA), weighing approximately 2 kg, were used in this study. These two groups of rabbits were used 3 months apart in order to verify reproducibility of the results in aminopeptidase activity. Rabbits were fasted overnight and then euthanized by a lethal injection of sodium pentobarbital solution (Eutha-6, Western Medical Supply Co., Arcadia, CA) into a marginal ear vein. Ten-centimeter segments of the jejunum and ileum devoid of Peyer's patches were excised, then rinsed in 0.9% NaCl, and the epithelial cells were scraped off with a No. 11 surgical blade. Additional 10-cm segments of the jejunum and ileum containing Peyer's patches were excised and treated similarly after trimming off the surrounding nonpatch tissues. Care was taken not to contaminate the Peyer's patch scrapings with columnar epithelial cells by trimming all non-Peyer's patch tissues off the Peyer's patch tissues. Each Peyer's patch averaged 0.9 cm^2 in size and could be distinguished from the surrounding

[1] Present address: Kyowa Hakko Kogyo, Pharmaceutical Research Laboratories, 1188 Shimotogari, Nagaizumi-cho Sunto-gun, Shizuoka-ken, Japan 411.

[2] Drug Targeting Research, Department of Pharmaceutical Sciences, University of Southern California, School of Pharmacy, Los Angeles, California 90033.

[3] To whom correspondence should be addressed at University of Southern California, School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California 90033.

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patch-free tissues by their oval, elevated, nodular appearance.

Preparation of Tissue Homogenates

Tissue scrapings were homogenized in 4–6 ml of a 0.05 *M* Tris-maleate buffer (pH 7.4) at 4°C using a Teflon–glass homogenizer. The homogenate was centrifuged at 3020*g* in a refrigerated (4°C) centrifuge (Sorvall RC-5B refrigerated superspeed centrifuge, Dupont Instruments, Newtown, CT) for 10 min to remove cellular and nuclear debris. The resulting supernatant, which contained cytosol and relevant plasma and intracellular membrane fractions, was assayed immediately for aminopeptidase activity against each of the four aminopeptidase substrates. Each supernatant was adjusted to a protein concentration of 2 mg ml^{-1} as determined by a dye-binding assay (18) using rabbit serum albumin as the standard.

Preparation of Stock Substrate Solutions

Stock solutions of the four aminopeptidase substrates, 30 m*M* each, were made in dimethylformamide, stored at −20°C, and used within 5 days of preparation. The final substrate concentration in an incubation mixture was 0.01, 0.02, 0.04, 0.1, and 1.0 m*M*. The final concentration of dimethylformamide in an incubation mixture was 3.3%, which was necessary to keep the substrates in solution.

Assay of Aminopeptidase Activity

Aminopeptidase activity was determined using an assay previously reported by Stratford and Lee (19). One hundred microliters of a tissue supernatant was preincubated in 2.8 ml of a 0.05 *M* Tris-maleate buffer, pH 7.4, for 15 min in a fluorescence cuvette thermostated at 37°C. The buffer contained one of the following aminopeptidase activators: 0.1 m*M* Mn^{2+} for leucine aminopeptidase, 0.1 m*M* Co^{2+} for aminopeptidase N, 10 m*M* Ca^{2+} for aminopeptidase A, and 0.1 *M* Cl^- for aminopeptidase B (20–24). The reaction was initiated by adding 100 μl of a stock substrate solution to the preincubated mixture. The linear increase in fluorescence intensity due to 4-methoxy-2-naphthylamine was monitored at an excitation wavelength of 342 nm and an emission wavelength of 426 nm for 5 min (Perkin-Elmer Model 650-10S spectrofluorometer, Norwalk, CT). Initial velocities were determined from the initial slopes of plots of fluorescence intensity vs time and are expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein after correcting for chemical hydrolysis. The Michaelis–Menten parameters, V_{max} and K_m, were obtained from these initial velocities by Lineweaver–Burk analysis. All incubations were performed at least in duplicate.

Aminopeptidase assays were also conducted with the alanine 4-methoxy-2-naphthylamide substrate in the presence of (a) an aminopeptidase inhibitor—0.01 m*M* amastatin, 0.01 m*M* bestatin, 0.1 m*M* puromycin, or 0.1 m*M* PCMB, or (b) a penetration enhancer—0.1 m*M* Na deoxycholate, 0.1 m*M* Na glycocholate, or 0.1 m*M* PLE. One hundred microliters of an inhibitor/penetration enhancer stock solution in buffer were added to 2.8 ml of tissue-buffer/activator preparation and incubated for 15 min at 37°C prior to initiating the reaction, as described earlier.

RESULTS

Rank Order of Aminopeptidase Activity in Various Mucosal Tissue Homogenates

The rank order of hydrolysis of all aminopeptidase substrates was ileum > jejunum > jejunal Peyer's patches = ileal Peyer's patches (Fig. 1). The aminopeptidase activity in the Peyer's patches was about one-third (jejunal) to one-fifth (ileal) that in the non-Peyer's patches (Table I). While the Peyer's patches in the jejunum and ileum were approximately equal in aminopeptidase activity, the non-Peyer's patches in the jejunum were 50% as rich in aminopeptidase activity as those in the ileum (Table I).

Pattern of Substrate Hydrolysis in Various Mucosal Tissue Homogenates

Both the Peyer's patches and the non-Peyer's patches behaved the same way toward the alanine and arginine substrates. On the basis of V_{max} (i.e., at high substrate concentrations), the alanine substrate was the most readily hydrolyzed, whereas the arginine substrate was the least readily hydrolyzed (Table II). The Peyer's patches and non-Peyer's patches, however, differed in how readily they hydrolyzed the leucine and the glutamic acid substrates. In the non-Peyer's patches, it was the glutamic acid substrate that was the more readily hydrolyzed; in the Peyer's patches, it was the leucine substrate that was the more readily hydrolyzed. Thus, the rank order of susceptibility of substrate to hydrolysis in the non-Peyer's patches was alanine > glutamic acid > leucine > arginine. In the Peyer's patches, the rank order was alanine > leucine > glutamic acid > arginine (Table II).

The rank order of substrate hydrolysis at low substrate concentrations (i.e., on the basis of V_{max}/K_m) differed from that at high substrate concentrations (Table II). In the jejunal non-Peyer's patches, the rank order was alanine = glutamic acid > leucine > arginine. In the ileal non-Peyer's patches, the rank order was glutamic acid > alanine = leucine > arginine. The rank order was the same in both jejunal and ileal Peyer's patches: leucine > alanine = glutamic acid > arginine.

The Peyer's patches and non-Peyer's patches were similar in the rank order of their K_m's toward the four substrates (Table II). The rank order was alanine > arginine > glutamic acid > leucine. Generally, the K_m in the Peyer's patches was lower than that in the non-Peyer's patches.

Effect of Aminopeptidase Inhibitors and Penetration Enhancers on Aminopeptidase Activity Against Alanine 4-Methoxy-2-Naphthylamide

The aminopeptidase activity in both the Peyer's patches and the non-Peyer's patches was most susceptible to inhibition by amastatin, followed by bestatin, PCMB, puromycin, Na deoxycholate, Na glycocholate, and PLE, in that order (Fig. 2). The three penetration enhancers were relatively ineffective against aminopeptidase activity in the jejunum and

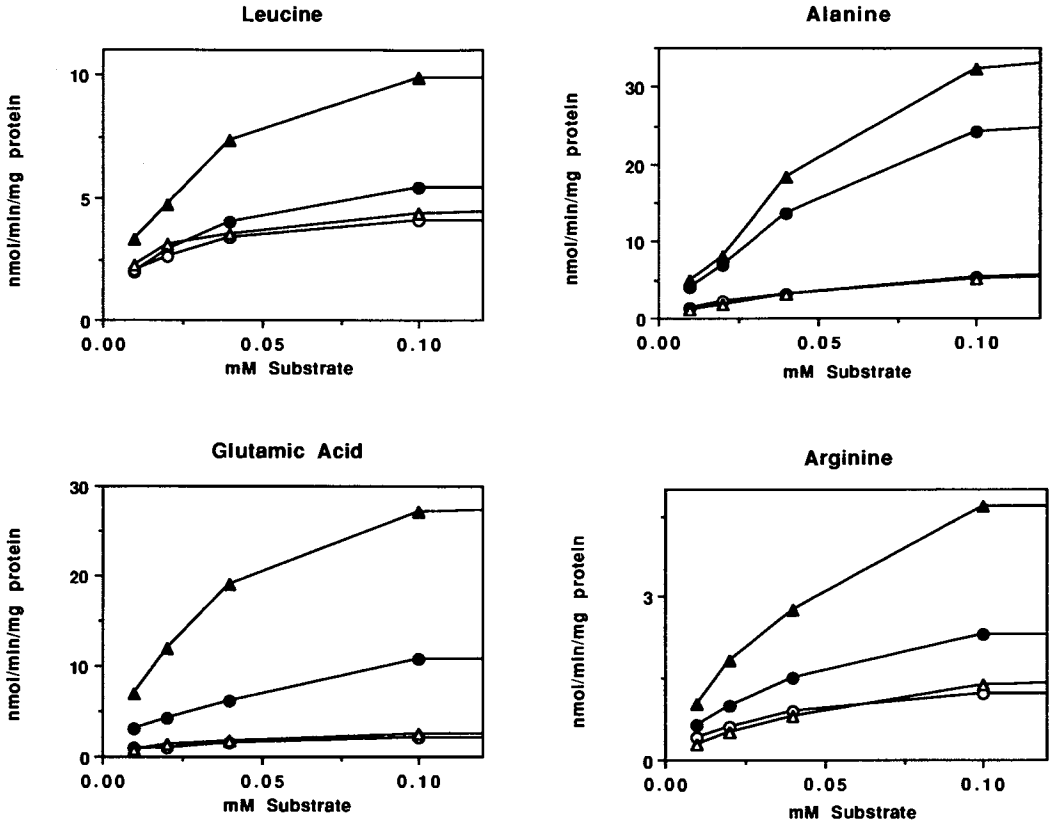

Fig. 1. Hydrolytic activities in the homogenates of the jejunum (●), jejunal Peyer's patches (○), ileum (▲), and ileal Peyer's patches (△) against 4-methoxy-2-naphthylamides of leucine, alanine, glutamic acid, and arginine. The error bars are smaller than the size of the symbols. The activities at 1 m*M* substrate concentration are omitted for clarity.

ileum ($P = 0.05$ by one-way ANOVA) and were only slightly effective against aminopeptidase activity in the Peyer's patches ($P < 0.05$ by one-way ANOVA). Compared with non-Peyer's patches, the aminopeptidase activity in the Peyer's patches was not as sensitive to inhibition by amastatin ($P < 0.05$ by Student's t test) but was equally as sensitive to inhibition by bestatin ($P = 0.05$ by Student's t test) and was more sensitive to inhibition by puromycin and PCMB ($P < 0.05$ by Student's t test). This is reflected in the ratios (J/JPP and I/IPP) of aminopeptidase activity between the non-Peyer's patches and Peyer's patches in the respective intestinal segment (Table III). Inhibition was generally due to both increases in K_m and decreases in V_{max} (Table IV), suggesting mixed competitive and noncompetitive inhibition.

DISCUSSION

An implicit assumption in this study was that both the M cells in the Peyer's patches and the enterocytes in the patch-free tissues were equally susceptible to disruption during homogenization. On this basis, it appears that the Peyer's patches in the jejunum and the ileum are only 20 to 30% as

Table I. Ratios of Aminopeptidase Activity Between the Jejunum and Its Peyer's Patches (J/JPP), the Ileum and Its Peyer's Patches (I/IPP), the Ileum and the Jejunum (I/J), and the Peyer's Patches in the Ileum and the Jejunum (IPP/JPP)

Substrate	J/JPP	I/IPP	I/J	IPP/JPP
Leucine	1.2 ± 0.2[a]	1.8 ± 0.3	1.8 ± 0.1	1.2 ± 0.1
Alanine	3.6 ± 0.7	5.2 ± 0.8	1.3 ± 0.2	0.9 ± 0.2
Glutamic Acid	4.0 ± 0.7	9.5 ± 1.5	2.6 ± 0.3	1.1 ± 0.2
Arginine	1.7 ± 0.2	3.3 ± 0.5	1.8 ± 0.2	1.0 ± 0.3
Overall	2.6 ± 1.3[b]	4.9 ± 3.0	1.9 ± 0.5	1.0 ± 0.2

[a] Mean ± SD of the ratios at five substrate concentrations.
[b] Mean ± SD of the means of the four substrates.

Table II. Michaelis–Menten Parameters of Aminopeptidase Activity in the Jejunum (J), the Ileum (I), and Their Peyer's Patches (JPP, IPP) Based on 4-Methoxy-2-Naphthylamides of Leucine (Leu), Alanine (Ala), Glutamic Acid (Glu), and Arginine (Arg) as Substrates

	K_m (μM)				V_{max} (nmol/min/mg protein)				V_{max}/K_m (10^2/min/mg protein)			
Substrate	J	JPP	I	IPP	J	JPP	I	IPP	J	JPP	I	IPP
Leu	22.8	11.9	25.2	13.2	6.4	4.4	11.5	5.2	9.0	11.9	14.7	12.6
Ala	105.8	49.0	138.5	83.9	46.5	8.1	72.6	9.9	14.2	5.4	16.9	3.8
Glu	30.6	18.8	46.0	34.4	12.1	2.5	39.2	3.6	12.8	4.3	27.5	3.4
Arg	36.8	26.2	51.7	62.9	2.9	1.5	6.4	2.1	2.6	1.8	4.0	1.1

active as the non-Peyer's patches in aminopeptidase activity (Table I). This lower aminopeptidase activity in the Peyer's patches can be due to inherently less active aminopeptidases in the Peyer's patches or to a smaller proportion of the soluble proteins in the Peyer's patch homogenates attributable to aminopeptidases. In either case, the lower aminopeptidase activity in the Peyer's patch homogenates is consistent with the trend of less enzymatic breakdown of horseradish peroxidase (25) and RU41740 (a glycoprotein immunomodulator) (26) in the patch-containing than in the patch-free epithelia of the piglet jejunum and the rabbit duodenum, respectively. Whether or not this lower aminopeptidase activity in the Peyer's patches resides exclusively in the M cells is as yet unknown, since no attempt was made in this study to separate M cells from the other cell types in the Peyer's patches.

The same four types of aminopeptidases are probably present in both the Peyer's and the non-Peyer's patches. They are leucine aminopeptidase, aminopeptidase N, aminopeptidase A, and aminopeptidase B. Among them, aminopeptidase N is probably the most abundant, whereas aminopeptidase B is the least. At substrate concentrations considerably above the K_m—80 μM for the Peyer's patches and 140 μM in the non-Peyer's patches (Table II), the alanine substrate is the most readily hydrolyzed, whereas the arginine substrate is the most stable (Table II). At substrate concentrations below the K_m, the leucine substrate is the most readily hydrolyzed in the Peyer's patches, whereas the alanine or glutamic acid substrate is the most readily hydrolyzed in the non-Peyer's patches (Table II). This subtle difference in rank order of substrate preference indicates that the relative proportions of aminopeptidases in the Peyer's patches and the non-Peyer's patches are different. Two additional lines of evidence supporting this hypothesis are (a) the nonidentity of the K_m's for a given substrate (Table II)—the K_m would have been the same from tissue to tissue if the

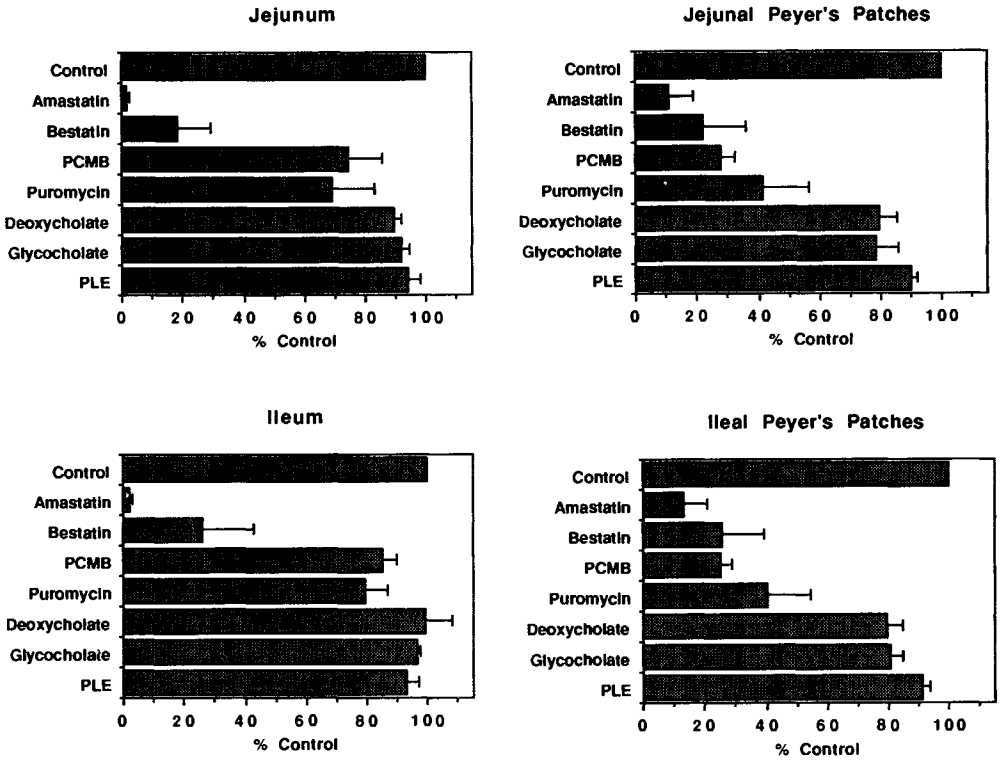

Fig. 2. Effect of various aminopeptidase inhibitors and penetration enhancers on the hydrolytic rates of alanine 4-methoxy-2-naphthylamide in the homogenates of the jejunum, jejunal Peyer's patches, ileum, and ileal Peyer's patches. Error bars represent standard deviation on the hydrolytic rates at five substrate concentrations.

Table III. Ratios of Aminopeptidase Activity Against Alanine 4-Methoxy-2-Naphthylamide Between the Jejunum and Its Peyer's Patches (J/JPP), the Ileum and Its Peyer's Patches (I/IPP), the Ileum and the Jejunum (I/J), and the Peyer's Patches in the Ileum and the Jejunum (IPP/JPP) in the Presence of Various Aminopeptidase Inhibitors and Penetration Enhancers

Condition	J/JPP	I/IPP	I/J	IPP/JPP
Control	2.2 ± 0.4[a]	3.2 ± 0.6	1.5 ± 0.1	1.0 ± 0.05
Amastatin	0.3 ± 0.04	0.5 ± 0.04	2.3 ± 0.4	1.3 ± 0.2
Bestatin	1.9 ± 0.3	3.1 ± 0.2	2.0 ± 0.2	1.2 ± 0.2
PCMB[b]	5.8 ± 0.5	10.9 ± 1.1	1.1 ± 0.2	0.9 ± 0.01
Puromycin	3.9 ± 0.8	6.6 ± 1.5	1.7 ± 0.2	1.0 ± 0.08
Deoxycholate	2.5 ± 0.4	4.0 ± 0.6	1.6 ± 0.1	1.0 ± 0.03
Glycocholate	2.6 ± 0.5	3.8 ± 0.7	1.5 ± 0.07	1.0 ± 0.04
PLE[c]	2.3 ± 0.4	3.2 ± 0.5	1.4 ± 0.1	1.0 ± 0.05

[a] Mean ± SD of the ratios at five substrate concentrations.
[b] Na *p*-chloromercuribenzoate.
[c] Polyoxyethylene-9-lauryl ether.

substrate were acted upon by a single protease or by the same proportion of proteases; and (b) the lower sensitivity of the aminopeptidase activity in the Peyer's patches to inhibition by amastatin and its higher sensitivity to inhibition by PCMB and puromycin (Fig. 2).

Although 1% Na glycocholate is very effective in inhibiting leucine aminopeptidase activity and in protecting insulin from proteolysis in the rat nasal mucosa (27), it is practically inert against the aminopeptidases in the Peyer's patches and is only slightly inhibitory against the aminopeptidases in non-Peyer's patches in the jejunum and the ileum. The same trend is observed in the other two enhancers, Na deoxycholate and PLE (Fig. 2). More potent inhibitors such as amastatin, bestatin, PCMB, and puromycin are required.

Aminopeptidase activity is only one factor influencing the absorption of peptides, susceptible to this family of proteases, from the Peyer's patches. The permeability characteristics of the Peyer's patches is another factor that is expected to play a role in absorption of peptides. Work by Ho *et al.* (10) has revealed no difference in the permeability of patch-containing and patch-free epithelia of the rabbit jejunum and ileum to passively transported compounds such as octanoic acid, salicylic acid, and hydrocortisone and to actively transported compounds such as *d*-glucose and taurocholic acid. Rubas *et al.* (28) have obtained similar results with mannitol, PEG900, and PEG4000. In spite of the early favorable results showing better penetration of horseradish peroxidase across the piglet jejunal Peyer's patches than across the non-Peyer's patches (9), work conducted since then in rabbits has revealed a lower transport rate of horseradish peroxidase (25,29) and RU41740 (26) across the Peyer's patches. Whether this discrepancy in results can be attributed entirely to species differences remains to be determined. To date, there is no information on the permeability characteristics of the Peyer's patches to peptide and protein drugs other than the three proteins mentioned earlier.

In summary, the aminopeptidase activity in the Peyer's patches of the jejunum and ileum is about 20–30% of that in the non-Peyer's patches. In addition, it is generally more sensitive to inhibition by aminopeptidase inhibitors rather than by penetration enhancers. Based on substrate preferences and on the relative sensitivity of aminopeptidase activity to inhibition by aminopeptidase inhibitors and penetration enhancers, the relative proportions of various aminopeptidases in the Peyer's patches and the non-Peyer's patches are possibly different. Further work is necessary to determine whether peptidases other than aminopeptidases, such as diaminopeptidases, carboxypeptidases, and various endopeptidases, are also low in activity in Peyer's patches and whether the low aminopeptidase activity in the Peyer's patches indeed favors the penetration of peptides susceptible to aminopeptidases.

Table IV. Effect of Aminopeptidase Inhibitors and Penetration Enhancers on the Michaelis–Menten Parameters of Aminopeptidase Activity in the Jejunum (J), the Ileum (I), and Their Peyer's Patches (JPP, IPP) Based on Alanyl 4-Methoxy-2-Naphthylamide as the Substrate

	K_m (μ*M*)				V_{max} (nmol/min/mg protein)				V_{max}/K_m (10^2/min/mg protein)			
Modulator	J	JPP	I	IPP	J	JPP	I	IPP	J	JPP	I	IPP
Control	105.8	49.0	138.5	83.9	46.5	8.1	72.6	9.9	14.2	5.4	16.9	3.8
Amastatin	125.7	121.1	382.5	330.3	0.5	1.2	2.8	2.8	0.12	0.33	0.23	0.27
Bestatin	231.8	129.7	478.6	388.6	12.8	2.6	42.8	6.7	1.8	0.64	2.9	0.55
PCMB[a]	110.9	107.9	175.1	245.3	34.2	3.9	73.7	5.3	10.0	1.2	13.6	0.70
Puromycin	84.3	186.5	227.7	152.5	25.2	8.1	85.8	5.3	9.6	1.4	12.2	1.1
Deoxycholate	118.0	56.5	92.2	98.2	45.0	7.0	53.4	8.6	12.3	4.0	18.7	2.8
Glycocholate	110.5	50.0	142.7	94.4	44.2	6.3	70.0	8.5	12.9	4.0	15.8	2.9
PLE[b]	130.3	51.4	108.4	92.1	51.2	7.5	54.3	9.5	12.7	4.7	16.2	3.3

[a] Na *p*-chloromercuribenzoate.
[b] Polyoxyethylene-9-lauryl ether.

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