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In situ characterization of nasal leucine enkephalin degrading aminopeptidase
Susceptibility of the nasal enzyme to boronic acids and phosphorus-containing peptide and amino acid isosteres

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Abstract

The N-terminal Tyr-Gly bond of leucine-enkephalin is specifically hydrolyzed during exposure to the nasal mucosa. Kinetic properties in situ indicate that this activity is due to a single enzyme which has a K_m^{app} of 0.4 mM for leucine-enkephalin. Analysis of initial rates of hydrolysis from earlier inhibition studies using boroalanine, boroleucine, and borovaline indicated that these inhibitors bind the nasal enzyme with K_i^{app} values of 0.009–0.02 μM. In addition, we have evaluated borophenylalanine ($K_i^{app} = 0.004$ μM) in this study. Similarly, H-PheΨ[P(O)(OH)CH_2]Phe-OMe binds the nasal aminopeptidase with a K_i^{app} of 0.2 μM. Comparison of these K_i values with those of cytosolic aminopeptidase and microsomal aminopeptidase derived from porcine kidney, indicates that the nasal enzyme closely resembles the microsomal enzyme in properties. Major distinctions between the enzymes are: (1) the greater dependence of the cytosolic enzyme on the nature of the amino acid residue in the primary site (2) a much greater preference of both the microsomal and nasal enzyme for H-PheΨ[P(O)(OH)CH_2]Phe-OMe over H-PheΨ[P(O)$(OH)_2$].

Keywords: Leucine-enkephalin degrading aminopeptidase; Nasal enzyme; Peptide; Leucine-enkephalin; Aminopeptidase

1. Introduction

Nasal delivery of peptide pharmaceuticals is an alternative to parenteral administration. However, one limitation is peptide hydrolysis by peptidases, specially exopeptidases associated with the nasal mucosa. An aminopeptidase which catalyzes the hydrolysis of neutral amino acid residues from the N-terminus of peptides appears to be the predominant activity (Hussain et al., 1989). We have focused on this activity using leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) as a model peptide and have shown with others that the Tyr-Gly bond is specifically hydrolyzed in situ in a nasal rat model. Initially, we demonstrated

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that alpha-aminoboronic acids were effective in blocking this rat nasal peptidase activity (Hussain et al., 1989). This group of compounds is also effective in the inhibition of both cytosolic and microsomal aminopeptidases (Shenvi, 1986). It is expected that these inhibitors are acting as transition state analogs mimicking the tetrahedral adduct formed during peptide hydrolysis. More recently, we have shown that the dipeptide phosphinate analog, H-PheΨ[P(O)(OH)CH_2]Phe-OMe, is effective in the inhibition of the nasal aminopeptidase (Hussain et al., 1992). This follows earlier observations (Giannousis and Bartlett, 1987) that phosphorus analogs are effective inhibitors of cytosolic leucine aminopeptidase and are also probably acting as transition state analogs. These particular phosphinate dipeptide analogs where -CO-NH- bonds are replaced by -P(O)(OH)-CH_2- have not been evaluated as aminopeptidase inhibitors previously.

In this study, we have further characterized the rat nasal aminopeptidase activity. We have determined its reactivity with a number of additional phosphorus-containing inhibitors and compared its susceptibility to inhibition with that of porcine kidney cytosolic and microsomal aminopeptidases. Our studies are directed toward understanding the specificity of the nasal enzymes and their control with synthetic inhibitors.

2. Experimental

2.1. Preparation of phosphorus-containing inhibitors

The syntheses of compounds **I** and **III** have been described previously (Hussain et al., 1992). Z-PheΨ[P(O)(OMe)CH_2]Phe-OMe was prepared by the Michael addition of Z-PheΨ[P(O)(OMe)(H)] (Baylis et al., 1984) to methyl benzacrylate using a previously described procedure (Parsons et al., 1988). This compound was used as an intermediate in the preparation of both **I** and **II**. Z-PheΨ[P(O)$(OPh)_2$] and H-PheΨ[P(O)$(OPh)_2$] were prepared by a previously described method (Oleksyszyn et al., 1979). **VI** was prepared from **IV** using the hydrolysis procedure described previously (Giannousis and Bartlett, 1987). Compounds **III–VI** were prepared as mixtures of enantiomers and were not resolved prior to testing.

2.1.1. H-PheΨ[P(O)(OMe)CH_2]Phe-OMe · HCl, II

Z-PheΨ[P(O)(OMe)CH_2]Phe-OMe (0.50 g, 0.98 mmol) was dissolved in 50 ml of methanol and hydrogenated on a Parr apparatus for 2 h in the presence of 0.5 g of 10% Pd/C and 1 equivalent of HCl. Catalyst was removed by filtration and the product was obtained as a foam (0.25 g) by evaporation of solvent. ^{1}H-NMR (CD_3OD): δ 1.3–2.4 (m, 2H), 2.7–3.3 (m, 4H), 3.6–3.9 (m, 7H), 7.1–7.5 (m, 10H). MS (FAB) calcd. for M ($C_{20}H_{28}NO_4P$) + H: 376.17. Found: 376.22.

It should be noted that even in the solid form, partial hydrolysis of the phosphonate ester was obtained on storage. **II** was separated from the hydrolyzed ester by HPLC using a 0.47 × 25 cm Vydec C4 column. Linear gradients from 10 to 45% acetonitrile in 0.1% aqueous trifluoroacetic acid were ran at a flow rate of 1.0 ml/min over a period of 20 min. Product eluted at 11.5–13.5 min while the corresponding acid eluted at 9.7 min.

2.1.2. H-PheΨ[P(O)(OPh)(OH)] · HCl, V

Z-PheΨ[P(O)$(OPh)_2$] (1.0 g, 2.0 mmol) was allowed to stir with 5 ml of 1.0 M NaOH for 1.5 h at room temperature. The mixture was heated for 30 min at 100°C to yield a complete solution. It was diluted to 100 ml with H_2O and extracted with ethyl acetate. The aqueous phase was acidified and the product was extracted into ethyl acetate. This solution was washed with 0.20 N HCl and with saturated aqueous NaCl. It was dried over Na_2SO_4, filtered, and evaporated to yield 0.78 g of Z-PheΨ[P(O)(OPh)(OH)]. ^{1}H-NMR (CD_3OD): δ 2.9 (m, 1H), 3.3 (m, 1H), 4.4 (m, 1H), 4.9 (q, 2H), 7.1–7.4 (overlapping m, 10H). FAB, MS calcd. for M ($C_{22}H_{22}NO_5P$) + H: 412.4. Found: 412.09.

The Z-protecting group was removed by dissolving Z-PheΨ[P(O)(OPh)(OH)] (0.30 g, 0.73 mmol) in 75 ml of methanol and hydrogenated for 2.5 h in the presence of 0.30 g of 10% Pd/C and 1.5 equivalents of HCl. Catalyst was removed by filtration, solvent was evaporated, and the residue triturated with ether to yield 0.11 g of the

desired product as a white solid. ^{1}H-NMR (CD_3OD): δ 3.1 (m, 1H), 3.2 (m, 1H), 3.9 (m, 1H), 7.1–7.5 (overlapping m, 10H). Anal. calcd for M ($C_{14}H_{16}NO_3P$) + H: 278.09. Found: 278.09.

2.1.3. H-PheΨ[P(O)(OPh)$_2$] · HBr, IV

Z-PheΨ[P(O)(OPh)$_2$] (1.0 g, 2.1 mmol) was dissolved in 5 ml of anhydrous HBr (30% w/w) in acetic acid and was allowed to stir for 1 h at room temperature. Solvent and excess HBr were removed by evaporation and the residue was triturated with ether to yield the desired product (0.87 g) as an amorphous white solid. ^{1}H-NMR (CD_3OD): δ 3.3 (m, 1H), 3.6 (m, 1H), 4.5 (m, 1H), 7.0–7.6 (overlapping m, 10H). FAB MS calcd for M ($C_{20}H_{20}NO_3P$) + H: 354.37. Found: 354.13.

2.1.4 H-PheΨ[P(O)(OH)$_2$] · HBr, VI

H-PheΨ[P(O)(OPh)$_2$] · HBr (0.50 g, 1.2 mmol) was suspended in 7 ml of glacial acetic acid and 7 ml of 48% aqueous HBr were added. The mixture was heated for 14 h at 115°C and evaporated to yield a solid. The product was triturated with hexane to give a solid, 0.19 g. ^{1}H-NMR (CD_3OD): δ 2.9 (m, 1H), 3.3 (m, 1H), 3.6 (m, 1H), 7.3–7.4 (m, 5H). Anal. calcd. for M ($C_8H_{12}NO_3P$) + e: 201.0514. Found: 201.0519.

2.2. Perfusion studies

Male rats (CD, Charles River) weighing approx. 300 g were used for all studies. The method for perfusion of the rat nasal cavity has been described previously (Hussain et al., 1989). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). An incision was made in the neck and the trachea was cannulated with a polyethylene tube (PE 240). A second tube, which served to introduce the perfusing solution, was inserted through the esophagus to the posterior part of the nasal cavity. The nasopalatine was closed with an adhesive to prevent drainage of the perfusing solution from the nasal to the oral cavity. Leucine-enkephalin solution in 0.10 M sodium phosphate, pH 7.4, was placed in a water-jacketed container thermostated at 37°C and was circulated, at a perfusion rate of 1.3 ml/min, through the nasal cavity of the anesthetized rats using a peristaltic pump.

2.3. In situ kinetic studies

Stock solutions of leucine-enkephalin were prepared in 0.05% acetic acid and stock solutions of inhibitors were prepared in dimethyl sulfoxide (DMSO).

Hydrolysis of leucine-enkephalin was determined by diluting this substrate into the perfusing solution (10 ml), removing aliquots (100 μl) at timed intervals, and diluting into 200 μl of 0.10 M citric acid, pH 2.3, to quench hydrolysis. The concentrations of leucine-enkephalin and its metabolite Des(Tyr)leucine-enkephalin were determined by high-performance liquid chromatography (HPLC) as described previously (Hussain et al., 1989).

Initial velocities for the hydrolysis of leucine-enkephalin were determined from plots of the concentrations of substrate remaining versus time using the initial linear portions of these plots. The apparent Michaelis constant, K_m^{app}, was determined from Lineweaver-Burk plots. Initial rates of hydrolysis were measured over the concentration range from 0.80 to 0.025 mM and were evaluated as double-reciprocal plots of velocity vs initial concentration.

A similar protocol was used to measure values of K_i^{app} for inhibition of leucine-enkephalin hydrolysis. Inhibitor (< 20 μl) was introduced in the perfusate with 0.10 mM leucine-enkephalin. Initial rates of substrate hydrolysis were determined. Data were evaluated graphically in plots of 1/velocity vs inhibitor concentration (Dixon and Webb, 1979). For perfusion studies, the assumption is made that the inhibitors are behaving as competitive inhibitors and values of K_i^{app} were calculated from individual levels of inhibitor. Here the ratio of rates of substrate hydrolysis in the presence and absence of inhibitor (v_s/v_0) and the corresponding Michaelis expressions ($V_mS/K_m(1 + I/K_i) + S$: $V_mS/K_m + S$) were used in obtaining Eq. 1:

$$K_i = \frac{I}{\dfrac{(K_m + S) - (Sv_s/v_0)}{K_m v_s/v_0} - 1} \qquad (1)$$

where v_0 is the velocity of the control in the absence of inhibitor, v_s denotes the velocity in the presence of inhibitor, I is the concentration of inhibitor, K_i represents the dissociation constant of enzyme-inhibitor complex, S is the concentration of substrate (leucine-enkephalin or leucine *p*-nitroanalide) and K_m denotes the Michaelis constant.

2.4. In vitro inhibition studies

The inhibition assays of cytosolic leucine aminopeptidase by the phosphorus-containing amino acid and peptide analogs were run at 25°C in 50 mM Tris buffer, pH 8.6, containing 5.0 mM $MgCl_2$. Porcine kidney cytosolic aminopeptidase (Sigma Type III-CP, 210 units/mg) was activated by incubation at 0.19 mg/ml for 2 h at 37°C in Tris buffer, pH 8.6, containing 1 mM $MnCl_2$. Values of K_i were determined by the Lineweaver-Burk method using leucine *p*-nitroanilide as a substrate. Assays were run in both the presence and absence of inhibitor over a substrate concentration range of 0.25–2.5 mM in 10% DMSO. In the absence of inhibitor, the K_m for substrate hydrolysis was 1.4 ± 0.2 mM. Reported K_i values are the average of at least duplicate determinations.

Assays of microsomal leucine aminopeptidase (Sigma L-6007) were conducted using an enzyme level of 2.0 μg/ml and leucine *p*-nitroanalide as a substrate in 50 mM sodium phosphate buffer, pH 7.2, containing 10% DMSO. The K_m for substrate hydrolysis measured over a substrate range of 0.20–2.0 mM was 0.47 ± 0.10 mM. Inhibitor binding was evaluated by the Lineweaver-Burk method and by the use of Eq. 1 for all inhibitors. Results, obtained over varied inhibitor levels and also over a varied substrate range (0.20–2.0 mM), are consistent with the compounds behaving as competitive inhibitors. Reported K_i values were determined from values of % inhibition measured for a minimum of two different levels of inhibitor and a substrate level of 0.71 mM.

Compound **II** was hydrolyzed to yield compound **I** even when stored as a solid at room temperature. The half-life for this process is approx. 20 days. This occurs much more rapidly in aqueous solution. K_i values in the range of 400 μM were measured for samples freshly prepared or purified by HPLC.

3. Results and discussion

We have focused on the activity of the rat nasal mucosal aminopeptidase responsible for the hydrolysis of the N-terminus residue of leucine-enkephalin and have partially characterized this activity in situ. We have determined its susceptibility to inhibition by phosphorus-containing inhibitors and compared the results with boronic acid inhibitors reported previously (Hussain et al., 1989). In turn, these inhibition data were compared with data for two well characterized aminopeptidases, porcine cytosolic leucine aminopeptidase and microsomal aminopeptidase, to further characterize this nasal enzyme.

3.1. Characterization of the nasal protease activities

In earlier studies, a number of different peptidase activities were detected in the homogenates of rabbit nasal mucosae (Stratford and Lee, 1986). In the present work, mucosal aminopeptidase activity was measured in situ in rats without disrupting the nasal membrane. A buffer solution of substrate was circulated through the nasal cavity of anesthetized rats and timed aliquots were removed to monitor substrate levels. Our results are consistent with the hydrolysis of leucine-enkephalin by a single enzyme associated with the nasal membrane. The presence of the other peptidase activities described previously (Stratford and Lee, 1986) was not detected with this substrate, but certainly must be a consideration for other substrates, particularly those with charged amino acid residues at the N-terminus. In our studies, the N-terminal Tyr-Gly bond in the Tyr-Gly-Gly-Phe-Leu sequence is hydrolyzed specifically as shown by the time-dependent disappearance of leucine-enkephalin with a corresponding appearance of Des(Tyr)leucine-enkephlin (Fig. 1). If the penultimate Gly-Gly bond is being hydro-

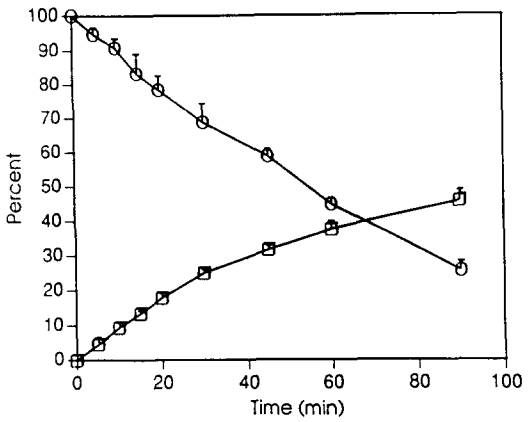

Fig. 1. In situ nasal disappearance of 0.10 mM leucine-enkephalin (○) and appearance of Des-Tyr-leucine-enkephalin (□) in the nasal perfusate (10 ml). Symbols represent the mean ± SD of three rats.

lyzed by aminopeptidases, it is at a much slower rate. The remaining portions of the molecule are not hydrolyzed by mucosal nasal carboxypeptidases or endopeptidases in this in situ model. It should be noted that this activity is similar to the aminopeptidase N-like activity previously observed (Stratford and Lee, 1986).

We have determined the effect of substrate concentration on rates of enzymatic hydrolysis by

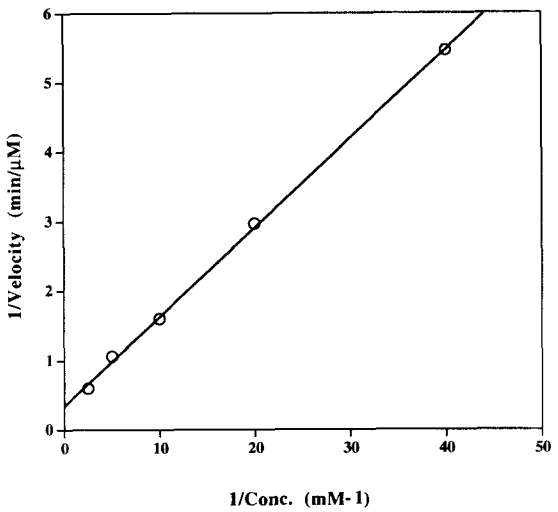

Fig. 2. Lineweaver-Burk plot for the hydrolysis of leucine-enkephalin by the nasal aminopeptidase in situ. Rates of hydrolysis were measured at pH 7.4 and 37°C according to the method described in section 2.

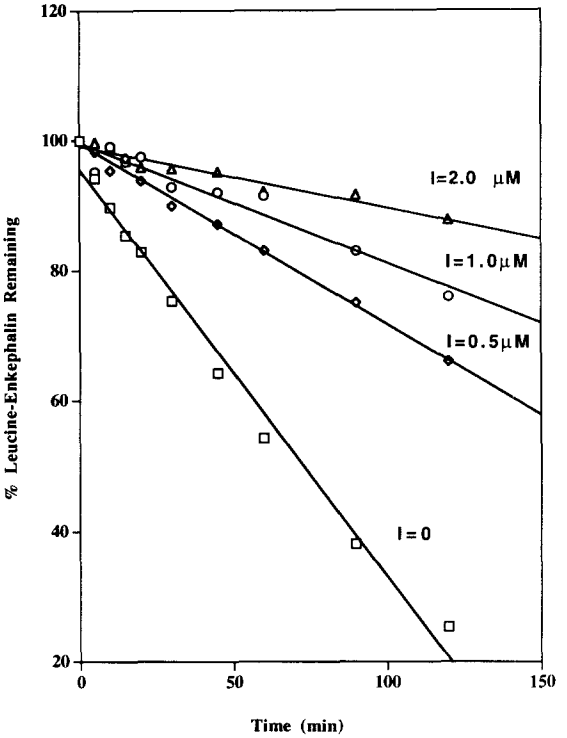

Fig. 3. Effect of H-PheΨ[P(O)(OH)CH_2]Phe-OMe I concentration on hydrolysis of 0.10 mM leucine-enkephalin in situ. Changes in the level of leucine-enkephalin with time were determined at the indicated concentration of I. Initial rates of hydrolysis were determined from the linear portions of the plots. These rates were used in the 1/velocity vs [I] plot in Fig. 4.

the rat nasal peptidase. As shown in Fig. 2, saturation kinetics was observed which follow Michaelis Menten kinetics as shown by the linear Lineweaver-Burk plot. The apparent Michaelis constant (K_m^{app}) for hydrolysis in situ is 0.4 mM.

Next, we determined the concentration dependence of the inhibitor, H-PheΨ[P(O)(OH)CH_2] Phe-OMe I, on the hydrolysis. Fig. 3 shows the time-dependent changes in the level of leucine-enkephalin in the presence of 0, 0.50, 1.0, and 2.0 μM I. The respective degrees of inhibition are 0, 64, 75, and 88%. These data readily fit the equation for competitive inhibition as shown by the linear plot of $1/v$ vs [I] (Fig. 4). From the intercept on the x-axis of the $1/v$ vs [I] plot and a K_m^{app} value of 0.4 mM, a K_i^{app} of 0.21 μM was calculated. In this analysis, we are assuming that the inhibitor is interacting with the enzyme in the

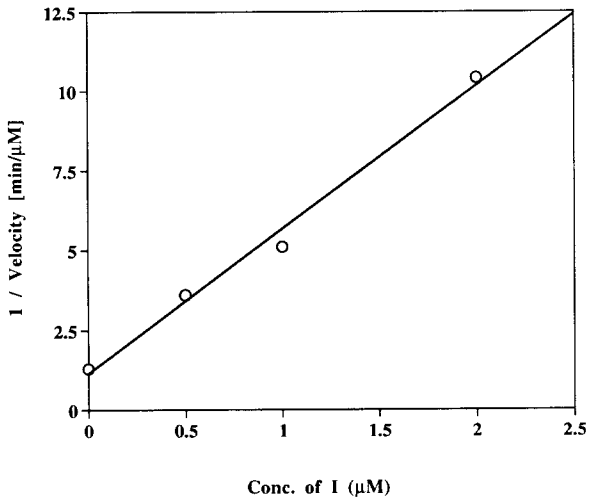

Fig. 4. Reciprocal velocity vs inhibitor concentration plot for H-PheΨ[P(O)(OH)CH_2] Phe-OMe [**I**]. Inhibition data were obtained from Fig. 3. Linear fit to the data is described by $y = 4.5x + 1.2$, $R^2 = 0.990$.

simplest manner, forming a 1:1 complex with the enzyme blocking the active site. We feel that this is reasonable considering the substrate like nature of the inhibitor and its high level of effectiveness.

A similar method of analysis was applied to inhibition data obtained with the most effective inhibitor of the nasal aminopeptidase, boroleucine (Hussain et al., 1989). Inhibitor levels of 0, 0.01, 0.03, and 0.10 μM yielded 0, 58, 76, and 87% inhibition, respectively.

The foregoing results are consistent with hydrolysis of leucine-enkephalin by a single peptidase. First, linear Lineweaver-Burk plots were obtained for the hydrolysis of this substrate and secondly, linear plots of 1/velocity vs inhibitor concentration were obtained for two chemically different types of inhibitors. Linear Lineweaver-Burk plots are not expected for multiple enzymes hydrolyzing the same substrate unless they have similar values of K_m and k_{cat} (Segel, 1975). Although this is unlikely, it is even more unlikely that multiple enzymes would bind two different inhibitors with the same affinities. This would be required for the linear 1/velocity vs inhibitor concentration plots which we observed with both the phosphorus inhibitor, **I**, and boroleucine. Note that both compounds inhibit the nasal enzymatic activity by approx. 90% at the higher levels tested.

In this in situ system, circulating leucine-enkephalin is being hydrolyzed by a membrane associated enzyme. Reproducible levels of activity were obtained from day to day. Since substrates appear to be readily accessible to the nasal enzyme, rates of substrate diffusion do not appear to be limiting factors. We feel values of K_m^{app} and

Table 1
Comparison of the binding of rat nasal aminopeptidase and porcine cytosolic and microsomal aminopeptidases to synthetic inhibitors [a]

No.	Inhibitor	K_i (μM)		
		Cytosolic AP	Microsomal AP	Nasal AP
I	H-PheΨ[P(O)(OH)CH_2]Phe-OMe	1.7 ± 0.2	0.13 ± 0.04	0.2
II	H-PheΨ[P(O)(OMe)CH_2]Phe-OMe	> 400 [b]	–	> 1
III	H-PheΨ[P(O)(OH)(H)]	240 ± 60	> 1000	> 1
IV	H-PheΨ[P(O)$(OPh)_2$]	180 ± 2	220 ± 40	> 1
V	H-PheΨ[P(O)(OPh)(OH]	28 ± 2	44 ± 5	> 1
VI	H-PheΨ[P(O)$(OH)_2$]	0.74 ± 0.32	49 ± 16	> 3
	H-boro-Phe	0.05 [c]	≈ 0.01 [d]	0.004 ± 0.001
	H-boro-Leu	0.13 [c]	≈ 0.01 [d]	0.009 ± 0.003
	H-boro-Ala	90 [c]	≈ 0.02 [d]	0.07
	H-boro-Val		0.01	0.02

[a] K_i values were determined using the procedure described in section 2 unless indicated otherwise.
[b] **II** is readily hydrolyzed to yield **I**. A K_i value of 400 μM was measured immediately following HPLC purification, but the presence of a trace of **I** in the sample could not be ruled out.
[c] K_i values for cytosolic aminopeptidase were reported previously (Shenvi, 1986).
[d] Values were calculated from reported slow-binding-inhibition data (Shenvi, 1986).

K_i^{app} for this system are good approximations of the behavior of the enzyme in solution. Even if these values are more complex than expected, they should provide good relative values for comparing inhibitors.

3.2. Susceptibility of the nasal aminopeptidase to inhibition

Establishing that most of the enzymatic activity which hydrolyzes leucine-enkephalin is due to a single neutral aminopeptidase has allowed us to treat our inhibition data in a more quantitative way. We have compared binding of different structural types of inhibitors with the nasal enzyme. In addition, we have compared this inhibition profile with the profile of two well characterized aminopeptidases, porcine kidney cytosolic and microsomal aminopeptidase. These results are shown in Table 1.

Compounds in the boronic acid series of inhibitors are the most effective for the nasal aminopeptidase. They have been evaluated previously except for H-boro-Phe which has been included in this study. We calculate values of K_i^{app} of 4–70 nM for these inhibitors. H-PheΨ[P(O)(OH)CH_2]Phe-OMe **I** was next in order of effectiveness inhibiting with a K_i^{app} of 0.2 μM. No significant inhibition was observed for the remaining phosphorus inhibitors at 1 μM. Most noticeable is the lack of effectiveness observed for H-PheΨ[P(O)$(OH)_2$] **VI** which did not give significant inhibition when tested at concentrations up to 3 μM.

Comparison of the inhibition profile of the nasal enzyme with porcine microsomal aminopeptidase indicates that the two enzymes are very similar. Distinct differences between these enzymes and the cytosolic aminopeptidase were obtained. Less than a 20-fold difference was observed for binding of boro-Phe, boro-Leu, and boro-Ala to the microsomal and nasal aminopeptidase. In contrast for the cytosolic enzyme, differences 3 orders in magnitude were observed. The cytosolic enzyme binds H-PheΨ[P(O)(OH)CH_2]Phe-OMe (**I**) and H-PheΨ[P(O)$(OH)_2$] (**VI**) with K_i values of 1.7 and 0.74 μM, respectively. The microsomal and nasal enzymes bind the dipeptide phosphinate much more tightly with K_i values of 0.13–0.20 μM, but only bind the simpler α-aminophosphonate weakly (K_i 50 μM for the microsomal aminopeptidase).

Our results for the binding of the phosphinate and phosphonate, **III** and **VI** agree fairly well with K_i values reported for the cytosolic enzyme (Giannousis and Bartlett, 1987). The reported K_i values are 59 and 0.42 μM and we measured 240 and 0.70 μM, respectively. However, data for binding of this series to the microsomal enzyme have not been reported and the much greater preference of the enzyme for the phosphinate dipeptide analog should be noted.

In conclusion, we have shown that hydrolysis of leucine enkephalin following nasal administration is primarily due to a single enzyme. This enzyme is very similar to porcine microsomal aminopeptidase in its susceptibility to inhibition by phosphorus containing and boronic acid inhibitors. Our expectation is that the catalytic properties of these two enzyme will also be similar. These studies suggest porcine microsomal aminopeptidase is a good model for the nasal aminopeptidase responsible for hydrolysis of peptides containing neutral residues. The susceptibility of peptides to hydrolysis by this enzyme may be predictive of their potential for nasal delivery.

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References

Baylis E.K., Campbell, C.D. and Dingwall, J.D., 1-Aminoalkylphosphonous acids: I. Isosteres of the protein amino acids. *J. Chem. Soc. Perkin Trans.*, 1 (1984) 2845–2853.

Dixon, M. and Webb, E., *Enzymes*, 3rd Edn, Academic Press, New York, 1979, p. 351.

Giannousis, P.P. and Bartlett, P.A., Phosphorous amino acid analogues as inhibitors of leucine aminopeptidase. *J. Med. Chem.*, 30 (1987) 1603–1609.

Hussain, M.A., Kim, M.S.L., Raghavan, K.S., Rogers, N.J., Hidalgo, R. and Kettner, C.A., A phosphinic acid dipeptide analogue to stabilize peptide drugs during their intranasal absorption. *Pharm. Res.*, 9 (1992) 626–628.

Hussain, M.A., Shenvi, A.B., Rowe, S.M. and Shefter, E., The use of α-aminoboronic acid derivatives to stabilize peptide drugs during their intranasal absorption. *Pharm. Res.*, 6 (1989) 186–189.

Oleksyszyn, J., Subotkowska, L. and Mastalerz, P., Diphenyl l-aminoalkanephosphonates. *Synthesis* (1979) 985–986.

Parsons, W.H., Patchett, A.A., Bull, H.G., Schoen, W.R., Taub, D., Davidson, J., Combs, P.L., Springer, J.P., Gadebusch, H., Weissberger, B., Valiant, M.E., Mellen, T.N. and Busch, R.D., Phosphinic acid inhibitors of D-alanyl-D-alanine ligase. *J. Med. Chem.*, 31 (1988) 1772–1778.

Segel, I.H., *Enzyme Kinetics*, Wiley, New York, 1975, pp. 64–72.

Shenvi, A.B., α-Aminoboronic acid derivatives: effective inhibitors of aminopeptidases. *Biochemistry*, 25 (1986) 1286–1291.

Stratford, R.E. and Lee, V.H.L., Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit: implications in peptide delivery. *Int. J. Pharm.*, 30 (1986) 73–82.