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Nasal pre-systemic metabolism of peptide drugs: substance P metabolism in the sheep nasal cavity

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Abstract

Although the nasal administration of peptide and protein drugs bypasses hepatic first-pass metabolism and the highly proteolytic environment in the gastrointestinal tract, the enzymes present in the nasal cavity may still limit the bioavailability of peptide and protein compounds. A knowledge of the degradation patterns in both the nasal secretions and homogenates is important to estimate bioavailability limitations resulting from pre-systemic metabolism. Substance P (SP) and its fragments SP(1–9), and SP(1–7) were incubated with both nasal secretions and homogenized mucosal tissues to investigate peptide metabolism in the sheep nasal cavity. The disappearance of the initial peptide compounds and appearance of their metabolites were analyzed by reverse phase HPLC. The metabolism patterns in the secretions were found to be similar to those in the homogenates. SP(1–9) and SP(1–7) were the two major fragments identified for the degradation of SP. SP(1–9) was also further metabolized to SP(1–7). The order of the degradation rates was SP $\approx$ SP(1–9) $>$ SP(1–7) in both the homogenates and secretions. No major C-terminal fragments, such as SP(2–11) and SP(3–11), were found which suggests that SP, SP(1–9) and SP(1–7) are resistant to the activity of the aminopeptidases present in the nasal cavity. Addition of phosphoramidon, a specific inhibitor of neutral endopeptidase 24.11 (NEP), to the reaction mixtures reduced the pseudo-first order rate constants by 72 and 90% in nasal homogenates and nasal secretions, respectively. Captopril, an inhibitor of angiotensin converting enzyme (ACE), did not inhibit SP metabolism in either secretions or homogenates.

Keywords: Substance P; Nasal secretions; Nasal homogenates; Metabolism; Angiotensin converting enzyme; Neutral endopeptidase-24.11; Captopril; Phosphoramidon

1. Introduction

Peptide and protein drug compounds generally require administration from parenteral routes in order to achieve clinically useful bioavailabilities (Lee, 1988). The low bioavailability from the oral route is due to a combination of rapid lumenal degradation, poor membrane permeability and significant hepatic first-pass metabolism. Nasal administration seems to be an ideal alternative for peptide and protein drug delivery; the nasal epithelium is considered to be leakier than other absorptive mucosae and the skin, and the proteolytic degradation is less than the oral route (Ver-

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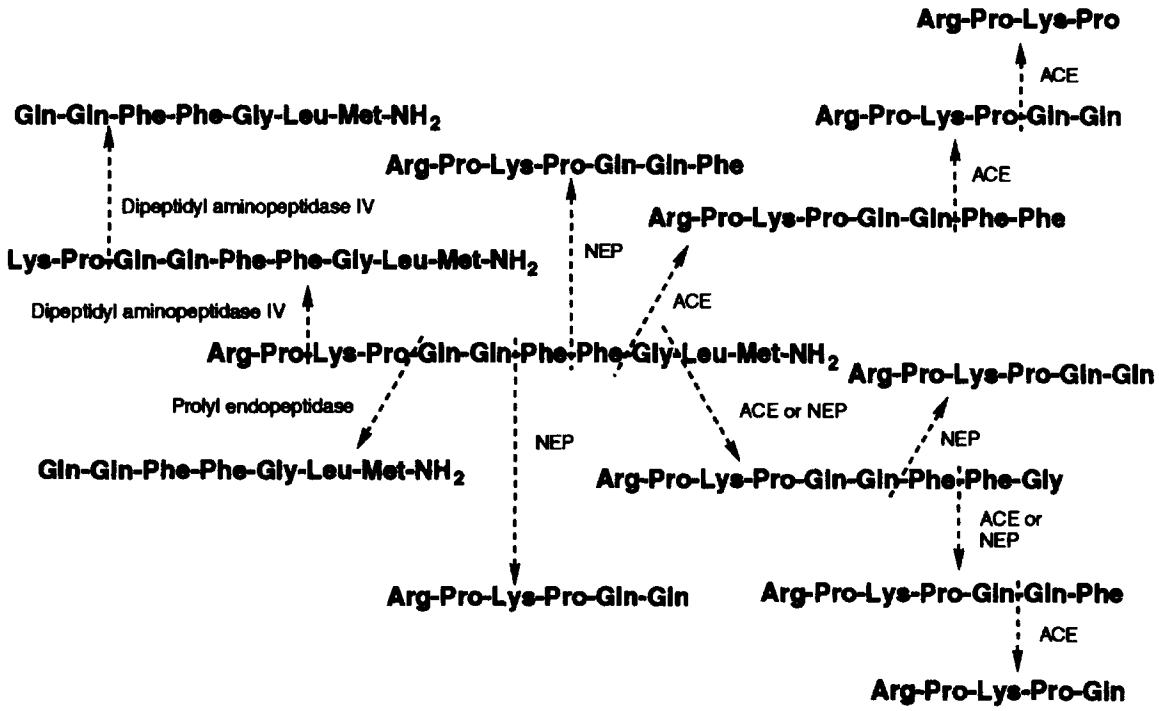

Fig. 1. Reported pathways of substance P metabolism (Blumberg et al., 1980; Lee et al., 1981; Conlon and Sheehan, 1983; Skidgel et al., 1984; Katayama et al., 1991).

hoef et al., 1990). The absorption of a wide variety of peptide and protein drugs has been evaluated following nasal administration and several have shown systemic bioavailability. These compounds include insulin, glucagon, growth hormone, vasopressin, enkephalin, somatostatin, bradykinin and calcitonin (Eppstein and Longenecker, 1988; Chien et al., 1989). Absorption from the nasal cavity is generally quite rapid; the time to peak plasma concentrations for intranasally administered peptides and proteins is usually < 10 min (Chang and Chien, 1992).

Although nasal administration can avoid the harsh environmental conditions in the gastrointestinal tract and bypasses the hepatic first-pass effect, the enzymatic systems present in the nasal cavity can still greatly limit the bioavailability of nasally administered peptide and protein drugs. Insulin, proinsulin, thyrotropin releasing hormone (TRH), along with enkephalin and related analogues have all been shown to be degraded by enzymes present in the nasal cavity (Kashi and Lee, 1986; Yamamoto et al., 1990; Jørgensen and Bechgaard, 1994), yet little information is known about the structural specificity of the enzymes present, or their intracellular and extracellular distribution patterns.

Substance P (SP), an undecapeptide with the amino acid sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, was used to investigate peptide degradation in the sheep nasal cavity. Substance P was selected as a compound to study metabolism patterns in the nasal cavity since it undergoes significant metabolism in the tissues of many species. SP has been reported to be metabolized by a variety of peptidases, including ACE (EC 3.4.15.1), NEP (EC 3.4.24.11), dipeptidyl aminopeptidase IV (EC 3.4.14.5), and prolyl endopeptidase (EC 3.4.99) (Blumberg et al., 1980; Lee et al., 1981; Conlon and Sheehan, 1983; Skidgel et al., 1984; Katayama et al., 1991). The degradation pathways of SP reported by pre-

vious investigators are summarized in Fig. 1. ACE is a peptidyl dipeptidase (also referred to as dipeptidyl carboxypeptidase) which can cleave the Gly^9-Leu^{10} bond of SP and further hydrolyze the Phe^7-Phe^8 bond of SP(1-9) (Skidgel et al., 1984). The products of these ACE mediated reactions will be SP(1–9) and SP(1–7). In addition, ACE can also act as a tripeptidase and cleave the Phe^8-Gly^9 bond of SP. NEP acts as an endopeptidase which can cleave the Gly^9-Leu^{10} and Phe^7-Phe^8 bonds of SP, as well as the Phe^7-Phe^8 bond of SP(1–9) (Katayama et al., 1991). Dipeptidyl aminopeptidase IV removes Arg^1-Pro^2 from SP (Conlon and Sheehan, 1983). Prolyl endopeptidase cleaves the Pro^4-Gln^5 bond of SP, yielding an *N*-terminal tetrapeptide SP(1–4) and a C-terminal heptapeptide amide SP(5–11) (Blumberg et al., 1980).

Metabolism in both the nasal secretions and nasal homogenates was investigated because they represent metabolism in different compartments of the nasal cavity. Metabolism of peptide drugs by extracellular enzymes can be estimated using the data from the nasal secretions, whereas metabolism in the nasal homogenates more likely characterizes the degradation due to both cytosolic and membrane-bound enzymes.

2. Materials and methods

2.1. Chemicals

SP, SP(1–9), SP(1–7), SP(1–4), SP(5–11), SP(6–11), SP(7–11), SP(8–11), captopril, phosphoramidon and *n*-butyl paraben were obtained from Sigma Chemical Company (St. Louis, MO) and used as received. Assay kits for protein concentration were also obtained from Sigma Chemical Company. HPLC grade solvents were used for HPLC analysis.

2.2. Collection of sheep nasal secretions

Sheep nasal secretions were collected by inserting 2.5 cm × 0.5 cm absorptive cotton pledgets into the nasal cavity of anesthetized sheep. The dimension of the cotton rolls is important in order to avoid damage to the nasal mucosa and subsequent blood contamination of the sample (Smith, 1975). The experimental procedures were approved by The University of Iowa Animal Care and Use Committee. The cotton pledgets were left undisturbed and were withdrawn after 10–20 min and placed into a 3 ml syringe. After placing pressure on the pledget, about 1 ml of nasal secretions could be recovered from each insert. Nasal secretions were stored at − 70°C until use. The protein content of the nasal secretions was measured using Sigma Diagnostics Micro Protein Kit # 610-2 employing brilliant blue G. Protein concentration was adjusted to 0.28 mg/ml with Krebs-Ringer buffer for incubation with SP and its fragments.

2.3. Preparation of sheep nasal homogenates

Sheep nasal mucosal tissue was obtained from local meatpackers. After opening the sheep nasal cavity along the septal midline, the nasal turbinates were removed and stored in ice-cold Krebs-Ringer bicarbonate buffer. The mucosal membranes were removed from the turbinates and washed in ice-cold Krebs-Ringer buffer several times to eliminate possible blood contamination. The tissues were homogenized in the buffer with a tissue homogenizer (Kinematica GmbH, Lucerne, Switzerland) at 25000 rpm. The homogenized tissues were centrifuged at 12700 × *g* for 10 min at 4°C (Micro-MB Centrifuge, International Equipment Company, Needham Heights, MA). The pellet (predominantly cell debris) was discarded. The protein content of the supernatant was measured using Sigma Diagnostics Micro Protein Kit # 610-2. The protein concentration was adjusted to 0.125 mg/ml with Krebs-Ringer buffer for incubation with SP. Diluted nasal homogenates were stored at − 70°C until use.

2.4. Peptide degradation

Substance P, SP(1–9) or SP(1–7) were incubated with sheep nasal secretions and nasal homogenates at 37°C for up to 60 min. The initial concentrations of these peptides in the reaction mixtures ranged from 10 to 20 μM. Aliquots (50

μl) of the incubation solutions were periodically removed, diluted immediately with 50 μl acetonitrile to quench further hydrolysis and analyzed by HPLC. *n*-Butyl paraben was added to the quenched sample as an internal standard prior to HPLC injection.

2.5. Inhibition of metabolism

Inhibition of SP degradation was measured in the presence of two different peptidase inhibitors at 37°C. The reaction mixtures consisted of 15–20 μM SP, sheep nasal secretions or homogenates, and 0.02 mM phosphoramidon or 0.015 mM captopril (Orawski and Simmons, 1989). Control incubates contained the same materials but without inhibitors. Samples were taken periodically, quenched and analyzed by HPLC.

2.6. Initial rate measurement

Substance P, at various concentrations, was incubated with sheep nasal homogenates at 37°C to measure the initial rate of degradation. Initial SP concentrations were 5, 10, 20, 50 and 200 μM. Reactions were quenched after a 1–3 min incubation by adding an equal volume of acetonitrile to the reaction mixture. In all cases, the degradation of the SP was limited to $< 10\%$ of the original amount present. Quenched samples were analyzed by HPLC, and the initial rate data were analyzed using an interactive fitting procedure to the Michaelis-Menten equation (KaleidaGraph™, Abelbeck Software, Reading, PA).

2.7. HPLC analysis

Substance P and its fragments were separated and quantified using HPLC analysis (Endo et al., 1989). The mobile phase consisted of acetonitrile and water each containing 0.05% TFA. The peptides were eluted using a gradient increasing from 4 to 34% acetonitrile over 20 min at a flow rate of 1 ml/min. The concentration of acetonitrile was increased to 54% during the next 5 min to elute the internal standard (*n*-butyl paraben). The HPLC system consisted of a SPD-6A UV spectrophotometric detector (210 nm) (Shimadzu USA, Columbia, MD), SP8700 solvent delivery system (Spectra-Physics, Fremont, CA), WISP 710B auto injector (Waters, Milford, MA), C-R6A Chromatopac® data recorder (Shimadzu USA, Columbia, MD) and a Vydac C_{18} peptide column (4.5 mm × 25 mm). Metabolite peaks were identified by comparing the retention times of the peaks with those of standard peptides obtained commercially, including SP, SP(1–9), SP(1–7), SP(1–4), SP(5–11), SP(6–11), SP(7–11) and SP(8–11). The concentrations of SP and its degradation fragments in the reaction mixtures were calculated from standard curves developed for each of the peptides using the commercially available fragments.

3. Results

The V_{max} and K_m values for SP metabolism were calculated from the initial degradation rates. They were found to be 34 $\mu g \cdot min^{-1}$ per mg protein and 33 μM, respectively. The initial concentration of SP for all of the metabolism studies in nasal homogenates and nasal secretions was kept below the K_m value to allow the SP disappearance rate to be treated using pseudo-first order kinetics. The estimated K_m value for SP degradation, 33 μM, is similar to the K_m's for SP degradation measured for purified NEP from pig kidney (32 μM) (Matsas et al., 1984a), recombinant human NEP (15 μM) (Katayama et al., 1991) and unpurified NEP in the longitudinal muscle layer of guinea pig small intestine (25 μM) (Nau et al., 1986). Since the SP metabolizing enzymes in the sheep nasal homogenates were not purified, the K_m value should not be treated as a kinetic parameter of a specific enzyme. However, when NEP overwhelmingly dominates the metabolism, the K_m value should be similar to that observed for the pure enzyme.

The degradation of SP in the sheep nasal homogenates yielded SP(1–9), SP(1–7) and SP(7–11) as the major metabolic fragments (Fig. 2a). The levels of SP(1–9) and SP(1–7) rose at the beginning of the incubation, but no SP(7–11) was detected during the first 5 min. Incubation of the peptide fragments showed that SP(1–7) was also

produced when SP(1–9) was incubated with the homogenates (Fig. 2b). SP(1–7) was further hydrolyzed by the enzymes present in the nasal homogenates, but the degradation rate was significantly slower than for either SP and SP(1–9) (Fig. 2c).

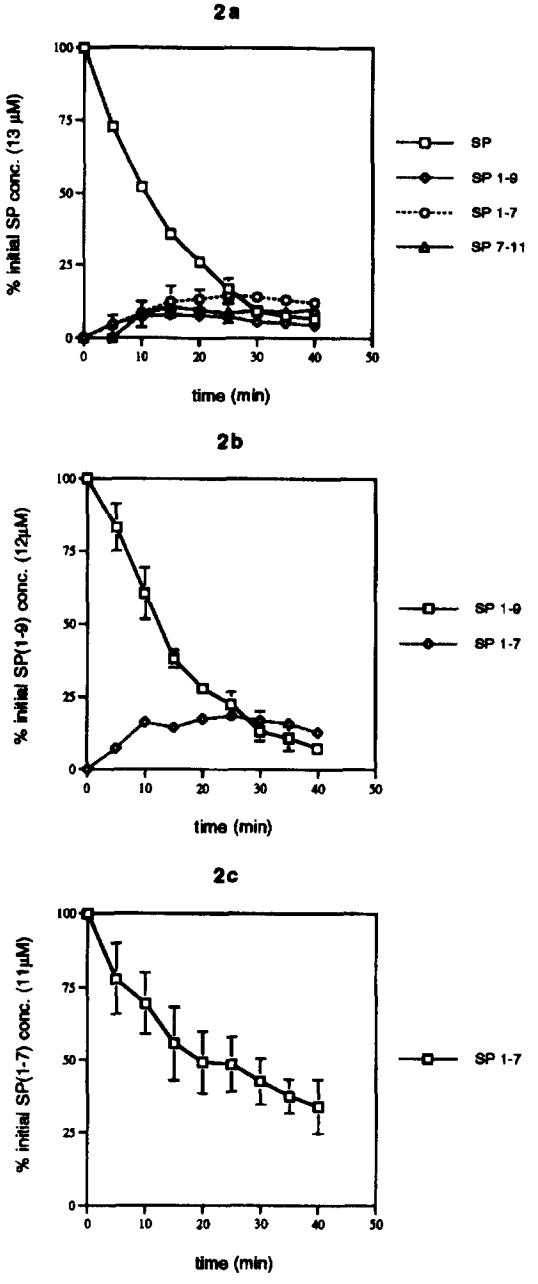

Fig. 2. Degradation of SP, SP(1–9) and SP(1–7) in sheep nasal homogenates. (2a) Substance P. (2b) Substance P(1–9). (2c) Substance P(1–7). Total protein concentration = 0.125 mg/ml. Data represent mean ± S.D., n = 3.

SP was also incubated with sheep nasal secretions; SP(1–9), SP(1–7), SP(7–11) and SP(8–11) were found in the reaction mixture (Fig. 3a). SP(1–9) and SP(1–7) were present throughout the incubation, but SP(7–11) and SP(8–11) were not measurable until time periods longer than 10 min. When SP(1–9) was incubated with the nasal secretions, SP(1–7) was the only metabolite identified in the reaction mixture (Fig. 3b). Further metabolism of SP(1–7) also occurred in the nasal secretions, but specific fragments could not be identified (Fig. 3c). As was observed with the homogenates, the degradation rate for SP(1–7) was significantly less than that for SP(1–9) or SP.

The use of specific peptidase inhibitors together with the observed metabolism patterns can assist in the identification of the enzymes primarily responsible for the degradation of SP. Two inhibitors were used to determine the relative contribution of ACE and NEP to the total degradation of SP in the nasal homogenates and nasal secretions. Angiotensin converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) have been reported to be involved in the metabolism of SP in other tissues (Grafford et al., 1983; Skidgel et al., 1984). Captopril, a specific inhibitor of ACE, had only a slight effect on the degradation of SP in either nasal secretions or nasal homogenates (Fig. 4a and Fig. 4b). When pseudo-first order degradation rate constants were calculated, captopril reduced the rate by approximately 10% in the nasal homogenates and secretions. In comparison, the degradation rate was greatly reduced by the addition of phosphoramidon, a specific inhibitor of NEP. The pseudo-first order rate constant was calculated to be 72% less than that of control in nasal homogenates. The rate constant was decreased by 90% in the nasal secretions.

The degradation of SP(1–9) in sheep nasal homogenates resulted in the formation of SP(1–7). This reaction can also be catalyzed by NEP. Therefore, the formation of SP(1–7) from SP could be due to the direct cleavage of Phe^7-Phe^8 bonds of SP or the cleavage of the Phe^7-Phe^8

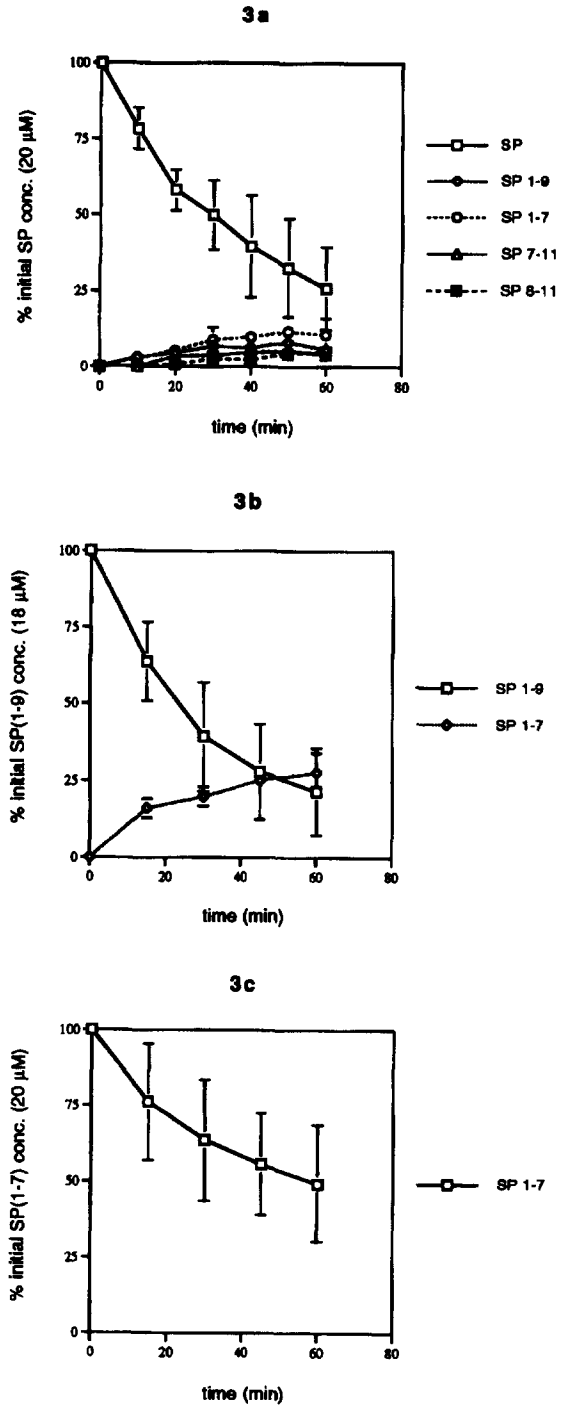

Fig. 3. Degradation of SP, SP(1–9) and SP(1–7) in sheep nasal secretions. (3a) Substance P. (3b) Substance P(1–9). (3c) Substance P(1–7). Total protein concentration = 0.28 mg/ml. Data represent mean ± S.D., n = 3.

bond of the SP(1–9) intermediate. SP(1–7) was further hydrolyzed by other peptidases. However, no individual fragments could be identified to further characterize the metabolic sequence.

Table 1 summarizes the rate constants for the metabolism of SP, SP(1–9), and SP(1–7) in the homogenates and secretions calculated assuming pseudo-first order kinetics. For comparative purposes, the rate constants have been normalized to the protein concentrations of the nasal secretions and homogenates. The rank order of the degradation rates for SP and its fragments in both sheep nasal homogenates and nasal secretions was SP $\approx$ SP(1–9) > SP(1–7). The degradation rates in nasal homogenates were greater than those in the nasal secretions in all cases. This suggests that the relative concentration or activity of SP metabolizing enzymes per unit weight of protein was higher in the homogenates than in the secretions.

4. Discussion

Previous investigators have demonstrated carboxypeptidase N, ACE, endopeptidase and aminopeptidase activity in rat, rabbit and sheep nasal cavities (Stratford and Lee, 1986; Lee, 1988; Yamamoto et al., 1990; Chung and Donovan, 1995a; Chung and Donovan, 1995b). In the nasal cavity, insulin and proinsulin are degraded by thiol protease and serine protease (Yamamoto et al., 1990), enkephalin is degraded by aminopeptidases (Stratford and Lee, 1986) and bradykinin is degraded by carboxypeptidase and endopeptidase (Chung and Donovan, 1995a; Chung and Donovan, 1995b). The metabolism pattern observed for SP is somewhat similar to the pattern for bradykinin; in both cases, since the Arg^1-Pro^2 sequence at the amino terminal is resistant to hydrolysis by most aminopeptidases, the degradation occurs exclusively from the C-terminal.

By comparing the metabolism patterns (Fig. 2a) with the known pathways shown in Fig. 1, it can be seen that the formation of SP(1–9) and SP(1–7) in the nasal homogenates is likely attributable to the activities of NEP or/and ACE present in the homogenates. Despite the ability of ACE to catalyze the formation of SP(1–9) and SP(1–7),

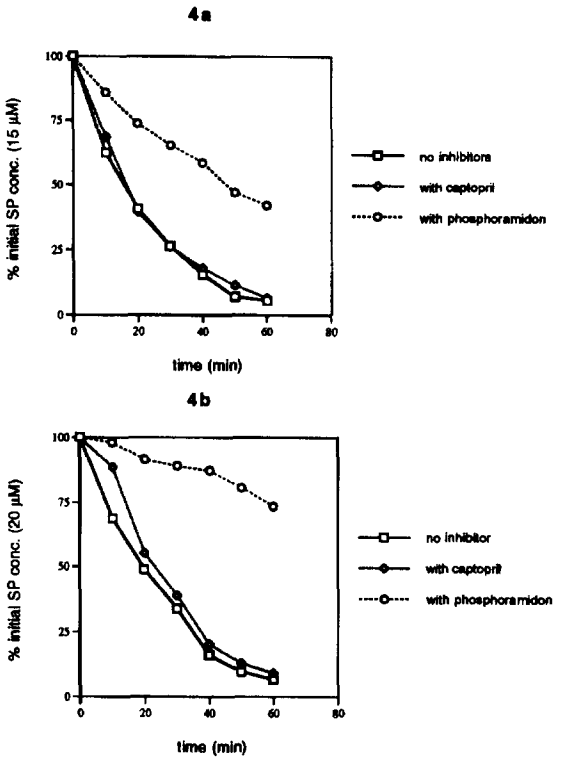

Fig. 4. Inhibition of substance P degradation in sheep nasal homogenates and nasal secretions by phosphoramidon and captopril. (4a) Inhibition in homogenate. (4b) Inhibition in secretions. $n = 1$.

captopril (an ACE inhibitor) reduced the total metabolism by only 10%. This indicates that ACE is not the major enzyme responsible for the SP degradation in sheep nasal homogenates. On the other hand, phosphoramidon (a NEP inhibitor) was able to inhibit 72% of the degradation. Therefore, NEP is likely to be one of the predominant enzymes involved in the metabolism of SP in the nasal cavity.

Similar metabolism patterns were found using nasal secretions in the incubates instead of homogenates. The appearance of SP(1–9) and SP(1–7) suggests that the metabolism was caused by NEP and/or ACE (Fig. 3a). But again, the inhibitor studies eliminated the possibility that ACE was actively involved in SP metabolism in sheep nasal secretions. NEP also appears to be a major SP metabolizing enzyme in the secretions, since phosphoramidon inhibited 90% of the SP degradation. As with the nasal homogenates, SP(1–9) was further degraded into SP(1–7). SP(1–7) was also further degraded in nasal secretions into unidentified fragments. From these results, it appears that the Gly^9-Leu^{10} and Phe^7-Phe^8 bonds of SP were cleaved by NEP in the secretions, and the initial fragment SP(1–9) was also further metabolized, likely at the Phe^7-Phe^8 bond.

The metabolism patterns of SP, SP(1–9) and SP(1–7) suggest that the degradation pathways of SP in the nasal secretions and nasal homogenates are very similar. However, the appearance of SP(8–11) only in the secretions demonstrates that there are some minor differences in the metabolism patterns between these two regions of the nasal cavity. Metabolism patterns in the nasal secretions and homogenates are not always the same for other peptide compounds, however, nor are they the same between animal species (Chung and Donovan, 1995a; Chung and Donovan, 1995b). The existence of differences between the enzymes in the nasal secretions and homogenates is quite reasonable. Degradation in the nasal secretions represents the activity of extracellular enzymes produced by local secretory cells or exuded from plasma (Shelhamer et al., 1984). The degradation in nasal homogenates is predominantly due to the activity of cytosolic and mem-

Table 1
Pseudo-first order rate constants (mean ± S.D., $n = 3$; corrected for protein concentration) for the degradation of substance P and its fragments in sheep nasal homogenates and nasal secretions

	SP	SP(1–9)	SP(1–7)
Nasal homogenates	0.592 (± 0.044)	0.576 (± 0.106)	0.208 (± 0.035)
Nasal secretions	0.096 (± 0.016)	0.125 (± 0.059)	0.046 (± 0.017)

Unit: $(\text{min} \cdot \text{mg/ml protein})^{-1}$.

brane-bound enzymes (Lee, 1989). The enzyme activities and populations in extracellular fluids, plasma and intracellular fluid are not likely to be identical. Similarly, the enzyme populations in the nasal secretions are not identical to those present in the nasal mucosal tissues, therefore, one should not predict rates of metabolism in the nasal secretions based upon the metabolism patterns in other tissues or fluids, or vice versa.

Peptide drugs can be absorbed through mucosal membranes via transcellular and/or paracellular pathways. Since many peptides are hydrophilic in nature, they are likely to be transported paracellularly (McMartin, 1989). Paracellularly transported peptides, while not exposed to intracellular enzymes, can still be degraded by extracellular enzymes. Previous reports have shown that carnosine (β-L-alanyl-L-histidine), vasopressin and its analogues, thyrotropin-releasing hormone (TRH) and its analogues, dipeptide inhibitors of angiotensin converting enzyme, and β-lactam antibiotics are transported via transcellular pathways (Humphrey, 1986; Vilhardt and Lundun, 1986; Gardner and Wood, 1989). Peptides transported transcellularly, in comparison, can be metabolized by both the extracellular enzymes in the secretions and by intracellular enzymes. If the residence time for both paracellularly and transcellularly transported peptides in the nasal cavity is sufficiently long, the contribution of extracellular metabolism to the total metabolism could be significant.

Although SP is not preferentially hydrolyzed by ACE, this does not imply that ACE is not present in the nasal cavity. In a previous report, ACE activity was detected in sheep nasal homogenates (Chung and Donovan, 1995b). The K_m value for the degradation of SP by purified ACE (25 μM) (Skidgel et al., 1984) is similar to the value for degradation by NEP (15–32 μM) (Matsas et al., 1984b; Nau et al., 1986; Katayama et al., 1991), however, the K_{cat} (225 min^{-1}; the number of catalytic processes the enzyme can catalyze in unit time), is much less than that for NEP (5062 min^{-1}) (Matsas et al., 1984a). Therefore, it is possible that ACE cannot compete efficiently with NEP for available SP. Similarly, SP is also a substrate for dipeptidyl aminopeptidase IV, but the high K_m (2.0 M) (Kato et al., 1978) for degradation by this enzyme suggests that this reaction may not be significant at the concentrations used in this study.

While the nasal cavity has been considered to be a less metabolically active site than the gastrointestinal tract, these results indicate that the peptidases present in the nasal cavity can still efficiently degrade peptide drugs prior to their absorption. Knowledge of degradation pathways, enzyme localization, enzyme activity, peptide sequence-degradation pattern relationships, and the ability to inhibit metabolism is needed in order to design effective strategies for the delivery of peptide and protein drugs via the nasal cavity.

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