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Effects of proteolytic enzyme inhibitors on nasal absorption of salmon calcitonin in rats

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Abstract

Proteolytic enzyme inhibitors were examined as absorption enhancers for the nasal absorption of salmon calcitonin (SCT) in rats. Bestatin, diprotinin, leupeptin, aprotinin, soybean trypsin inhibitor and camostat mesilate were used as enzyme inhibitors. The nasal absorption of SCT was evaluated by measuring its hypocalcemic effects. The peptidase activities of rat nasal mucosal tissue were high and found to be in the following order: leucine aminopeptidase (2.72 nmol/min per mg protein) > dipeptidyl aminopeptidase (1.84 nmol/min per mg protein) > cathepsin (650 pmol/min per mg protein) > trypsin.(4.61 pmol/min per mg protein). Nasal administration of SCT (10 IU/kg, pH 7.0) showed low pharmacological availability (3.2%). Coadministration with bestatin (aminopeptidase inhibitor, 0.01–1 mM) or diprotinin A (dipeptidyl peptidase inhibitor, 0.1–1 mM) did not change the hypocalcemic effects. Coadministration with aprotinin (trypsin inhibitor, 10^3–10^4 KIU/ml), camostat mesilate (aminopeptidase and trypsin inhibitor, 0.1–10 mM) or leupeptin (trypsin and cathepsin B inhibitor, 0.1–1 mM) enhanced the hypocalcemic effects and, thus, the nasal absorption of SCT. The hypocalcemic effects of SCT at various pH values (pH 4.0, 7.0 and 8.0) with or without aprotinin were the highest at pH 4.0. The pharmacological availabilities after nasal administration of SCT (10 IU/kg) at pH 4.0 and 7.0 were increased from 5.4 to 7.5% and from 3.2 to 6.9% by aprotinin (10^4 KIU/ml), respectively. Therefore, inhibitors which have a trypsin inhibitory activity are useful for enhancing nasal absorption of SCT.

Keywords: Nasal absorption; Salmon calcitonin; Proteolytic enzyme inhibitor; Peptide absorption; Absorption enhancer

1. Introduction

The systemic delivery of therapeutic drugs such as peptide and protein drugs through the transmucosal route has several potential advantages including rapid absorption, avoidance of first-pass gastrointestinal and hepatic metabolism. The intranasal route has received a great deal of attention (Chien et al., 1989). A large number of studies in human and animals have revealed that many drugs including hydrophilic compounds (molecular mass up to approx. 1000 Da) can be absorbed through the nasal membrane (McMartin et al., 1987) This is due to the large surface area of the nasal cavity and the highly

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vascularized nature of nasal mucosa. The low mucosal permeability of peptides and protein (molecular mass above approx. 1000 Da) and marked mucosal endopeptidase activity require a suitable absorption enhancer of low toxicity to improve systemic delivery (McMartin et al., 1987). We previously reported that camostat mesilate, an aminopeptidase and trypsin inhibitor, was useful as an absorption enhancer for the nasal delivery of vasopression (AVP, molecular mass 1084 Da) and desmopressin (DDAVP, molecular mass 1069 Da) in rats (Morimoto et al., 1991).

Calcitonin (CT, molecular mass 3500 Da), which is a cyclic 32 amino acid peptide, has a physiological role in the regulation of calcium homeostasis and is a potent inhibitor of osteoclastic bone resorption. Salmon, human and eel CT have been used in the treatment of Paget's disease, osteoporosis and hypercalcemia (Meyer et al., 1991). Intranasal spray preparations containing salmon calcitonin (SCT) have been shown to be effective in metabolic bone diseases (Thamsborg et al., 1991). Unfortunately, nasal bioavailability is lower than that by injection. In the present study, the effects of various types of proteolytic enzyme inhibitors as possible absorption enhancers were examined to characterize the enzymatic barrier in the nasal absorption of SCT in rats. Bestatin (aminopetidase inhibitor), diprotinin A (dipeptidyl peptidase inhibitor), leupeptin (trypsin and cathepsin B inhibitor), aprotinin (trypsin inhibitor), soybean trypsin inhibitor (trypsin inhibitor) and camostat mesilate (aminopeptidase and trypsin inhibitor) were used as proteolytic enzyme inhibitors.

2. Materials and methods

2.1. Materials

Synthesized salmon calcitonin (SCT) was supplied by Teikokuzoki (Tokyo). Proteolytic enzyme inhibitors, bestatin, diprotin A, leupeptin, aprotinin and soybean trypsin inhibitor (STI) were purchased from Sigma Chemical Co. (St. Louis, MO). Camostat mesilate was supplied by Ono Pharm. Co., Ltd (Osaka). 6-Carboxyfluorescein (CF) was purchased from Eastman Kodak Co. (Rochester, NY). All other chemicals were of reagent grade.

2.2. Preparations

SCT (20 IU (2.9 μg)/ml) or CF (20 mg/ml) was dissolved in isotonic buffer solutions (pH 4.0, 7.0 and 8.0) containing 0.1% w/v BSA. The isotonic buffer solutions were made by mixing 0.236 M citric acid/0.123 M disodium phosphate buffer (pH 4.0) and 0.171 M potassium phosphate/0.144 M sodium bicarbonate (pH 7.0 and 8.0). Proteolytic enzyme inhibitors were dissolved in this SCT solution at each concentration. The final pH of each preparation was adjusted by adding HCl or NaOH solution.

2.3. Nasal absorption of SCT

The nasal absorption of SCT was evaluated by its hypocalcemic effects. Male Wistar rats (230–250 g) were fasted for 20 h before experiments, while allowing access to water ad libitum. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), with additional doses given intraperitoneally as necessary. The SCT preparations (0.5 ml/kg body weight) were administered into the nasal cavity through a tubing by a syringe, as described by Hirai et al. (1981). The dose of SCT was 10 IU/kg. Blood samples (0.2 ml) were obtained with a heparinized syringe from the femoral vein 5 min before administration and 0.5, 1, 1.5, 2, 3, 4 and 5 h after administration. The plasma was separated by centrifugation at 3000 rpm. The plasma calcium levels were determined using the Calcium C Test Wako™ (Wako Pure Chemicals Ind., Osaka). In a comparative study, SCT was administered intravenously to rats at a dose of 0.15 IU/kg. The area above the time course of the hypocalcemic effect (AAC) was calculated by means of trapezoidal integration using the program MULTI. Pharmacological availability was calculated as $(AAC_{nasal}/AAC_{i.v.}) \times (D_{i.v.}/D_{nasal}) \times 100\%$, where D is the dose of SCT.

2.4. Nasal absorption of CF

CF solution (0.5 ml/kg body weigt) was administered into the nasal cavity of rats using the same method as described for the administration of SCT. Blood samples (0.2 ml) were obtained from the femoral vein. The plasma concentration of CF was measured according to the method of Oshima et al. (1989).

2.5. Peptidase activities

Rat nasal mucosal tissue was homogenized in 0.25 M sucrose in a glass homogenizer cooled by ice water. The homogenate was centrifuged at $9000 \times g$ for 5 min at 5°C and the supernatant was used for analysis. The peptidase activity and the inhibition effects of proteolytic enzyme inhibitors on the peptidase activity were determined using peptidyl-4-methylcoumaryl-7-amide (peptidyl-MCA) as described by Komada et al. (1985). The 7-amino-4-methylcoumarin (AMC) liberated from the peptidyl-MCA substrate by the proteolytic enzyme reaction was assayed by fluorescence intensity and enzyme activities were calculated.

2.6. Data analysis

Statistical significance was evaluated using the Student's paired t-test.

3. Results

3.1. Peptidase activity

Peptidase activities on the nasal mucosal tissue of rats and the effects of various proteolytic enzyme inhibitors on its peptidase activities are summarized in Table 1. The peptidase activities were high and conformed to the following order: leucine aminopeptidase (LAP) > dipeptidyl aminopeptidase IV (DPP IV) > cathepsin B > trypsin. LAP activity was considerably reduced by bestatin (0.1 and 1 mM), diprotin A (1 mM), STI (1.25 mM) and camostat mesilate (0.1 and 1 mM) and slightly reduced by leupeptin (1 mM). DDP

Table 1
Effects of various protease inhibitors on the protease activities in nasal mucosal tissue of rats

Protease inhibitor		Protease activity [a]			
		LAP (nmol min^{-1} mg $protein^{-1}$)	DPP IV	Trypsin (pmol min^{-1} mg $protein^{-1}$)	Cathepsin B
Without inhibitor		2.72 ± 0.21	1.84 ± 0.10	4.61 ± 0.22	650.91 ± 15.23
Bestatin	0.1 mM	1.07 ± 0.10 [c]	1.64 ± 0.02	4.10 ± 0.43	567.95 ± 11.73 [b]
	1.0 mM	0.35 ± 0.04 [d]	1.65 ± 0.02	3.34 ± 0.05	321.23 ± 19.15 [d]
Diprotin A	0.1 mM	3.25 ± 0.26	1.04 ± 0.07 [c]	4.01 ± 0.29	549.52 ± 17.05 [b]
	1.0 mM	3.33 ± 0.06 [b]	0.72 ± 0.02 [d]	2.91 ± 0.58	238.47 ± 14.39 [d]
Aprotinin	200 KIU/ml	2.05 ± 0.16	1.67 ± 0.21	N.D.	666.20 ± 20.67
	1000 KIU/ml	1.65 ± 0.33	1.61 ± 0.01	N.D.	627.55 ± 10.19
STI	0.25 mM	2.63 ± 0.08	1.71 ± 0.02	N.D.	535.72 ± 30.68 [b]
	1.25 mM	0.73 ± 0.11 [c]	–	–	397.96 ± 32.33 [b]
Camostat	0.1 mM	1.54 ± 0.07 [c]	1.79 ± 0.08	0.36 ± 0.22 [d]	614.17 ± 11.30
mesilate	1.0 mM	0.53 ± 0.04 [d]	1.28 ± 0.11 [b]	N.D.	612.41 ± 15.37
Leupeptin	0.1 mM	2.35 ± 0.27	1.65 ± 0.09	2.42 ± 0.79	N.D.
	1.0 mM	1.99 ± 0.15 [b]	1.38 ± 0.17	N.D.	N.D.

[a] Substrates used for in vitro evaluation of leucin aminopeptidase (LAP), dipeptidyl aminopeptidase IV (DPP IV), trypsin and cathepsin B activities were Leu-7AMC, Gly-Pro-7AMC, Gly-Pro-7AMC, Z-Arg-7AMC and Z-Phe-Arg-7AMC, respectively.
[b] Significantly different from without inhibitor at $p < 0.05$.
[c] Significantly different from without inhibitor at $p < 0.01$.
[d] Significantly different from without inhibitor at $p < 0.001$.
N.D., not detectable; –; not tested; STI; soybean trypsin inhibitor. Each value represents the mean ± S.E. of three experiments.

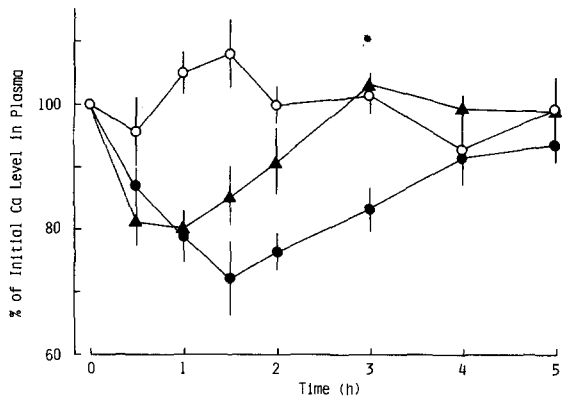

Fig. 1. Change in plasma calcium levels after nasal (IN) and intravenous (IV) administration of SCT in rats. (○) Buffer solution without SCT (IN, pH 7.0); (●) SCT IN (10 IU/kg, pH 7.0); (▲) SCT IV (0.15 IU/kg). Each value represents the mean ± S.E. of four animals.

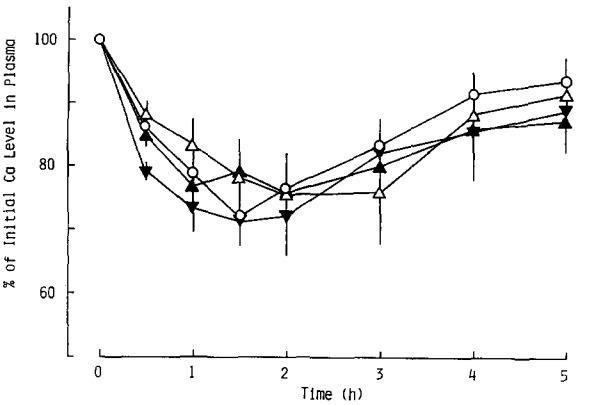

Fig. 2. Effects of bestatin on the change in plasma calcium levels after nasal administration of SCT (10 IU/kg, pH 7.0) in rats. (○) Without bestatin. Concentrations of bestatin: (△) 0.01, (▲) 0.1, (▼) 1.0 mM. Each value represents the mean ± S.E. of four animals.

IV activity was markedly reduced by diprotin A (0.1 and 1 mM) and slightly reduced by camostat mesilate (1 mM). Trypsin activity was remarkably reduced by aprotinin (200 and 1000 KIU/ml), STI (0.25 mM), camostat mesilate (0.1 and 1 mM) and leupeptin (1 mM). Cathepsin B activity was significantly reduced by bestatin (1 mM), diprotin A (1 mM) and leupeptin (0.1 and 1 mM) and slightly reduced by bestatin (0.1 mM) and diprotin A (0.1 mM).

3.2. Nasal absorption of SCT

Fig. 1 shows the change in plasma calcium levels after nasal (pH 7.0, dose of SCT 10 IU/kg) and intravenous (dose of SCT 0.15 IU/kg) administration of SCT in rats. The AAC values after nasal and intravenous administration of SCT were 77.9 and 36.9% h, respectively. The pharmacological availability of the nasal administration of SCT was 3.2%.

Fig. 2 demonstrates the effects of bestatin (0.01, 0.1 and 1 mM), an aminopeptidase B and LAP inhibitor, on the hypocalcemic effects after nasal administration of SCT (pH 7.0) in rats. Bestatin did not significantly change the hypocalcemic effects after administration of SCT at any concentration studied. Furthermore, diprotin A (0.1 and 1 mM), a DPP IV inhibitor, slightly enhanced the hypocalcemic effects after nasal administration of SCT (pH 7.0) in rats (data not shown). However, the AAC values of SCT with diprotin A (0.1 and 1 mM) were not significantly different from those estimated in the absence of the proteolytic enzyme inhibitor.

Fig. 3 shows the effects of aprotinin (10^3 and 10^4 KIU/ml) and soybean trypsin inhibitor (0.125 and 1.25 mM) on the hypocalcemic effects after

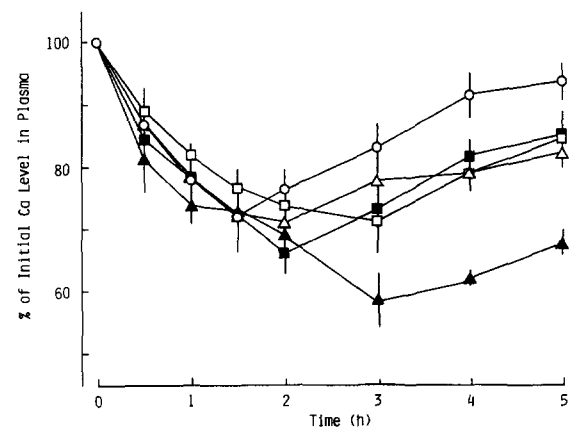

Fig. 3. Effects of aprotinin and soybean trypsin inhibitor (STI) on the change in plasma calcium levels after nasal administrations of SCT (10 IU/kg, pH 7.0) in rats. (○) Without inhibitor; (△) 10^3 KIU/ml aprotinin; (▲) 10^4 KIU/ml aprotinin; (□) 0.125 mM STI; (■) 1.25 mM STI. Each value represents the mean ± S.E. of four animals.

nasal administration of SCT (pH 7.0) in rats. Aprotinin (10^3 KIU/ml) and STI (0.125 and 1.25 mM) slightly prolonged the hypocalcemic effects after administration of SCT. However, these AAC values were not significantly different from those estimated in the absence of inhibitors. Aprotinin (10^4 KIU) significantly enhanced the hypocalcemic effects after administration of SCT. There was a peak enhancing effect at 3 h after after aprotinin administration.

Fig. 4 depicts the effects of camostat mesilate (an aminopeptidase and trypsin inhibitor) and leupeptin (a trypsin and cathepsin B inhibitor) on the hypocalcemic effects after nasal administration of SCT (pH 7.0) in rats. The increasing concentration in these inhibitors led to greater hypocalcemic effects. The AAC values of SCT (126.6% h) with camostat mesilate (10 mM) and leupeptin (1.0 mM) (138.9% h) were significantly different from that without inhibitors (77.9% h), $p < 0.05$ and $p < 0.01$, respectively.

Fig. 5 shows the effects of aprotinin and bestatin at various pH values (4.0, 7.0 and 8.0) on the pharmacological availabilities after nasal administration of SCT in rats. The pharmacological availability at pH 4.0 was the highest compared to those at pH 7.0 and 8.0 in the presence or absence of inhibitors. Aprotinin enhanced the hypocalcemic effects at all pH values studied. The pharmacological availabilities at pH 4.0 and 7.0 were increased from 5.4 to 7.5% and from 3.2 to 6.9% by aprotinin (10^4 KIU/ml), respectively. The degree of enhancement by aprotinin was the highest at pH 7.0. However, bestatin did not

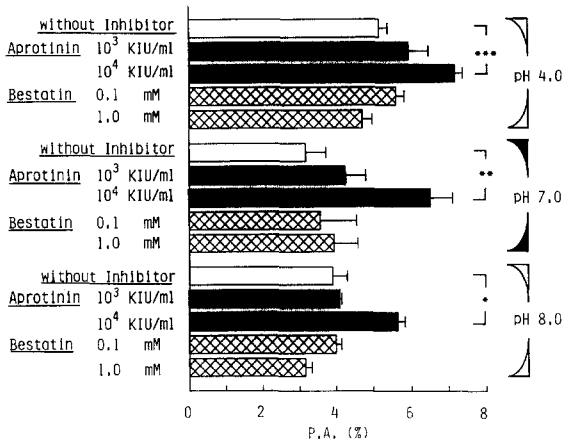

Fig. 5. Pharmacological availabilities (P.A.) after nasal administration of SCT (10 IU/kg) containing bestatin and aprotinin at various pH values and concentrations in rats. Significantly different from respective value without inhibitor at * $p < 0.01$, ** $p < 0.05$, *** $p < 0.001$. Each value represents the mean ± S.E. of four animals.

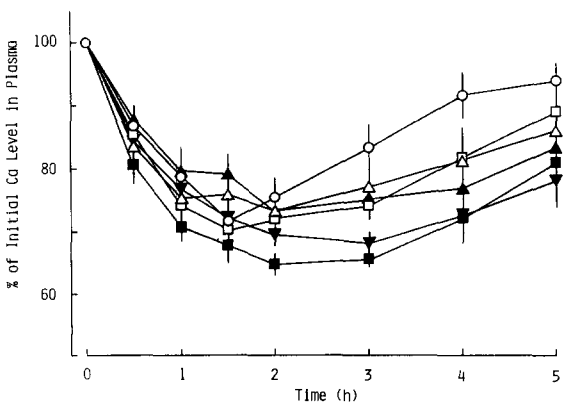

Fig. 4. Effects of camostat mesilate and leupeptin on the change in plasma calcium levels after nasal administrations of SCT (10 IU/kg, pH 7.0) in rats. (○) Without inhibitor; (△) 0.1 mM camostat mesilate; (▲) 1 mM camostat mesilate; (▼) 10 mM camostat mesilate; (□) 0.1 mM leupeptin; (■) 1 mM leupeptin. Each value represents the mean ± S.E. of four animals.

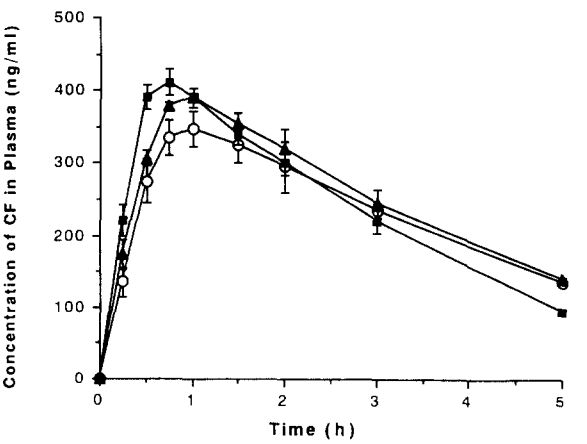

Fig. 6. Plasma concentrations of CF after nasal administration of CF (1 mg/kg, pH 7.0) containing protease inhibitors in rats; (○) without inhibitor, (▲) aprotinin (10^4 KIU/ml), (■) camostat mesilate (10 mM). Each value represents the mean ± S.E. of four animals.

enhance the absorption of SCT regardless of pH studied.

3.3. Nasal absorption of CF

CF is a poorly absorbing drug due to its high water solubility and lack of lipophilic properties. The effects of camostat mesilate and aprotinin on the nasal absorption of CF were evaluated in rats (Fig. 6). The time courses for the plasma CF concentration-time profile of CF after administration remained unchanged on treatment with camostat mesilate (10 mM) or aprotinin (10^4 KIU/ml).

4. Discussion

The transport barrier of nasal epithelium is considered to weaker for the absorption of peptides and proteins than that of the intestinal epithelium (McMartin et al., 1987). However, the nasal transepithelial electrical resistance (60–260 Ω/cm^2) suggests equal or slightly less permeability than intestine (Rojanasakul et al., 1992). Fisher et al. (1991) reported that the bioavailabilities of nasally administered dextran of average molecular mass of 1625 and 2300 Da were high (21.9 and 26.7%), respectively. However, the bioavailability of nasally administered polypeptides, of molecular mass greater than 1000 Da, is less than that by parenteral administration, which may mainly be attributed to degradation by proteolytic enzyme in the nasal mucosa (McMartin et al., 1987). In this study, the pharmacological availabilities of nasally administered SCT (molecular mass 3500 Da) were relatively low, yielding values of 5.4, 3.2 and 3.9% at pH 4.0, 7.0 and 8.0, respectively. These values are very comparable to those found with nasally administered secretin (molecular mass 3052 Da) in rats (Owaki et al., 1987).

We recently reported that camostat mesilate (aminopeptidase and trypsin inhibitor), but not aprotinin and STI, enhanced the nasal absorption of AVP and its analogue (Morimoto et al., 1991). Hussain et al. (1989) reported that the aminopeptidase inhibitor was useful for improving the bioavailability of nasally administered leucine enkephalin. In this study, bestatin (aminopeptidase B and LAP inhibitor), diprotinin (DPP IV inhibitor), and STI (trypsin inhibitor) did not enhance the nasal absorption of SCT. However, camostat mesilate, aprotinin and leupeptin (which all show trypsin inhibitory activity) also significantly enhanced the nasal absorption of SCT. Parsons et al. (1979) reported that the plasma levels of porcine calcitonin subcutaneously injected into chicks were enhanced with aprotinin. This is in contrast to the non-enhancing effect exerted by STI, since this inhibitor (molecular mass 8000 Da) might not permeate into the nasal epthelial cells.

The Cys-1-Cys-7 ring of SCT, a 32-residue peptide, is in close association with the helix between 8 and 22, while the C-terminal is known to fold toward the core, forming a loose loop (Meyer et al., 1991). Camilleri et al. (1991) reported that SCT was degraded to four fragments (1–11, 12–18, 19–24 and 25–32) by trypsin. Iwakawa et al. (1984) reported that exoproteases such as aminopeptidase and carboxypeptidase did not cause significant degradation of elcatonin (eel calcitonin analogs), and incubation with endopeptidase such as trypsin or chymotrypsin caused a rapid loss of immunoreactive elcatonin. These data suggest that nasally administered SCT is probably degraded by trypsin activity. The degree of absorption enhancement of SCT by trypsin inhibitors was lower than that of vasopressin and of leucine enkephalin by aminopeptidase inhibitors (Hussain et al., 1989; Morimoto et al., 1991). This phenomenon may be due to the relatively high molecular mass of SCT which renders low permeation and increases degradation susceptibility of SCT by other proteolytic enzymes.

Cremaschi et al. (1991) suggested that the active transport of elcatonin in parallel with passive permeation (possibly a leak through intercellular junctions), which does not appear to be related to nonspecific pinocytosis, may be via receptor-mediated endocytosis. Leupeptin, which is an inhibitor of lysosomal enzyme such as cathepsin B, enhanced the nasal absorption of SCT. However, bestatin, diprotinin A and STI, which demonstrated a weak inhibitory effect on cathepsin B activtiy in nasal tissue, did not enhance the nasal

absorption of SCT. SCT, if transported by receptor-mediated endocytosis, may then be degraded by lysosomal enzymes such as cathepsins.

The pharmacological availabilities of SCT at various pH values (pH 4.0, 7.0 and 8.0) with or without aprotinin were greatest at pH 4.0. The nasal absorption of secretin was also higher at lower pH. The effects of pH on nasal absorption of SCT may be due to changes in the structure or electrical charge of the mucosal membrane, and/or to a conformational change in the peptide. The pharmacological availability of SCT at pH 7.0 was low and the degree of enhancement by aprotinin was highest at pH 7.0. This phenomenon may be due to fact that the trypsin-like activity on the surface of nasal epithelial membrane may be highest at near physiological pH and its activity may be mostly inhibited by aprotinin at pH 7.0.

In order to examine the effects of camostat mesilate and aprotinin on the permeation barrier of the nasal mucosal membrane, several studies were carried out in rats. Camostat mesilate (10 mM) and aprotinin (10^4 KIU/ml) did not enhance the nasal absorption of CF (molecular mass 376 Da, $pK_a = 6.4$), a hydrophilic compound. Therefore, this particular observation suggests that camostat mesilate and aprotinin did not affect the paracellular permeation barrier of nasal epithelium.

In conclusion, aprotinin, camostat mesilate and leupeptin enhanced the nasal absorption of SCT. Therefore, SCT during absorption may be degraded by trypsin-like activity in the membrane of the nasal epthelium. These findings show that the enzymatic barrier for nasal absorption of SCT may be one factor requiring consideration when designing a nasal drug delivery system.

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