Study on Pulmonary Delivery of Salmon Calcitonin in Rats: Effects of Protease Inhibitors and Absorption Enhancers

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Effects of protease inhibitors and absorption enhancers on the absorption of salmon calcitonin (sCT) were evaluated after intratracheal coadministration to rats using the plasma Ca level as an index. Remarkable absorption enhancement could be attained with unsaturated fatty acids such as oleic acid and polyoxyethylene oleyl ether (absorption enhancers) and with chymostatin, bacitracin, potato carboxypeptidase inhibitor and phosphoramidon (protease inhibitors). sCT degrading enzymes had four times higher activity per total protein in membrane fraction of lung homogenates than the activity in cytosol fraction. These enzymes are thought to be serine proteases and metalloenzymes from the *in vitro* action profile of protease inhibitors. A good correlation between the *in vitro* activity of protease inhibitors and the *in vivo* enhancing effect on sCT activity suggested that membrane enzymes are responsible for the inactivation of sCT. Metabolic degradation and low permeability of sCT may be possible barriers to the absorption of sCT.

KEY WORDS: salmon calcitonin; rat alveolar absorption; protease inhibitor; absorption enhancer.

INTRODUCTION

The pulmonary route has attracted attention in addition to nasal, dermal and rectal routes for its potential for noninvasive systemic administration of peptide and protein drugs (1,2). The present study was undertaken to evaluate the effect of protease inhibitors and absorption enhancers on the absorption of salmon calcitonin (sCT) in rats after intratracheal coadministration. The possible absorption barrier and enzymatic barrier of the alveolar epithelial mucosa are also discussed.

MATERIALS AND METHODS

Reagents

Synthetic sCT and protease inhibitors [chymostatin, potato carboxypeptidase inhibitor (pCPI), phosphoramidon, antipain, leupeptin, bestatin, foroxymithin, amastatin, pepstatin, Tos-Lys-chloromethylketone (TLCK), Tos-Phe-chloromethylketone (TPCK), 3,4-dichloroisocumarin (3,4-DCI), trans-epoxysuccinyl-leucylamido(4-guanido)butane (E-64)] were purchased from Sigma Chemical Co. Other protease inhibitors used were D-Tyr-Pro-Arg-chloromethylketone (CK) purchased from Bachem Co., (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), soy bean trypsin inhibitor (STI), diisopropyl fluorophosphate (DFP), bacitracin and benzamidine from Wako Pure Chemical, and aprotinin from Behringwerke AG. Fatty acids and surfactants were purchased from Nihon Oil & Fats, which had been synthesized from highly pure oleic acid (purity: $\geq 95\%$).

As dilution buffers for reagents, phosphate buffered saline (PBS) and 0.1M Tris-HCl buffer (pH 7.5) were used for animal and *in vitro* studies, respectively. sCT was dissolved in purified water to prepare a high conc. solution, which was diluted with the dilution buffers containing the below mentioned absorption enhancers/protease inhibitors. Pepsin and chymostatin were dissolved in 0.1N HCl; TPCK and 3,4-DCI, in dimethyl sulfoxide (DMSO); and other protease inhibitors, in purified water all at high concentrations. These high conc. solutions were diluted with the dilution buffer. The final concentration of DMSO in the drug solution was $\leq 1\%$. As absorption enhancers, surfactants and fatty acids were also dissolved or suspended in the dilution buffer, followed by 2-min sonication. Exceptionally, palmitic acid was melted at 60°C before the addition of an adequate amount of the buffer. Palmic acid suspension was obtained by 2-min sonication at 37°C. All fatty acid/surfactant solutions or suspensions were prepared just before use.

Animal Studies

Male Sprague-Dawley rats, 7–9 weeks of age and weighing 200–300 g, were fasted for 18–20 hr prior to the experiment. Intratracheal (i.t.) administration was done according to the method of Enna and Schanker (3). The rats were anesthetized with urethane and the trachea was exposed. A polyethylene tube was inserted through a tracheal incision. The drug solution was instilled through the polyethylene tube with a 100-μl microsyringe (100 μl/300 g body weight). The animals were fixed in a supine position on a plate kept at 37°C. In the intramuscular (i.m.) administration group, the same volume of the drug solution was injected into the thigh muscle. The control animals received PBS solution without sCT.

Determination of sCT Biological Activity

Blood (100 μl) was collected from the tail vein using a heparinized capillary tube at designated intervals for 5 hr after the administration and centrifuged at 1000 xg to separate plasma. Plasma Ca levels were measured with Calcium Test Wako C (Wako Pure Chemical). The Ca reduction curve was drawn with reduction rates of plasma Ca levels at 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr and 5 hr postadministration against the plasma Ca level just before the administration. The area between the plasma Ca level versus time curves of the sCT administered groups and that of the control (PBS) group for 0–5 hr was calculated by the trapezoidal method. The obtained value (*A*rea of *C*a *R*eduction: ACR) was used as an index of sCT biological activity. Each dose group con-

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sisted of 4 to 6 rats. Student's t-test was used for the statistical analysis.

Degradation of sCT in Lung Homogenate

The lungs of 6 rats were pooled, washed with cool saline solution, and homogenized with a 4-fold volume of 0.1M Tris-HCl (pH 7.4) containing 0.2M sucrose per gram of the tissue weight with ice cooling. The homogenate was centrifuged at 4°C, 4500 xg for 30 min. The supernatant was further centrifuged at 50,000 xg for 30 min, and the resultant supernatant was used as the cytosol fraction. The precipitate was washed with the same buffer twice by centrifugation in a similar manner, and the resultant precipitate was used as the membrane fraction.

The substrate solution was prepared with 0.1M Tris-HCl buffer (pH 7.4) to a final sCT concentration of 50 μM. To 100 μl of the substrate solution, 25 μl of purified water or an enzyme inhibitor/absorption enhancer solution/suspension and 25 μl of enzyme solution were added in this order. The mixture was allowed to react at 37°C for 30 min.

The reaction was stopped with 10 μl of 10% trifluoroacetic acid. After centrifugation at 7000 xg for 3 min to remove proteins, the residual sCT concentration in the supernatant was determined by HPLC to calculate the enzyme activity. HPLC conditions were: column, Vydac Protein & Peptide 0.4 × 15 cm; measurement wavelength, at 215 nm; mobile phase, 32% acetonitrile solution containing 0.1% trifluoroacetic acid; and flow rate, 1.7 ml/min. Under these conditions, an sCT peak appeared at a retention time of about 7 min. The protein content of the enzyme solution was determined with a Protein-Assay Kit (Bio-Rad).

RESULTS

Intramuscular Administration

Fig. 1 shows the reduction rate of plasma Ca levels after i.m. injection of sCT. At 0.2 and 0.4 mg/kg, a maximum reduction rate of 10–15% was observed at 1 hr postadministration, and then the plasma Ca level increased gradually to reach a normal level at 5 hr. At 1, 2, 4 and 8 μg/kg, the Ca level decreased until 5 hr postadministration in a similar fashion for each dose showing the saturation of Ca reduction effect. The control group showed no change in the plasma Ca level.

Intratracheal Administration

Fig. 2 shows the reduction rate of plasma Ca levels after i.t. administration of sCT. Plasma Ca levels decreased dose-dependently. At 4 and 8 μg/kg, a similar reduction pattern to that after i.m. injection of 1 μg/kg or higher was observed. No change of the plasma Ca level was seen at 0.2 μg/kg and in the control group.

Comparison of sCT Activity Between Intramuscular and Intratracheal Administration

In Fig. 3, the ACR is plotted against each dose level. In the i.m. injection group, a linear relationship was observed between ACR and the logarithm of doses at 0.2, 0.4, 1 and 2 μg/kg. In the i.t. administration group, a linear relationship was obtained at 0.4, 1, 2 and 4 μg/kg. The relative i.t. bioavailability (BA) against i.m. administration was calculated to be 30%.

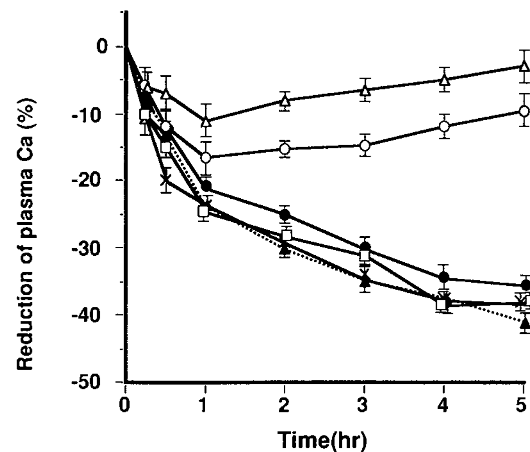

Fig. 1. Efficacy of salmon calcitonin after intramuscular administration in rats. Doses were given at 0.2 μg/kg (△), 0.4 μg/kg (○), 1 μg/kg (●), 2 μg/kg (□), 4 μg/kg (x) and 8 μg/kg (▲). Each point represents the mean ± S.E. of plasma Ca reduction (%) (n = 6).

Enhancing Effect of Fatty Acid and Surfactant on sCT Absorption in Intratracheal Administration

Free unsaturated fatty acids of oleic acid, palmitoleic acid and linoleic acid exhibited very strong enhancing effect (Fig. 4). Sodium oleate was also effective to the same degree as oleic acid. POE oleyl ether showed very excellent enhancing activity. In contrast, sorbitan trioleate, POE sorbitan monooleate and POE sorbitan trioleate exhibited moderate enhancing effect, and the effect of glycerol trioleate, ethyl oleate, oleyl alcohol, palmitic acid and stearic acid was poor.

Enhancing Effect of Protease Inhibitor on sCT Absorption in Intratracheal Administration

As shown in Fig. 5, bacitracin, chymostatin, pCPI and phosporamidon exhibited remarkable enhancing effect. Antipain, leupeptin, DFP and TLCK promoted Ca reduction to

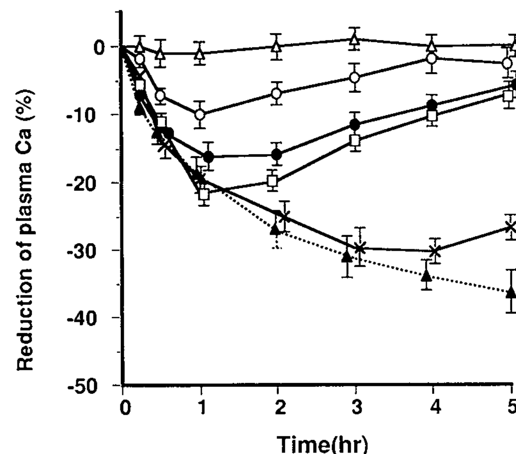

Fig. 2. Efficacy of salmon calcitonin after intratracheal administration in rats. Doses were given at 0.2 μg/kg (△), 0.4 μg/kg (○), 1 μg/kg (●), 2 μg/kg (□), 4 μg/kg (x) and 8 μg/kg (▲). Each point represents the mean ± S.E. (n = 6).

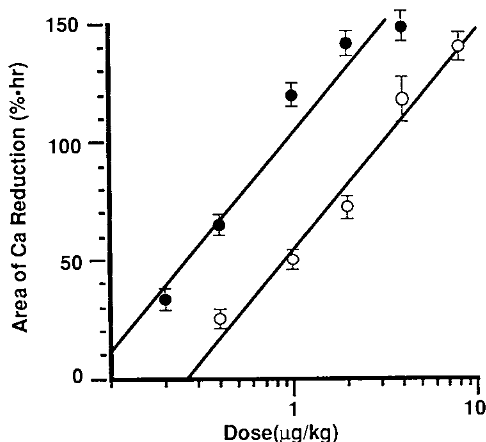

Fig. 3. Dose-efficacy curves of salmon calcitonin after intramuscular (●) and intratracheal (○) administration in rats. Each point represents the mean ± S.E. (n = 6). Minimum square regression equations are y = 105.3 + 93.8 log (x) (r = 0.949) for intramuscular administration and y = 55.2 + 92.3 log (x) (r = 0.974) for pulmonary administration.

a moderate degree. The other inhibitors affected the BA of sCT only slightly.

Degradation of sCT in Lung Homogenate and Its Inhibition

Measurement of the sCT-degrading enzyme activity in the homogenate indicated that the enzyme activity per total protein in the membrane fraction was four times higher than that in the cytosol fraction.

Inhibition of sCT Degradation by Enzyme Inhibitor

The inhibitory effect of each protease inhibitor against sCT degrading enzymes is shown in Fig. 6. The inhibition

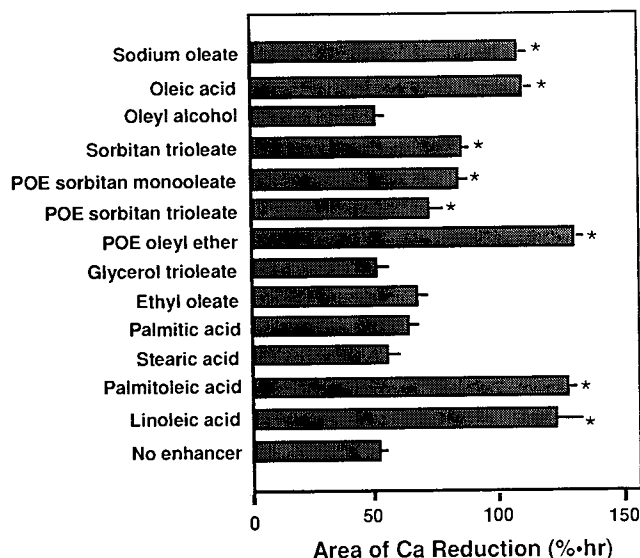

Fig. 4. Absorption enhancing effects of fatty acids and surfactants on the pulmonary absorption of salmon calcitonin in rats. The enhancer was given at 0.5% simultaneously with salmon calcitonin (1 μg/kg). Each point represents the mean ± S.E. (n = 4 ~ 6). The mark * indicates a statistically significant difference from the control group ($P < 0.01$).

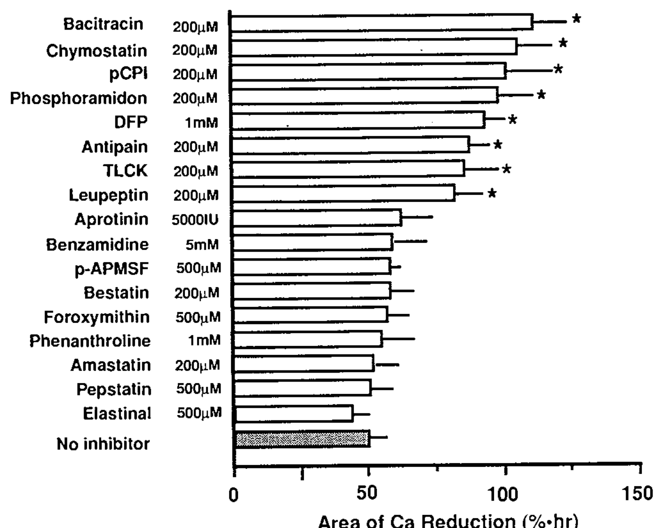

Fig. 5. Absorption enhancing effects of protease inhibitors on the pulmonary absorption of salmon calcitonin in rats. The inhibitor was given simultaneously with salmon calcitonin (1 μg/kg). Each point represents the mean ± S.E. (n = 4 ~ 6). The mark * indicates a statistically significant difference from the control group ($P < 0.01$).

ratios of serine protease inhibitors (chymostatin, antipain, leupeptin, DFP, D-Tyr-Pro-Arg-CK and TLCK) were 60% or more against membrane enzymes. In particular, effects of antipain and leupeptin were specific to membrane enzymes. On the contrary, the inhibitory effect of serine proteases (p-APMSF, aprotinin, 3,4-DCI and STI) was much stronger against cytosol enzymes than against membrane enzymes. A metal protease inhibitor, phosphoramidon, inhibited membrane enzyme activity specifically, while phenanthroline and EDTA showed stronger inhibitory effect against cytosol enzymes than membrane enzymes. The inhibitory action of bacitracin or pCPI was very strong on both membrane and cytosol enzymes. A thiol protease inhibitor, E-64, showed a weak inhibitory action only on membrane enzymes. The other types of protease inhibitors [pepstatin (aspartic protease inhibitor), foroxymithin (ACE inhibitor), bestatin and amastatin (amino peptidase inhibitor) and elastinal (elastase inhibitor)] showed no inhibitory activity in the membrane and cytosol fractions.

Fig. 7 shows the relationship between the *in vivo* enhancing effect of protease inhibitors and the *in vitro* inhibitory effect of sCT degrading enzymes. A relatively favorable correlation was observed for membrane enzymes, whereas such a relationship was not shown for cytosol enzymes. Particularly, phosphoramidon, phenanthroline, benzamidine, p-APMSF and aprotinin deviated from the correlation curve for the cytosol enzymes.

Inhibitory Effect of Fatty Acid and Surfactant Against sCT Degradation

The fatty acids and surfactants used here did not inhibit nor accelerate the activity of membrane and cytosol enzymes.

DISCUSSION

Recently it was reported that the i.t. BA of insulin solution was 13% in rats in the same experimental system as

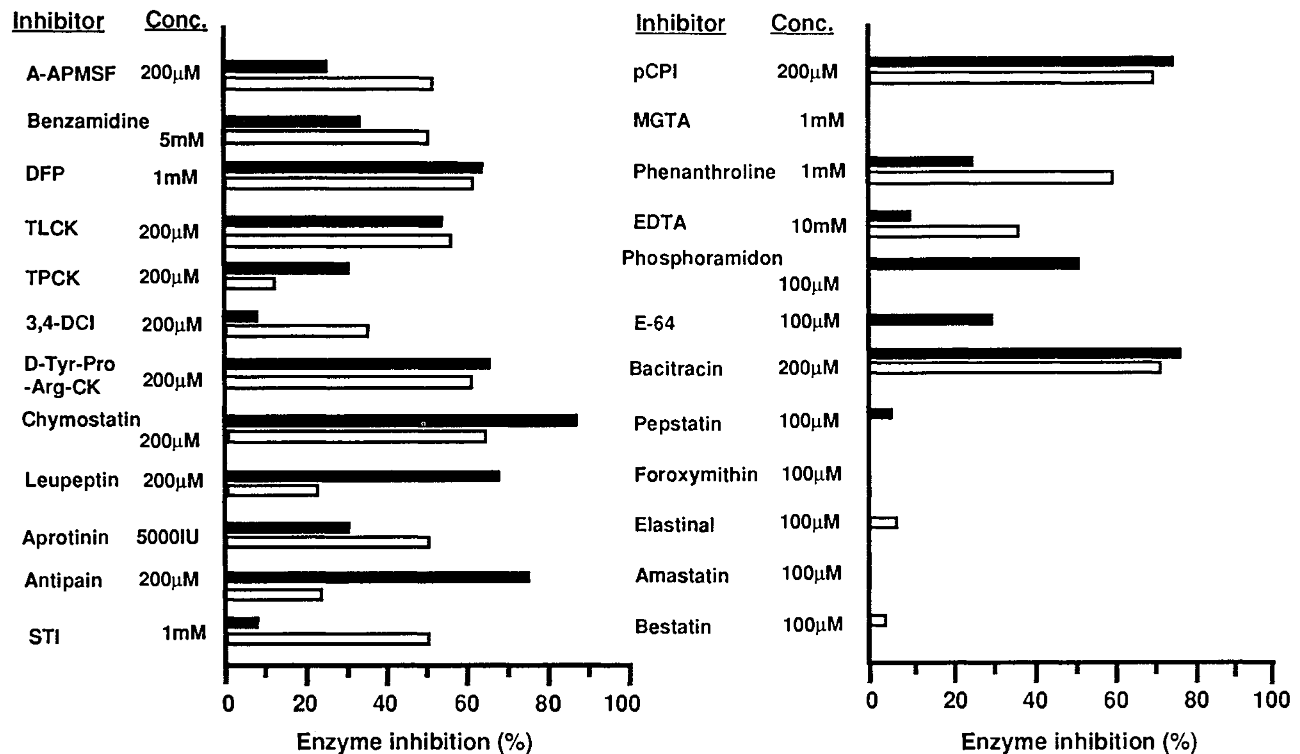

Fig. 6. Inhibitory effects of protease inhibitors on salmon calcitonin degradation by membrane enzyme (closed bar) and cytosol enzyme (open bar). Each bar represents the mean of duplicate assays.

employed here (4). While the i.t. BA of sCT was as high as 30% in our study, any comparisons should be made with caution. Since ACRs (Area of Calcium Reduction) in the sCT high dose groups were calculated based on plasma Ca levels which did not recover to initial levels even after 5 hr of sCT injection, the sCT activity may have been underestimated. The effects of protease inhibitors or absorption enhancers observed here suggested that the incomplete i.t. BA of sCT might be attributable to both enzymatic degradation and an absorption barrier in the pulmonary mucosa. The membrane fraction of the lung tissue homogenate showed four times higher sCT degradation activity, and the correlation between the *in vitro* inhibitory effect of protease inhibitors and the i.t. BA was better than that obtained with the cytosol fraction. These results indicate that the membrane fraction largely accounts for the inactivation of sCT at the

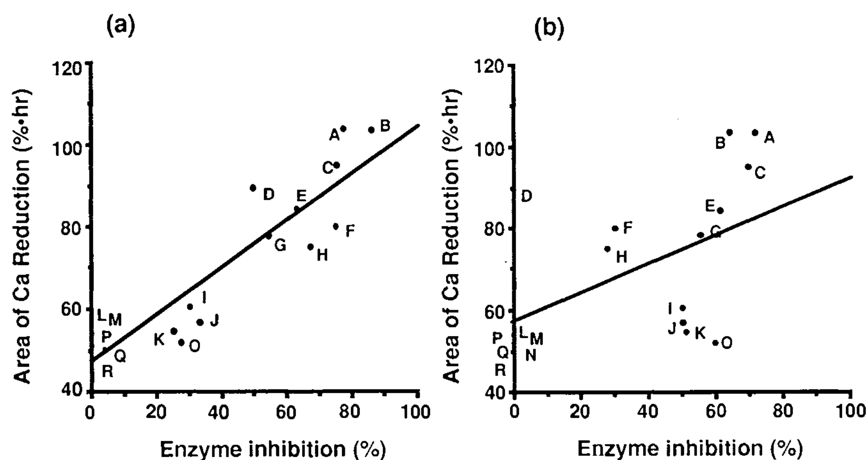

Fig. 7. Relationship between absorption enhancing activity and salmon calcitonin-degrading enzyme inhibition by protease inhibitors: (a), membrane enzyme ($y = 47.8 + 0.569x$, $r = 0.825$); (b), cytosol enzyme ($y = 55.9 + 0.440x$, $r = 0.345$). Alphabets represent bacitracin (A), chymostatin (B), pCPI (C), phosphoramidon (D), DFP (E), antipain (F), TLCK (G), leupeptin (H), aprotinin (I), benzamidine (J), p-APMSF (K), bestatin (L), foroxymithin (M), amastatin (N), phenanthroline (O), pepstatin (P), elastinal (Q) and no inhibitor (R).

absorption site. However, intracellular incorporation of an inhibitor is prerequisite for the inhibition of cytosol enzymes *in vivo*. This fact may explain the inconsistent *in vivo* and *in vitro* inhibitory effects. In contrast to our results, insulin has been reported to be degraded primarily by aminopeptidases in the cytosol fraction of the lung *in vitro* (5). It is of interest that major sCT degrading enzyme activity is located in a different cellular fraction from the one responsible for insulin metabolism in the lung.

There are reports on the enzymatic metabolism of peptides in the lung (5,6,7), but little has been reported on sCT degrading enzymes. Calcitonin absorption is accelerated by aprotinin and benzamidine in the nasal mucosa (8) and by bestatin and pepstatin in the rectal mucosa (9). In our study, all of these inhibitors did not accelerate the pulmonary absorption of sCT, possibly as a result of the presence of different calcitonin degrading enzymes among mucosal tissues. The present study indicated that sCT is metabolized mainly by serine protease enzymes and metalloenzymes. The inhibitory activity of bacitracin and pCPI might be nonspecific as a peptide substrate. Bacitracin activity is not always specific (10). Okumura *et al.* reported that bacitracin did not accelerat insulin absorption in rats after i.t. administration (4), which is different from our results on sCT. Nonspecificity of pCPI is suggested by two findings: (1) MGTA, a synthetic carboxypeptidase inhibitor, did not show any inhibitory activity and (2) the carboxypeptidase-resistant sCT derivative having D-proline amide at the C-terminus instead of L-proline amide was easily inactivated (data not shown). However, we should take notice of the fact that there are some limitations to the interpretation of the results obtained with lung tissue homogenate because of the heterogeneity of cell types and a possible activation or inactivation of enzymes by homogenation.

The absorption enhancing effect of surfactants and fatty acids in various mucous membranes has been reported (8,11). Niven *et al.* have reported that the pulmonary absorption enhancing effect is potent in the order of oleic acid > sorbitan trioleate = oleyl alcohol in the isolated perfused lung of rats (12). This is in good agreement with our results. Oleic acid and surfactants, *etc.* are very potent enhancers as evidenced from the present study but some of them are known to cause edema of the lung (13). For clinical application of these absorption enhancers, toxicological investigations would be indispensible.

The pulmonary administration of sCT was suggested to be worthy of further investigations.

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