

INSULIN AND PROINSULIN PROTEOLYSIS IN MUCOSAL HOMOGENATES OF THE ALBINO RABBIT: IMPLICATIONS IN PEPTIDE DELIVERY FROM NONORAL ROUTES

Akira Yamamoto,¹ Eiji Hayakawa,² and Vincent H.L. Lee

University of Southern California, School of Pharmacy
1985 Zonal Avenue, Los Angeles, California 90033

(Received in final form October 25, 1990)

Summary

The objective of this study was to determine (a) the rate of hydrolysis of insulin and proinsulin in homogenates of various non-oral absorptive mucosae relative to the ileal mucosa, and (b) the inhibitory effect of various protease inhibitors on the degradation of these peptides. Overall, insulin was somewhat more susceptible to hydrolysis than proinsulin in all mucosal homogenates. Proteolytic activity was highest in the nasal and rectal homogenates, followed by the ileal, vaginal, conjunctival and buccal homogenates in that order. The rate of insulin and proinsulin proteolysis differed by a factor of about 6 to 7 between the least and the most proteolytically active mucosa. A 5-fold maximum reduction in proteolytic rate was seen in the presence of Na glycocholate, aprotinin and p-chloromercuriphenylsulfonic acid (PCMPS). The rank order of effectiveness was Na glycocholate (1%) > aprotinin > PCMPS in all but the conjunctival and vaginal homogenates. Extrapolating the above *in vitro* findings to *in vivo*, both insulin and proinsulin are expected to be degraded to varying degrees during absorption across the various mucosae. Absorption may therefore be enhanced in the presence of protease inhibitors such as aprotinin, PCMPS, or Na glycocholate.

Insulin, which is derived from proinsulin through cleavage by intracellular trypsin-like and carboxypeptidase B-like proteases (1), has been used in the treatment of diabetes mellitus since its discovery in 1922. Presently, insulin cannot be given orally due to extensive hydrolysis in the gastrointestinal tract (2-4). Consequently, alternative routes which are presumed to be deficient in protease activity are being investigated for insulin delivery. These routes include the nasal (5-7), buccal (8-10), rectal (11-13), vaginal (14,15), pulmonary (16), ocular (17-18), and transdermal routes (19). Results to date indicate that the bioavailability of insulin from such routes is indeed considerably improved over the oral route, but it is still 2 to 30 times less than after parenteral administration (20). For instance, Aungst et al. (21) found that insulin bioavailability in the rat was only 0.4-2% from the nasal route, 3.2-17% from the rectal route, and

¹Department of Basic Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan, 606. ²Kyowa Hakko Kogyo, Pharmaceutical Research Laboratories, Shizuoka, Japan, 411.

0.7-3.6% from the buccal route. A possible reason for this low bioavailability is that, as is the case in the gastrointestinal tract, insulin is hydrolyzed by proteases both before and during absorption.

In an earlier study (22), we reported that the pentapeptides leucine and methionine enkephalins were rapidly degraded in various mucosal homogenates of the albino rabbit. Proteolysis was mediated primarily by aminopeptidases, whose activity did not differ greatly among various mucosae (23). Aminopeptidases, however, do not appear to play an important role in degrading insulin and proinsulin. Insulinase (24,25), glutathione insulin transhydrogenase (26-28), and insulin degrading enzyme (29-31) are mainly responsible for the degradation of insulin, while trypsin-like and carboxypeptidase B-like proteases are primarily responsible for the cleavage of proinsulin to insulin and C-peptide (1). There is, however, no information on whether these proteases follow the same rank order of activity as aminopeptidases among the various absorptive mucosae.

Thus, the objectives of the present study were: (a) to determine whether insulin and proinsulin were degraded in various mucosal homogenates in the albino rabbit, (b) to determine the rank order of insulin and proinsulin proteolysis among the various mucosae, and (c) to determine the extent to which aprotinin, p-chloromercuriphenylsulfonic acid, and Na glycocholate would protect insulin and proinsulin from degradation in various absorptive mucosal homogenates.

Materials and Methods

Materials

Crystalline porcine Zn insulin and human proinsulin were a gift from Lilly Research Laboratories and were used as received. Na glycocholate, aprotinin, and p-chloromercuriphenylsulfonic acid (PCMPS) were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and water were of HPLC grade. All other reagents and solvents were of analytical grade.

Preparation of Mucosal Tissue Homogenates

Mucosal tissue homogenates were prepared as previously described (32). Twelve female albino rabbits (Irish Farm, Los Angeles, CA), weighing 2-3 kg, were used. They were euthanized by an injection of Na pentobarbital solution (Eutha-6, Western Medical Supply Co., Arcadia, CA) into a marginal ear vein. Mucosae were removed immediately by scraping the epithelial cell layers with a #11 blade in this order: nasal, conjunctival, buccal, ileal, rectal, and vaginal mucosae. These specimens were pooled by tissue type and stored at -70°C. Immediately before each experiment, a mucosal specimen was thawed at room temperature for about 10 minutes, rinsed twice in isotonic KCl, and then homogenized in 1-2 ml of isotonic phosphate buffer (pH 7.4) at 4°C using a Tissumizer (Ultra-Turrax, Tekmar Co., Cincinnati, OH). The homogenate was centrifuged at 3,020x g in the refrigerated (4°C) centrifuge for 10 min to remove cellular and nuclear debris. The resulting supernatant was diluted with isotonic phosphate buffer to a protein concentration of 10 mg/ml. The protein concentration in each tissue supernatant was determined using a dye-binding assay with bovine serum albumin as the standard (33).

Degradation of Insulin and Proinsulin in Various Mucosal Homogenates

The degradation of insulin and proinsulin was studied by incubating, in triplicate, 100 µL of a tissue supernatant, which had been preincubated at 37°C for 15 min with 100 µL of a given concentration of Na glycocholate (0.1% and 1%), aprotinin (0.01%), or PCMPS (0.05 mM), with 300 µL of an

insulin solution (32). The insulin solution was prepared by dissolving porcine Zn insulin first in 0.2 ml of 0.005 N HCl, followed by dilution with an isotonic phosphate buffer (pH 7.4) to a final insulin concentration of 10 μ M in the incubation mixture. The proinsulin solution was prepared in the same manner as the insulin solution. At predetermined times up until 120 min, 50 μ L was withdrawn from the incubation mixture, to which was added 100 μ L of acetonitrile to precipitate tissue proteins thereby terminating the reaction. Fifty microliters of a 8 μ g/ml tryptophan solution, the internal standard, were then added. The resulting mixture was centrifuged for 10 min to remove the precipitated protein. The precipitate was washed first with 20 μ L of phosphate buffer and then with 80 μ L of acetonitrile and centrifuged for another 10 min. The supernatants from the two centrifugations were combined, and 5 μ L of propylene glycol was added. After evaporating off the acetonitrile under nitrogen, 95 μ L of 0.2% Na glycocholate solution was added. Fifteen microliters of the concentrate were injected, in duplicate, into the HPLC.

In pilot experiments, it was found that insulin and proinsulin remained in solution at the concentration of acetonitrile used, that propylene glycol was necessary to keep both polypeptides in solution during evaporation, and that Na glycocholate was required to improve the shape and height of the polypeptide peaks in the chromatogram. Moreover, control experiments revealed no chemical or physical loss of either polypeptide other than through proteolysis during the time course of incubation.

Assay of Insulin and Proinsulin.

Insulin and proinsulin were assayed by a reversed phase HPLC procedure on a Vydac protein and peptide C18 column (250 x 4.6 mm, 5 μ m). The HPLC consisted of two Shimadzu model LC-6A programmable solvent metering pumps, a Shimadzu model SCL-6A system controller, a Rheodyne model 7125 sample injector equipped with a 100 μ L loop, a Shimadzu model SIL 6A autoinjector, and a Shimadzu model C-R3A integrator. The mobile phase was a mixture of acetonitrile and water containing 0.6% ethanolamine adjusted to pH 3 with phosphoric acid. The proportion of acetonitrile in the mobile phase was increased linearly from 10 to 34% during the first 23 min, maintained at 34% for the next 11 min, and increased linearly to 40% for another 6 min. The flow rate was 1.1 ml/min. These conditions were designed to resolve insulin from chain A and chain B, which were monitored at 210 nm using a Shimadzu SPD-6A variable wavelength UV detector. Under the above conditions, proinsulin, insulin, chain A, and chain B were eluted at 26.6 ± 0.2 min, 25.8 ± 0.2 min, 17.5 ± 0.1 min, and 26.8 ± 0.2 min, respectively. The retention time of tryptophan, the internal standard, was 4.4 ± 0.2 min.

Results

Typical concentration-time profiles for the disappearance of insulin and proinsulin in the various mucosal homogenates of the albino rabbit are shown in Fig. 1. Disappearance followed first order kinetics. There was, however, no evidence for the formation of insulin from proinsulin over the time course of incubation in any mucosal homogenate. Fig. 2 shows the times for insulin and proinsulin to lose 10% of their initial activity, hereafter to be denoted as $t_{0.9}$. These values were calculated from the first order rate constants obtained from the semilogarithmic plots shown in Fig. 1. Except in the conjunctival and ileal homogenates, insulin was about 1.5 times more susceptible to hydrolysis than proinsulin. For insulin, the rank order of proteolytic activity among the mucosal homogenates was nasal > rectal > ileal > vaginal > conjunctival > buccal, there being a 6-fold difference in activi-

ty between the least and the most active mucosae. A similar rank order was seen in proinsulin, namely, ileal > nasal > rectal > vaginal > conjunctival > buccal, there being a 7-fold difference in activity between the least and the most active mucosae.

The effect of Na glycocholate, aprotinin, and PCMPS on the rates of insulin proteolysis in various mucosal homogenates is shown in Fig. 3. The rank order of insulin proteolysis among the mucosal homogenates was not markedly affected by these protease inhibitors. As can be seen, 1% Na glycocholate was the most effective of the three. The $t_{0.9}$ for insulin proteolysis was increased 2-5 times. Their rank order of effectiveness in protecting insulin from proteolysis in all but the conjunctival and vaginal homogenates was 1% Na glycocholate > 0.01% aprotinin > 0.05 mM PCMPS. Increasing the concentration of Na glycocholate from 0.1% to 1% elicited larger changes in insulin proteolysis (2-3 times) in the nasal, rectal, and conjunctival homogenates than in the vaginal, buccal, and ileal homogenates. Overall,

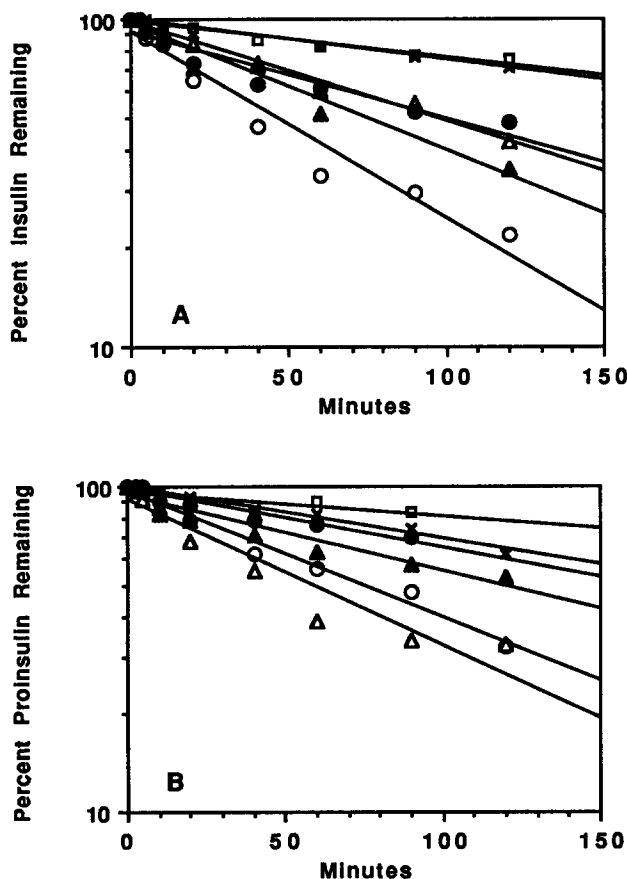


FIG. 1.

Semilogarithmic plots on time course of (A) insulin and (B) proinsulin hydrolysis in homogenates of various absorptive mucosae in the albino rabbit at 37°C. Key: (O) Nasal, (▲) Rectal, (Δ) Ileal, (●) Vaginal, (×) Conjunctival, (□) Buccal. Error bars are omitted for clarity.

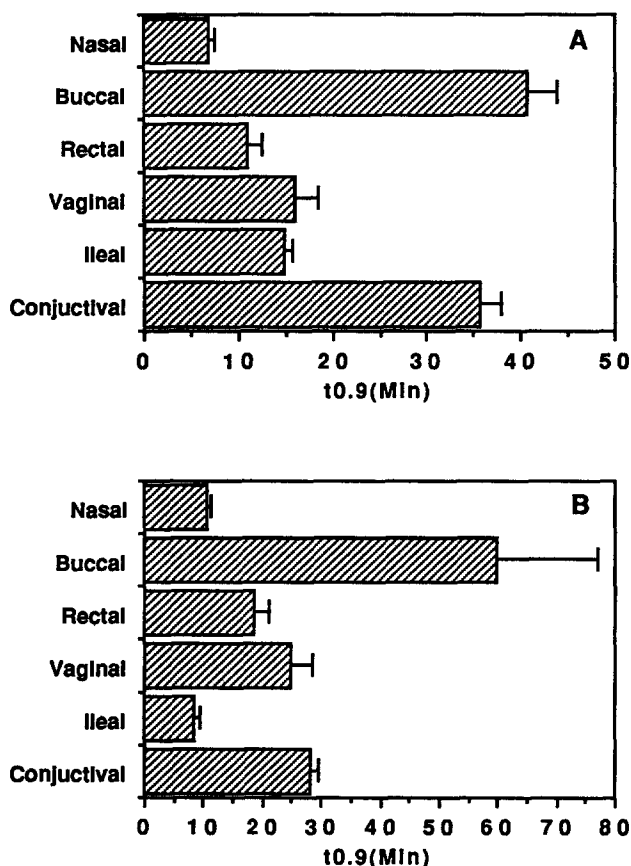


FIG. 2

Rank order of susceptibility of (A) insulin and (B) proinsulin to proteolysis, expressed as $t_{0.9}$, in various mucosal homogenates. Error bars represent standard deviations.

insulin proteolysis in the nasal, rectal, and vaginal homogenates was more sensitive to these protease inhibitors than the conjunctival, buccal, and ileal homogenates. The $t_{0.9}$ for insulin proteolysis was increased 1.4-4.9 times in the former group and 1.2-2.8 times in the latter group.

The effect of protease inhibitors on the rate of proinsulin hydrolysis in mucosal homogenates is shown in Fig. 4. The rank order of effectiveness afforded by the inhibitors in retarding proinsulin proteolysis in all but the ileal and conjunctival homogenates was 1% Na glycocholate > 0.1% aprotinin > 0.05 mM PCMPs, there being a 1.8-2.2 fold difference in effectiveness between Na glycocholate and the last two inhibitors. The most effective inhibitor was aprotinin in the case of the ileal homogenate and was PCMPs in the case of the conjunctival homogenate. Compared to insulin, proinsulin was as sensitive to Na glycocholate but was 1.1-1.5 times less sensitive to aprotinin and PCMPs.

The effect of varying PCMPs concentration on nasal insulin and proinsu-

lin proteolysis is shown in Fig. 5. A dose-dependent inhibitory effect was seen. At PCMPS concentrations less than 0.01 mM, proinsulin was more stable than insulin towards proteolysis. The opposite was observed at higher PCMPS concentrations. Indeed, at 1 mM PCMPS, insulin was completely stable to proteolysis whereas the rate of proinsulin hydrolysis was reduced by only 21 times (data not shown).

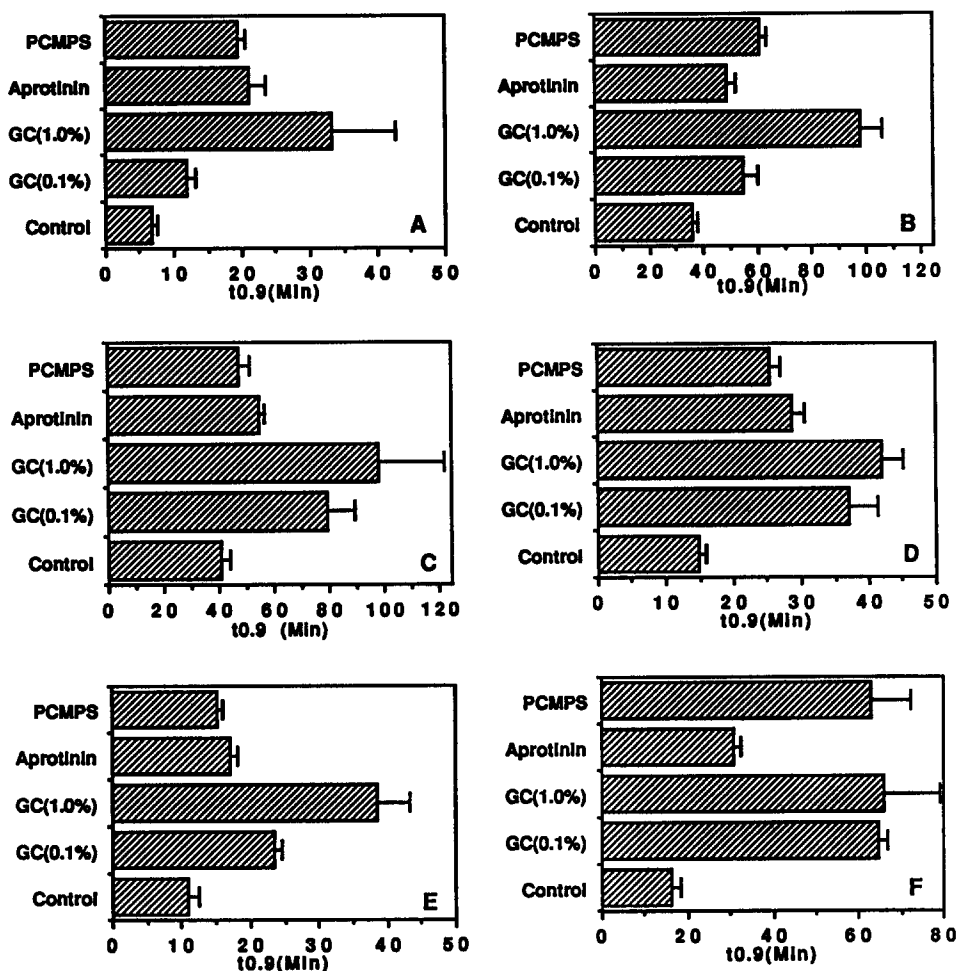


FIG. 3

Effect of Na glycocholate (GC), aprotinin, and PCMPS on the susceptibility of insulin to proteolysis, expressed as $t_{0.9}$, in homogenates of various absorptive mucosae of the albino rabbit. Error bars represent standard deviation for triplicate incubation. Key: A, Nasal; B, Conjunctival; C, Buccal; D, Ileal; E, Rectal; F, Vaginal.

Discussion

The above findings indicate that both insulin and proinsulin would be degraded to varying degrees when administered by the nasal, buccal, oral, rectal, vaginal, and ocular routes. Insulin was about 1.5 times more susceptible to proteolysis than proinsulin in mucosal homogenates (Fig. 2), a finding which is consistent with the 10 times faster degradation rate of insulin than proinsulin in homogenates of the liver, kidney, pancreas, and other organs in the rat (24,25). That this enzymatic barrier plays some

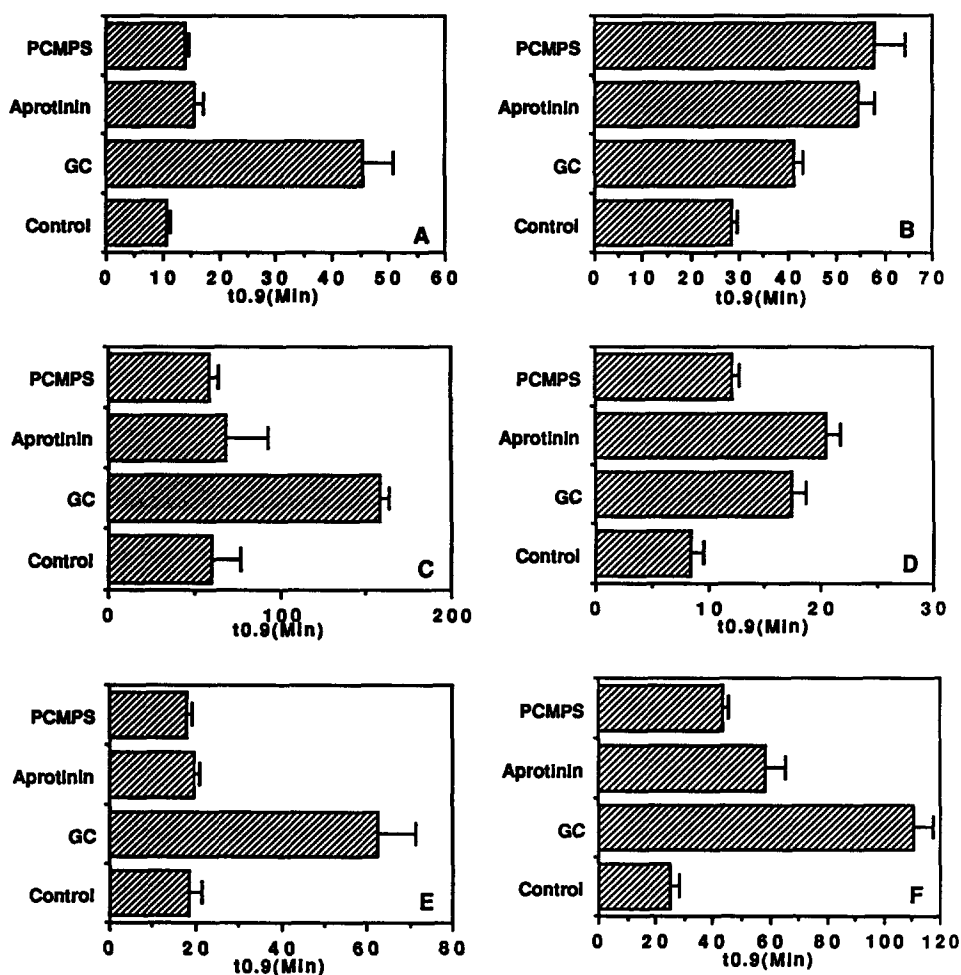


FIG. 4

Effect of Na glycocholate (GC), aprotinin, and PCMPS on the susceptibility of proinsulin to proteolysis, expressed as $t_{0.9}$, in homogenates of various absorptive mucosae of the albino rabbit. Error bars represent standard deviation for triplicate incubation. Key: A, Nasal; B, Conjunctival; C, Buccal; D, Ileal; E, Rectal; F, Vaginal.

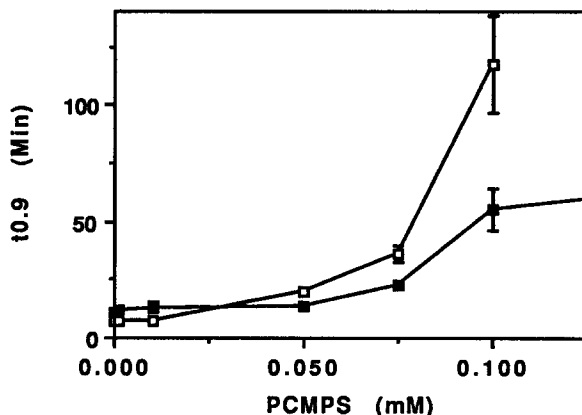


FIG. 5

Effect of varying concentrations of PCMPS on the susceptibility of insulin ($\square$) and proinsulin ($\blacksquare$) to proteolysis, expressed as $t_{0.9}$, in the nasal homogenate of the albino rabbit. Error bars represent standard deviation for triplicate incubation.

role in limiting insulin absorption across the nasal and rectal mucosae can be seen upon comparing the susceptibility of insulin to proteolysis observed in the present study (Fig. 2) to the extent of absorption reported by Aungst et al. (21). Provided that the rank order of proteolytic activity in the rat is the same as in the rabbit, the more extensive absorption of insulin across the rectal than the nasal mucosa (of the rat) is consistent with the lower proteolytic activity in the rectal than the nasal mucosa (of the rabbit), although differences in contact area and contact time cannot be ruled out. Unlike the nasal and rectal mucosae, however, the enzymatic barrier in the buccal mucosa plays a limited role in restricting insulin absorption. This is because despite the low proteolytic activity against insulin in the buccal mucosa, insulin absorption across this mucosa was much less when compared with the proteolytically more active nasal and rectal mucosae. Clearly, the relative importance of the enzymatic and transport barriers to mucosal peptide absorption is mucosal route dependent.

Compared with enkephalins (22), insulin and proinsulin were about 2-33 times more stable to proteolysis. Insulin and proinsulin further differed from enkephalins in the rank order of susceptibility to proteolysis among the various mucosae. The rank order of enkephalin hydrolysis was rectal > buccal > ileal = nasal > vaginal (22). While the vaginal mucosa is consistently low and the rectal mucosal is consistently high in proteolytic activity against all three peptides, the rank order of proteolytic activity between the nasal and buccal mucosae varies with the peptide. The above two findings can be taken to suggest that different principal proteases were involved in the degradation of enkephalins, insulin, and proinsulin.

Doddakashi and Lee (22) have demonstrated that aminopeptidases are the principal proteases mediating the degradation of enkephalins. In light of the sensitivity of insulin and proinsulin proteolysis to aprotinin (a serine protease inhibitor (34)) and PCMPS (a thiol protease inhibitor (30)) (Figs. 3 and 4), the principal proteases are probably serine and thiol proteases in

the case of insulin and proinsulin. Although neither A nor B chain was detected as was the case in plasma and subcutaneous tissues (35), it cannot be concluded that insulinase (24,25), glutathione insulin transhydrogenase (26-28) and insulin degrading enzyme (29-31), all of which played some role in degrading insulin in the liver, kidney, skeletal muscle and other organs, did not contribute to the degradation of insulin in any absorptive mucosa. This is because neither glutathione nor another reduced thiol, which is needed by glutathione insulin transhydrogenase to separate the A and B chains in insulin for degradation by various proteases, was not in the incubation medium. On the other hand, a more definitive statement can be made on the probable non-involvement of the proteases that liberate insulin from proinsulin, since no insulin was recovered in any mucosal tissue homogenate. Whatever the nature of the proteases, however, their relative proportion was possibly different in each absorptive mucosa. This possibility is suggested by the different sensitivity of each mucosa to treatment by the protease inhibitors Na glycocholate, aprotinin, and PCMPS (Figs. 3 and 4). Similarly, the different sensitivity of insulin and proinsulin proteolysis in the nasal homogenate to changes in PCMPS concentration, as shown in Fig. 5, suggests that the principal protease responsible for the initial cleavage of insulin and proinsulin was probably different.

Na glycocholate is a much investigated penetration enhancer in mucosal peptide and protein absorption. Since its protease inhibitory activity relative to nasal insulin absorption in the rat was first reported by Hirai et al. (6), there has been no report on its protease inhibitory activity either in other absorptive mucosae or against other peptides. Clearly, as seen in Figs. 3 and 4, this penetration enhancer is also active against insulin-degrading proteases in absorptive mucosae other than the nasal mucosa and is effective in protecting proinsulin in addition to insulin from proteolysis. Indeed, at the concentration typically used in penetration enhancement (1%), Na glycocholate is generally more effective in protecting insulin and proinsulin against proteolysis than 0.1% aprotinin and 0.5 mM PCMPS (Figs. 3 and 4).

In summary, proteases exist in the nasal, buccal, rectal, vaginal, and ocular mucosae to degrade insulin and proinsulin as is the case in the ileal mucosa. The role of this proteolytic barrier in limiting peptide and protein absorption, however, appears to be dependent on the mucosa and on the nature of the peptide and protein drug. Regardless of the mucosa, such a barrier can be circumvented to varying extents by the protease inhibitors Na glycocholate, aprotinin, and PCMPS. Whether this intervention will lead to improved peptide and protein absorption and the extent to which it contributes to improved peptide and protein bioavailability will be the subject of a future communication.

Acknowledgements

This work was supported in part by a grant from the American Diabetes Association, Los Angeles, California, by an unrestricted gift from Lilly Research Laboratories, Indianapolis, Indiana, and by the Gavin S. Herbert Professorship (VHLL).

References

1. D.F. STEINER and H.S. TAGER, in: "Endocrinology, vol. 2," L.J. De Groot, ed., Grune & Stratton, 1979.
2. V.H.L. LEE, Pharm. Int. 7 208-212, 1986.
3. V.H.L. LEE, CRC Crit. Rev. Ther. Drug Carrier Syst. 5 69-97, 1988.

4. V.H.L. LEE and A. YAMAMOTO, *Adv. Drug Deliv. Rev.* 4 171-207, 1990.
5. S. HIRAI, T. YASHIKI and H. MIMA, *Int. J. Pharm.* 9 165-172, 1981.
6. S. HIRAI, T. YASHIKI and H. MIMA, *Int. J. Pharm.* 9 173-184, 1981.
7. G.S. GORDON, A.C. MOSES, R.D. SILVER, J.S. FLIER and M.C. CAREY, *Proc. Natl. Acad. Sci. USA* 82 7419-7423, 1985.
8. M. ISHIDA, Y. MACHIDA, N. NAMBU and T. NAGAI, *Chem. Pharm. Bull.* 29 810-816, 1981.
9. W.A. RITSCHER, G.B. RITSCHER, H. FORUSZ and M. KRAELING, *Res. Commun. Chem. Pathol. Pharmacol.* 63 53-67, 1989.
10. B.J. AUNGST and N.J. ROGERS, *Int. J. Pharm.* 53 227-235, 1989.
11. K. ICHIKAWA, I. OHATA, M. MITOMI, S. KAWAMURA, H. MAENO, and H. KAWATA, *J. Pharm. Pharmacol.* 32 314-318, 1980.
12. H. BAR-ON, E.M. BERRY, A. ELDOR, M. KIDRON, D. LICHTENBERG and L. CALD WELL, *Brit. J. Pharmacol.* 73 21-24, 1981.
13. T. NISHIHATA, J.H. RYTTING, A. KAMADA, T. HIGUCHI, M. ROUTH AND L. CALD WELL, *J. Pharm. Pharmacol.* 35 148-151, 1983.
14. K. MORIMOTO, T. TAKEEDA, Y. NAKAMOTO and K. MORISAKA, *Int. J. Pharm.* 12 107-111, 1982.
15. H. OKADA, I. YAMAZAKI, T. YASHIKI and H. MIMA, *J. Pharm. Sci.* 72 75-78, 1983.
16. H. YOSHIDA, K. OKUMURA and R. HORI, *J. Pharm. Sci.* 68 670-671, 1979.
17. A. YAMAMOTO, A.M. LUO, S. DODDASHI and V.H.L. LEE, *J. Pharmacol. Exp. Ther.* 249 249-255, 1989.
18. G.C.Y. CHIOU and C.Y. CHUANG, *J. Pharm. Sci.* 78 815-818, 1989.
19. J.C. LIU, Y. SUN, O. SIDDQUI, Y.W. CHIEN, W-M. SHI and J. LI, *Int. J. Pharm.* 44 197-204, 1988.
20. V.H.L. LEE, in: "Delivery Systems for Peptide Drugs," S.S. DAVIS, L. ILLUM and E. TOMLINSON, eds., Plenum Press, New York, 1986.
21. B.J. AUNGST, N.J. ROGERS. and E. SHEFTER, *J. Pharmacol. Exp. Ther.* 244 23-27, 1988.
22. S. DODDASHI and V.H.L. LEE, *Life Sci.* 38 2019-2028, 1986.
23. R.E. STRATFORD and V.H.L. LEE, *Int. J. Pharm.* 30 73-82, 1986.
24. A.E. KITABCHI, F.B. STENTZ and M.A.B. MEMPHIS, *Diabetes* 21 1091-1101, 1972.
25. W.C. DUCKWORTH, F.B. STENTZ, M. HEINEMANN and A.E. KITABCHI, *Proc. Natl. Acad. Sci. USA* 76 635-639, 1979.
26. P.T. VARANDANI, L.A. SHROYER and M.A. NAFZ, *Proc. Natl. Acad. Sci. USA* 69 1681-1684, 1972.
27. M.L. CHANDLER and P.T. VARANDANI, *Biochim. Biophys. Acta* 286 136-145, 1972.
28. P.T. VARANDANI, *Biochim. Biophys. Acta* 295 630-636, 1973.
29. K. YOKONO, Y. IMAMURA, K. SHII, N. MIZUNO, H. SAKAI and S. BABA, *Diabetes*, 29 856-859, 1980.
30. I.D. GOLDFINE, J.A. WILLIAMS, A.C. BAILEY, K.Y. WONG, Y. IWAMOTO, K. YOKONO, S. BABA and R.A. ROTH, *Diabetes* 33 64-72, 1984.
31. K. YONEZAWA, K. YOKONO, S. YASO, J. HARI, K. AMANO, Y. KAWASE, T. SAKAMOTO, K. SHII, Y. IMAMURA and S. BABA, *Endocrinology*, 118 1989-1996, 1986.
32. E. HAYAKAWA, A. YAMAMOTO, Y. SHOJI and V.H.L. LEE, *Life Sci.* 45 167-174, 1989.
33. M. BRADFORD, *Anal. Biochem.* 72 248-254, 1976.
34. N. BEROVA, I. PETKOV, and V.C. ANDREEN, *Br. J. Dermatol.* 90 431-434, 1974.
35. B. GIVEN, R. COHEN, B. FRANK, A. RUBENSTEIN, and H. TAGER, *Clin. Res.* 33 569A, 1985.