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A comparison of aminopeptidases from excised human buccal epithelium and primary cultures of hamster pouch buccal epithelium

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Abstract—Aminopeptidase activity associated with human buccal tissue and primary cultures of hamster buccal epithelium homogenates was assayed fluorometrically using 4-methoxy-2-naphthylamides of leucine, alanine, and arginine. Kinetic parameters, K_m and V_{max}, for all substrates were estimated. Aminopeptidase parameters for human tissue were similar to those for the in-vitro system and reported literature values for rodent buccal tissue aminopeptidases. Aminopeptidases of both tissues were also found to be similarly sensitive to typical inhibitors, bestatin and puromycin. Overall results suggest that appropriate in-vitro systems derived from animal tissues may be useful in assessing the role and localization of peptidases associated with buccal tissue.

Because of the significant proteolytic activity encountered along the gastrointestinal tract following oral administration of peptides, recent attention has focused on systemic delivery of these agents by intranasal, ocular, rectal, transdermal and buccal routes (Banga & Chien 1988; Audus et al 1990). As an alternative, the application of peptides in certain dosage forms on buccal epithelium has been suggested for the systemic delivery of selected peptide hormones (Ishida et al 1981; Nagai & Machida 1985; Merkle et al 1990) but a major limitation to adequate bioavailability appears to be the absorption process (Gupta et al 1990). Another possible limiting factor in the buccal delivery of peptides, may be degradation due either to interactions with secreted peptidases in the oral cavity or to epithelial peptidases. The possible role of peptidases in buccal delivery has been suggested from rodent studies (Kashi & Lee 1986; Stratford & Lee 1986; Garren & Repta 1988; Tavakoli-Saberi & Audus 1989a; Garren et al 1989) and the enhancement of the permeation of aminopeptidase labile substrates across rodent buccal tissues in-vitro (Garren et al 1989).

In this study, we have examined aminopeptidases in excised human buccal tissue. The results obtained were compared with an in-vitro system comprised of primary cultures derived from buccal epithelium of a representative animal model (hamster) for studying the buccal route of administration (Tavakoli-Saberi & Audus 1989a, b).

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Materials and methods

Materials. Aminopeptidase substrates, 4-methoxy-2-naphthylamides, and inhibitors, bestatin and puromycin, were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest grade commercially available.

Buccal tissue preparation. Superficial human buccal tissues were surgically removed postmortem from tissue donors at the VA Medical Center, Kansas City, MO. Anonymous donors chosen were of any age, either sex, but were non-smokers without oral disease. Tissues from individuals were collected within about 4-5 h of death and transported to the laboratory within 1 h in ice-cold phosphate buffered saline, pH 7·4 (PBS). The tissue probably included some minor components of underlying tissues. Based on the observation of Garren et al (1989) that aminopeptidase activity resides predominantly in the epithelial cell layers, further dissection of the tissues was not made.

Homogenous populations of hamster buccal epithelial cells were isolated and grown in primary culture (Tavakoli-Saberi & Audus 1989a, b). Expression of aminopeptidases has been shown to be retained in such primary cultures when compared with freshly excised hamster tissue (Tavakoli-Saberi & Audus 1989a). The viability of the tissue cultures was 100% as estimated by trypan blue exclusion before collection and homogenization for aminopeptidase assays.

Homogenate preparations. Either excised human buccal tissue or primary cultures of hamster buccal epithelial cells grown in 100 mm culture dishes (Tavakoli-Saberi & Audus 1989a, b) were rinsed three times with phosphate buffered saline, pH 7·4 (PBS). Hamster epithelial cells grown in 100 mm dishes were scraped from the dishes and collected in 2-3 mL of PBS containing 0·32 M sucrose. Excised human buccal tissues were also placed in PBS containing 0·32 M sucrose and cut into small cubes (1 mm).

Suspended tissues were freeeze-thawed at −70°C, homogenized for 1 min, and then were centrifuged at 1000 g for 5 min. The resulting supernatant was used immediately for the aminopeptidase assays described below and protein determination by the method of Lowry et al (1951).

Aminopeptidase assays. The method of Stratford & Lee (1986) was used to assay aminopeptidases in the tissues. Briefly, 4-methoxy-2-naphthylamides (leucine, alanine, arginine) were added to a reaction mixture consisting of 150 μL tissue supernatant and 2·8 mL of 0·05 Tris-maleate buffer, pH 7·4, in duplicate or triplicate 3 mL quartz cuvets. Before addition of the substrate to initiate the reaction, the tissue-buffer suspension was preincubated at 37°C for 15 min. The reaction mixture was incubated at 37°C, the sample excited at 342 nm, and the emission at 426 nm observed for 10 min with an SLM-Aminco 4800 Spectrofluorometer (Urbana, IL). Blanks consisted of a reaction mixture prepared as above with tissue supernatant that had been boiled. Where enzyme inhibitors were used, either bestatin or puromycin 0·1 or 0·01 mM was added to tissue-buffer mixtures 15 min before a 4-methoxy-2-naphthylamide substrate.

Mean enzyme velocities in nmol mg^{-1} min^{-1} were calculated from standard curves for fluorescence intensity vs moles of β-naphthylamine and from plots of fluorescence intensity vs time from either three or four separate experiments (cell culture or excised tissue preparations). Michaelis-Menten kinetic parameters, K_m and V_{max}, were estimated with a nonlinear regression data analysis program, ENZFITTER (Elsevier-Biosoft, Cambridge, UK).

Results

Aminopeptidases associated with homogenate preparations of excised human and cultured hamster buccal epithelial cells hydrolysed the 4-methoxy-2-naphthylamide substrates in a manner consistent with Michaelis-Menten kinetics. Relationships between the release of β-naphthylamine and concentration of 4-methoxy-2-naphthylamide substrates were similar for both tissues and all substrates tested. Fig. 1 illustrates the typical relationship. Tables 1 and 2 summarize the apparent K_m and V_{max} parameters for each substrate and in each tissue. Comparisons between the results in Tables 1 and 2 indicated that apparent K_m and V_{max} parameters were relatively similar for both tissues.

The release of β-naphthylamine from the naphthylamide substrates by aminopeptidases was further investigated with a general aminopeptidase inhibitor, bestatin, and an arylamidase inhibitor, puromycin. As shown (Figs 2, 3) inhibition profiles of the aminopeptidases were similar in both types of tissue homogenates.

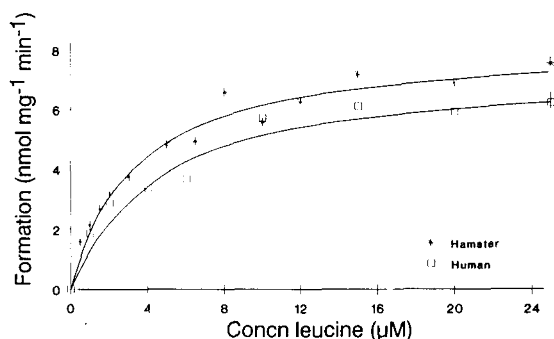

FIG. 1. Concentration-dependent hydrolysis of a representative 4-methoxy-2-naphthylamide substrate to naphthylamine (nmol mg^{-1} min^{-1}) in homogenates of human buccal tissue (open square) and primary cultures of hamster buccal epithelium (cross). Data points represents the means of triplicate experiments (tissues).

Table 1. Michaelis-Menten kinetic parameters for 4-methoxy-2-naphthylamide aminopeptidase substrates in homogenates of excised human buccal epithelium.

Substrate	Enzyme	K_m ± s.d.* (μM)	V_{max} ± s.d.* (nmol mg^{-1} min^{-1})
Leucine	Leucine aminopeptidase (EC 3.4.11.1)	3·5 ± 0·6	7·0 ± 0·3
Alanine	Aminopeptidase N (EC 3.4.11.2)	12·3 ± 5·0	30·4 ± 3·1
Arginine	Aminopeptidase B (EC 3.4.11.6)	3·5 ± 0·2	6·6 ± 0·3

* Data presented are the means of triplicate experiments (tissues) ± s.d.

Table 2. Michaelis-Menten kinetic parameters for 4-methoxy-2-naphthylamide aminopeptidase substrates in homogenates of primary cultures of hamster buccal epithelial cells.

Substrate	Enzyme	K_m ± s.d.* (μM)	V_{max} ± s.d.* (nmol mg^{-1} min^{-1})
Leucine	Leucine aminopeptidase (EC 3.4.11.1)	3·3 ± 0·4	8·2 ± 0·3
Alanine	Aminopeptidase N (EC 3.4.11.2)	10·3 ± 0·7	19·4 ± 0·6
Arginine	Aminopeptidase B (EC 3.4.11.6)	0·6 ± 0·1	11·3 ± 0·3

* Data presented are the means of triplicate experiments (tissues) ± s.d.

Significant loss of tissue viability could have resulted in loss of enzymatic activity. Since human buccal tissues were removed at varying times postmortem, the possibility of an underestimation of its aminopeptidase activity existed but was not resolved.

Discussion

The peptidase activity of buccal tissues has been described as significant relative to that of intestinal tissues (Kashi & Lee 1986; Stratford & Lee 1986; Garren & Repta 1988). Generally, those studies have been conducted with rodent tissues. A direct knowledge of human buccal tissue peptidase activity has been lacking. In the present study, excised human buccal tissue was surveyed for aminopeptidase activity and compared with primary cultures of hamster buccal epithelium, an alternative in-vitro system for studying buccal epithelial transport and metabolism characteristics (Tavakoli-Saberi & Audus 1989a, b). Aminopeptidases, which represent a principal class of enzymes for peptide and protein degradation (Turner et al 1987), were assayed in the tissues. The substrates used, derivatives of 4-methoxy-2-naphthylamides, are considered specific for aminopeptidases (Sylven & Bois 1962; Stratford & Lee 1986).

The aminopeptidase activity (V_{max}) associated with excised human buccal homogenates was found to be in good agreement with values in rat homogenates (Stratford & Lee 1986), excised hamster pouch homogenates (Garren & Repta 1988; Tavakoli-Saberi & Audus 1989a) and in the primary culture hamster buccal epithelial cell homogenates examined herein. Apparent affinities of the 4-methoxy-2-naphthylamide substrates for enzymes (K_m values) associated with the human buccal homogenates were comparable with values from hamster buccal homogenates. Apparent K_m values have not been previously reported for buccal epithelial cell aminopeptidases. These results suggest that aminopeptidase expression was similar in human and

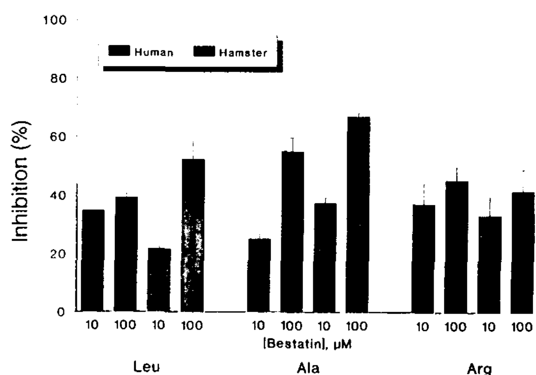

FIG. 2. Bestatin inhibition of aminopeptidases associated with homogenates of excised human buccal tissue or primary cultures of hamster buccal epithelial cells. A 100 μM final concentration of 4-methoxy-2-naphthylamide derivative of leucine (Leu), alanine (Ala), or arginine (Arg) was used to assay aminopeptidase activity in the presence of the indicated concentration of bestatin as described in Materials and methods. Data points represent the means of triplicate (tissues) ± s.d.

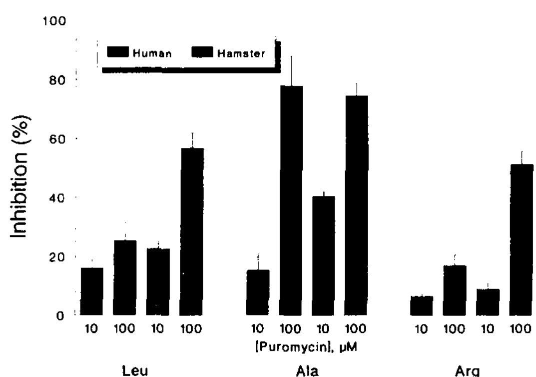

FIG. 3. Puromycin inhibition of aminopeptidases associated with homogenates of excised human buccal tissue and primary cultures of hamster buccal epithelial cells. A 100 μM final concentration of 4-methoxy-2-naphthylamide derivative of leucine (Leu), alanine (Ala), or arginine (Arg) was used to assay aminopeptidase activity in the presence of the indicated concentration of puromycin as described in Materials and methods. Data points represent the means of triplicate (tissues) ± s.d.

rodent buccal tissues but the results for human tissue may be affected by degradative processes occurring in the time between death and assay.

The hydrolysis of all three 4-methoxy-2-naphthylamide substrates by both homogenates was sensitive to puromycin and bestatin (Hui et al 1983); our results suggested that the enzyme activity observed was attributable to aminopeptidases, probably of more than one type. Bestatin, for example, strongly inhibits relatively pure leucine aminopeptidase, but puromycin does not (Stratford & Lee 1986). We found both inhibitors attenuated enzyme activity assayed with a leucine aminopeptidase substrate suggesting the possibility of contributions to hydrolysis of the substrate by other aminopeptidases. These observations were consistent with the conclusion of multiple aminopeptidase involvement in the hydrolysis of a given 4-methoxy-2-naphthylamide substrate in rabbit buccal tissue homogenates (Stratford & Lee 1986). However, literature results using the in-vitro model and rodent tissues reflected a pattern of substrate handling similar to that found by us with human buccal tissues.

The use of tissue homogenates facilitated comparison between this study and previous literature values. The role of aminopeptidases in degrading peptides exposed to intact human buccal tissue, thus, remains to be established. Garren et al (1989) observed that the rate of degradation of leucine aminopeptidase substrates exceeded the diffusion of the substances across intact hamster buccal tissue. This observation suggests that aminopeptidases assayed in hamster buccal epithelial cell homogenates may play a significant role in degrading enzyme labile peptides.

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