NEW SUBSTRATES OF TRYPSIN AND SOME TRYPSIN-LIKE ENZYMES

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SUMMARY

The results of the investigation of two new naphthyl substrates of trypsin and the trypsin-like enzyme(s) in human mast cells, carbobenzyloxy-L-arginine and benzoyl-L-arginine β-naphthylamide, have led directly to the synthesis and study of a further substrate, carbobenzyloxy-L-arginine methyl ester. The activity of trypsin against the carbobenzyloxy ester is greater than that previously described biochemically for other synthetic substrates. The inhibition of the amidase and esterase activity of trypsin and the tryptic enzyme in human mast cells by ϵ-aminocaproic acid derivatives is characterized and evidence is presented to show that this enzymic activity in human mast cells is more closely related to trypsin than to plasmin (fibrinolysin).

INTRODUCTION

Within the past several years a series of naphthyl amide and ester substrates have been introduced for the histochemical (5, 6, 9, 10, 12) and biochemical (1, 2, 17) estimation of the activity of trypsin and trypsin-like enzymes. Shalitin (18) and Shapiro and Doherty (19) demonstrated that ϵ-aminocaproic acid esters were substrates of trypsin and evidence has been presented (9) that ϵ-aminocaproic acid is a competitive inhibitor of tryptic hydrolysis. During a previous study of naphthyl substrates of trypsin (9), we noted that ϵ-aminocaproyl β-naphthylamide was a better competitive inhibitor than ϵ-aminocaproic acid of trypsin and the trypsin-like enzyme(s) in human mast cells. The purpose of this report is to introduce two further naphthyl derivatives, the N$^{\alpha}$-Cbz-L-arginine[2] and N$^{\alpha}$-benzoyl-L-arginine β-naphthylamides, as well as N$^{\alpha}$-Cbz-L-arginine methyl ester as substrates of trypsin and trypsin-like enzymes and to evaluate the relative effectiveness of two ϵ-aminocaproic acid derivatives, ethyl ϵ-aminocaproate and ϵ-aminocaproyl amide, as inhibitors of trypsin and the human mast cell enzyme(s).

MATERIALS AND METHODS

Reagents: N$^{\alpha}$ - benzoyl - L - arginine β - naphthylamide hydrochloride: To a solution of L-arginine β-naphthylamide dihydrochloride (7) in dimethylformamide was added a molar equivalent of triethylamine. The solution was cooled to 0°C and an equimolar amount of benzoyl chloride added slowly with stirring over a 30 minute period. The precipitated triethylamine hydrochloride was removed by filtration and the filtrate evaporated to an oil, washed with ether and cold distilled water and triturated with ethyl acetate. The product was crystallized from ethanol-ethyl acetate, m.p. 219–220. Calculated for $C_{23}H_{25}N_5O_2 \cdot HCl$: C, 62.8; H, 5.8; N, 15.9 Found: C, 62.7; H, 6.0; N, 16.0. $[\alpha]_D^{20}$ $-38.7 \pm 1.0°$ (c, 1.0 in pyridine). Reported for the D-enantiomorph, m.p. 219–220; $[\alpha]_D^{20}$ $+38.6 \pm 1.0°$ (c, 1.0 in pyridine) (10).

An alternative synthetic route also gave the desired product: N$^{\alpha}$-Cbz-N$^{\omega}$-nitro-L-arginine β-naphthylamide was formed from N$^{\alpha}$-Cbz-N$^{\omega}$-nitro-L-arginine and β-naphthylamine by the mixed anhydride method using ethyl chloroformate in dioxane. The product was crystallized from ethyl acetate, m.p. 173. Calculated for $C_{24}H_{26}N_6O_5$: N, 17.6. Found: N, 17.6. N$^{\alpha}$-benzoyl-N$^{\omega}$-nitro-L-arginine β-naphthylamide: the product was then treated with hydrogen bromide in glacial acetic acid and the resulting hydrobromide collected as an oil on the addition of ether and triturated with ethyl acetate. The resulting product was dissolved in dimethylformamide-ethyl acetate (2:1) to which two equivalents of tri-n-butylamine were added, cooled in an ice bath, and an excess of

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[2] Abbreviations: Cbz, carbobenzyloxy; L and DL-BANA, L and DL isomers of benzoyl-arginine β-naphthylamide HCl; L-CANA, Cbz-L-arginine β-naphthylamide HCl; CAME, Cbz-L-arginine methyl ester HCl; TAA, tosyl-L-arginine amide; TAME, tosyl-L-arginine methyl ester; L-TANA, tosyl-L-arginine β-naphthylamide HCl; ϵACA, ϵ-aminocaproic acid; ϵACA-AS, Naphthol AS ϵ-aminocaproate HBr.

benzoyl chloride slowly added with shaking. The solution was evaporated to dryness, the residue redissolved in hot ethyl acetate, washed with water, 1 *N* HCl and 1 *N* sodium bicarbonate, and the product crystalized from solution on reduction of volume, m.p. 254–256. Calculated for $C_{23}H_{24}N_6O_4$: N, 18.7. Found: N, 18.4. The final product was obtained by hydrogenation of the N^ω-nitro derivative as a suspension in glacial acetic acid containing an equivalent quantity of 1 *N* HCl. During hydrogenation the compound went into solution and was recovered on evaporation of the solvent *in vacuo*. Crystallized from ethanol-ethyl acetate, m.p. 218–220.

Ethyl ε-aminocaproate hydrochloride: The compound was prepared by the introduction of HCl gas into an ethanolic solution of ε-aminocaproic acid at 0°, evaporation of the solution to a small volume *in vacuo*, and crystallization on the addition of ether, m.p. less than 30°. Calculated for $C_8H_{17}NO_2 \cdot HCl$: C, 49.1; H, 9.3; N, 7.2. Found: C, 49.1; H, 9.2; N, 7.4.

ε-Aminocaproyl amide hydrobromide: Following the formation of a mixed anhydride of Cbz-ε-aminocaproic acid with ethyl chlorocarbonate in methylene chloride, the solution was gassed with ammonia and a crystalline precipitate which immediately formed was collected, m.p. 123–125. On treatment with hydrogen bromide in glacial acetic acid the final product was obtained as an oil on addition of ether and was crystallized from ethanol-ether, m.p. 142–143. Calculated for $C_6H_{14}N_2O \cdot HB4$: C, 34.1; H, 7.2; N, 13.3; Found: C, 34.0; H, 7.0; N, 13.6.

N^α-carbobenzyloxy-L-arginine methyl ester hydrochloride hemihydrate: To a 100 ml solution of 3.45 g of N^α-Cbz-L-arginine HCl in methanol cooled to −20°C, ten ml of methanol saturated with HCl gas was added and the solution maintained for two hours at −20°C and then evaporated to dryness *in vacuo*. The residue was dissolved in methanol and crystallized on the addition of ether. Recrystallized from methanol-ether, m.p. 62–63. The crystalline product was then dried at 50°C overnight *in vacuo* with the weight loss of 2.37%. Calculated for $C_{15}H_{22}O_4N_4 \cdot HCl$: C, 50.2; H, 6.5; N, 15.6. Found: C, 49.9; H, 6.7; N, 15.7 $[\alpha]_D^{20}$ −17.0 ± 1.0 (c, 1.0 in methanol).

N^α-Cbz-L-arginine β-naphthylamide hydrochloride and L-arginine β-naphthylamide dihydrochloride (7) were generously supplied by Cyclo Chemical Corp., Los Angeles, Calif.; N^α-tosyl-L-arginine β-naphthylamide hydrochloride was a gift of Dr. H. Nesvadba, Vienna; DL-BANA, εACA, TAME and TAA were obtained from Mann Research Lab., N. Y. The synthesis of Naphthol AS ε-aminocaproate hydrobromide has been previously reported (9).

TABLE I

Stock substrate solution	L-BANA concentration	Buffer solution	Stock enzyme solution
ml	*mM*	*ml*	*ml*
2.00	0.30	0.80	0.20
1.33	0.20	1.47	0.20
1.00	0.15	1.80	0.20
0.67	0.10	2.13	0.20

Biochemical Studies: The enzymes used were crystalline trypsin and chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.). For studies with the naphthyl substrates a trypsin stock solution was freshly prepared with a concentration of 0.45 mg/ml in 0.1 *mM* hydrochloric acid. Because of the low solubility of many of these substrates, for comparative purposes all of them were first dissolved in methanol (10 μmoles in 5 ml), and then diluted to ten ml with distilled water for studies on the relative substrate hydrolysis rates in which the following incubation medium was used:

Phosphate buffer, 0.1 *M*, pH 7.0....	1.8 ml
Stock substrate solution, 1 *mM*....	1.0 ml
Stock enzyme solution, 0.45 mg/ml..	0.2 ml

For the inhibitor studies using DL-BANA as substrate the stock enzyme solution was prepared at a concentration of 0.9 mg/ml in 0.1 *mM* hydrochloric acid and the stock substrate solution at a concentration of 0.9 *mM* (0.45 *mM* of each enantiomorph) in 0.1 *M* phosphate buffer, pH 7.0. Stock solutions of the inhibitors were made at concentrations of 20 *mM* in 0.1 phosphate buffer, pH 7.0. For the determination of the inhibition constants graded concentrations of the substrate solution was used and the buffer replaced by a suitable quantity of inhibitor stock solution (Table I). In the control tubes the enzyme was replaced by water. Incubation was performed at 37°C and the reaction followed at one min. intervals for 15 min. The reaction was then stopped by the addition of 1 ml of 1 *M* acetate buffer, pH 4.2 containing 0.2 mg of fast garnet GBC to form the azo dye and 10% Tween 20 to disperse the dye product. Standard curves were prepared from β-naphthylamine under identical conditions and the optical densities measured at 525 mμ using a Beckman DU spectrophotometer. The values of the kinetic constants were determined according to Lineweaver and Burk (13).

Comparative evaluation of the carbobenzyloxy and tosyl-L-arginine methyl esters as substrates of trypsin was performed using a Cary recording spectrophotometer. The incubating medium consisted of:

TAME or CAME, 0.1 *M* in water...	0.4 ml
Tris-HCl buffer, 0.08 *M*, pH 8.1, containing 0.01 *M* calcium chloride...	2.0 ml
Water...	1.6 ml

and to this solution 10 μl of a 1.0 mg/ml solution of trypsin was added at zero time. TAME hydrolysis rate was measured as the increase in absorbancy of the acid at 247 mμ using a 0.1 cm quartz cuvette (11). CAME hydrolysis rate was measured as the loss in absorbancy of the ester bond at 224 mμ using a 1.0 cm cuvette. Hydrolysis of the ε-aminocaproic acid derivatives by trypsin was evaluated by incubating 0.02 mmol of the amide or ester in 1 ml of 0.1 *M* phosphate buffer, pH 7.0 containing 1 mg of trypsin for 2 hours at 37°C, and detection of hydrolysis by paper chromatography of a 5 μl aliquot of the reaction mixture using 77% ethanol as the solvent phase and 0.2% ninhydrin in acetone as the color developer.

Antifibrinolytic activity: These assays were performed by Dr. Inga Marie Nilsson and co-workers, Malmö, Sweden. Anti-tissue activator assays were performed as described by Nilsson *et al.* (16) and anti-plasmin assays according to Sjoerdsma and Nilsson (20), ε-aminocaproic acid being used as the reference compound. The inhibitory effect of ε-aminocaproic acid and the ethyl ester and amide derivatives on plasminogen activation by urokinase and streptokinase was tested in a clot system at pH 7.4 at 37°C. Each tube contained the following components in the order of their addition: 0.1 ml plasminogen (0.1 mg/ml of 0.9% sodium chloride) plus 0.5 ml Tris buffer, pH 7.4 and ionic strength 0.15 containing a sufficient amount of the compound to be tested to give final concentrations shown in Table IV plus 0.2 ml of 0.5% bovine fibrinogen in Tris buffer plus 0.1 ml urokinase solution (60 Ploug units/ml or 0.1 ml streptokinase (50 units/ml)) plus 0.1 ml thrombin solution (20 N.I.H. units/ml of 0.9% sodium chloride). Lysis time was determined from the time urokinase or streptokinase was added.

Histochemical studies: Human skin obtained at necropsy was used as the test object and was frozen on dry ice and cut at 6 μ on a rotary microtome in a cryostat. Both DL-BANA (4 mg/ml) and ε-aminocaproic acid and its derivatives (0.1 mg/ml) were dissolved in distilled water, while, due to their difficult aqueous solubility, an equal quantity of the other naphthylamide substrates were first dissolved in either 1 ml of methanol or dimethylsulfoxide and water added to the final concentration. In view of the extreme insolubility of L-CANA and L-TANA, 1% bovine albumin was included in the buffer solution to prevent the precipitation of the substrate from solution. The ester substrate, εACA-AS, was made up as a stock solution (0.01 mg/ml) in 1 *mM* HCl. Fast garnet GBC was used as the diazonium salt component (10 mg/ml) freshly prepared in distilled water, and tetra-*n*-butylammonium iodide (0.02 *M*) was used as activator (9). The final incubating solution consisted of:

Phosphate buffer, 0.1 *M*, pH 7.0....	5.0 ml
Activator solution...	3.0 ml
Substrate solution...	1.0 ml
Fast garnet GBC solution...	1.0 ml

Incubation time varied from 5 min to 30 min. In studies on the effects of inhibitors, no activator was used, its volume being replaced by the inhibitor solution and/or distilled water. The inhibitor studies were carried out according to the principles previously presented (10) with the time for the initial appearance of visible mast cells in the microscopic field being recorded for a particular substrate and inhibitor concentration. Knowing that with smaller reaction velocities, longer times are necessary for the visualization of mast cells, the length of time needed was taken as an expression of the degree of inhibition.

RESULTS

Biochemical Studies: The results with the series of β-naphthylamide compounds as substrates of trypsin are recorded in Table II. The singular findings were the exceedingly rapid hydrolysis rate of the Cbz-L-arginine derivative (about 500%) and the rather slight increase in the hydrolysis

TABLE II

Rate of the Hydrolysis of the Present Substrates by Trypsin

Tested in 0.1 *M* phosphate buffer pH 7.0, enzyme concentration 30 μg/ml, substrate concentration 0.33 *mM*. Expressed in mμmoles of β-naphthylamine liberated/mg of enzyme/min.

Substrate	Reaction Rate
L-CANA	725
L-BANA	167
L-TANA	162
DL-BANA	124

rate (less than 35%) of the L-enantiomorph of the benzoyl arginine derivative as compared to an equimolar concentration of the BANA racemate.

The biochemical substrate CAME was found to be a more sensitive indicator of trypsin activity than TAME. The rates of hydrolysis (Vm) in μmoles/min/ml/mg enzyme were CAME, 580; TAME, 234 [lit. 288 under slightly different conditions (8)]. The K_m for CAME was found to be approximately 7.8×10^{-5} M, comparing closely with that reported for TAME, approximately 8.1×10^{-5} M (8).

TABLE III

Inhibition of the Tryptic Hydrolysis of Benzoyl-DL-arginine β-Naphthylamide by ε-Aminocaproic Acid, its Amide and Ethyl Ester

Phosphate buffer 0.1 M, pH 7.0. Inhibitor concentration 3.3 mM for εACA and εACA-NH_2 and 1.1 mM for εACA-EE.

Inhibitor	$K_i(M/l)$
εACA	14.4×10^{-3}
εACA-amide	8.9×10^{-3}
εACA-ethyl ester	1.7×10^{-4}

The findings with the ε-aminocaproic acid derivatives (Table III and Fig. 1) as inhibitors of tryptic hydrolysis of DL-BANA demonstrate that the amide is a better and the ethyl ester, a very markedly better inhibitor than ε-aminocaproic acid, itself. This is consistent with the initial observations that ε-aminocaproic acid alone and its β-naphthylamide derivative were inhibitors of tryptic hydrolysis (9) and the finding of a much more rapid hydrolysis rate of εACA-AS than the β-naphthylamide substrates for trypsin (9, 10). After a 2 hour incubation, hydrolysis by trypsin of the ethyl ester, but not of the amide derivative, of ε-aminocaproic acid was detected by paper chromatography (Fig. 2).

Fibrinolysin and activator studies: When ε-aminocaproic acid was compared to its ester and amide derivatives (Table IV), it was evident that, though the ester and amide were better inhibitors of urokinase activation of plasminogen at high concentration, they were no more effective at low

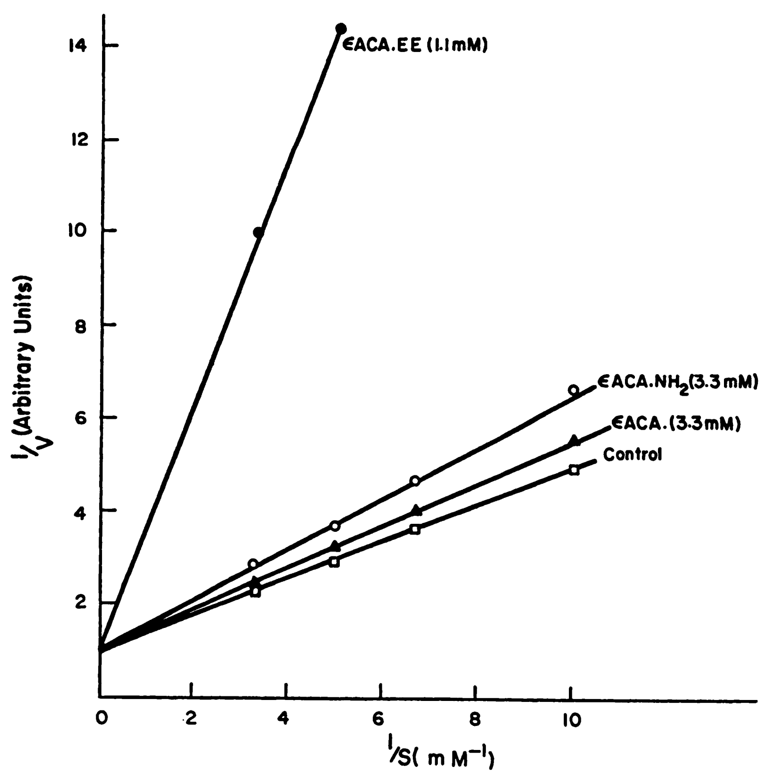

FIG. 1. Lineweaver-Burk plot demonstrating the inhibition of the tryptic hydrolysis of benzoyl-DL-arginine β-naphthylamide by ε-aminocaproic acid (εACA), its amide (εACA·NH_2) and ethyl ester (εACA·EE).

concentration than ε-aminocaproic acid. Further, they showed neither anti-plasmin nor anti-tissue activator activity.

Histochemical studies: Both L-CANA and L-BANA gave excellent localization of all human mast cells in 15 min., a rate of hydrolysis about equal to that of DL-BANA. TANA, however, gave a very slight but definite reaction only after a 30 min. incubation.

When the ε-aminocaproic acid derivatives were used as inhibitors of DL-BANA hydrolysis by the mast cell enzyme (Table V), the ester was more effective than the amide which in turn was more effective than the free acid. Only the ester could be shown to be effective as an inhibitor when εACA-AS was used as substrate. Neither tosyl-L-arginine amide nor the methyl ester was as effective as inhibitor as the corresponding ε-aminocaproic acid derivative.

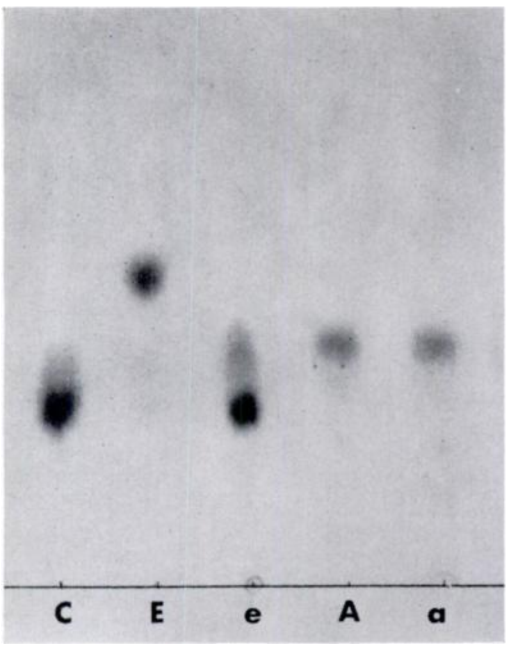

FIG. 2. Chromatographic evidence of the hydrolysis by trypsin of the methyl ester but not of the amide derivative of ε-aminocaproic acid during a two hour incubation. C, εACA control; E, methyl ester control; e, methyl ester incubation; A, amide control; a, amide incubation.

TABLE IV

Inhibitory Effect of εACA Derivatives on the Plasminogen Action by Urokinase

Inhibitor	Concentration and Lysis Times in Minutes				
	10^{-2} *M*	2×10^{-2} *M*	10^{-3} *M*	10^{-4} *M*	Tris buffer
εACA	77	57	24	20	18.5
εACA-amide	19	18	18	18	
εACA-ethyl ester	198	93	25	20	--

TABLE V

Inhibition of the Hydrolysis of DL-BANA and εACA-AS by the Human Mast Cell Enzyme

In terms of the time needed for the histochemical visualization of the colored product in the mast cells. Substrate concentration 0.11 *mM* of DL-BANA and 2.2 *μM* of εACA-AS. Concentration of Fast Garnet GBC, 1.0 mg/ml.

Inhibitor Concentration	Time
	min
DL-BANA as substrate:	
No inhibitor	6–7
εACA, 0.01 *M*	11–12
εACA-amide, 0.01 *M*	17–18
εACA-ethyl ester, 0.01 *M*	No cells
εACA, 0.005 *M*	9–10
εACA-amide, 0.005 *M*	10–11
TAA, 0.005 *M*	8–9
εACA, 0.001 *M*	6–7
εACA-amide, 0.001 *M*	7–8
εACA-ethyl ester, 0.001 *M*	No cells
TAME, 0.001 *M*	15–16
εACA-AS as substrate:	
No inhibitor	5–6
εACA, 0.01 *M*	5–6
εACA-amide, 0.01 *M*	5–6
εACA-ethyl ester, 0.01 *M*	18–19

DISCUSSION

Although the carbobenzyloxy group has been used to replace benzoyl and *p*-toluenesulfonyl (tosyl) groups as N^α substituents in substrates for chymotrypsin and carboxypeptidase, we are not aware of an attempt to utilize N^α-carbobenzyloxy derivatives of arginine or lysine amides and esters as substrates for trypsin. Our studies indicated that at the same substrate concentration the carbobenzyloxy group confers an increase over the benzoyl and tosyl N^α-acylated arginine β-naphthylamides in the hydrolysis rate by trypsin. As a consequence of these results, Cbz-L-arginine methyl ester was synthesized and the hydrolysis rates by trypsin compared to the corresponding tosyl compound, the most sensitive trypsin substrate so far described (8, 15). The Cbz derivative was found to be hydrolyzed at approximately twice the rate of the tosyl compound. These results confirm that the N^{α} substituent or R group plays a significant role in the attachment of the substrate to the active center of trypsin (15), the carbobenzyloxy substituent apparently more nearly simulating both sterically and electronically an aromatic amino-acid side-chain than the tosyl or benzoyl substituents.

The finding that benzoyl-L-arginine β-naphthylamide was only slightly more sensitive than its racemate as a substrate of trypsin and the trypsin-like activity in human mast cells may be predicated upon the rather high K_m of the racemate and the even higher K_i of benzoyl-D-arginine β-naphthylamide (9). It is unlikely that this result is due to activation of hydrolysis of the L-isomer by the D-enantiomorph in the racemate (22), since the D-isomer is not hydrolyzed by trypsin (9, 17), but acts as a competitive inhibitor of hydrolysis in the racemic mixture (9). The finding that hydrolysis by trypsin of ϵ-aminocaproyl amide could not be detected, yet under the experimental conditions used, it acted as a competitive inhibitor of tryptic activity, is consistent with results reported for the β-naphthylamide derivative (9). We are unaware of a report of any ester or amide compound having these characteristics in their effect on tryptic activity, though it is probable that other amides of this type, e.g., β-alanylglycine amide (9, 18), will be found to exhibit similar properties.

We have previously reported the hydrolysis in human mast cells of a series of trypsin substrates including the benzoyl-DL-arginine- and lysine β-naphthylamides, the Naphthol AS and AS-D ϵ-aminocaproates and Naphthol AS β-alanylglycinate (5, 6, 9, 10). The purpose of these studies was to define the relative substrate specificity and hydrolytic characteristics of this species-limited mast cell activity (5), with the object of utilizing this approach as a prototype for the evaluation of quantitative enzymic characteristics in a histochemical system (9, 10). The hydrolysis of tosyl-L-arginine β-naphthylamide in human mast cells had not previously been noted by us until the method of maintaining this difficultly soluble substrate in solution by the use of albumin and dimethylsulfoxide was initiated. The finding of hydrolysis of the tosyl derivative indicates that the human mast cell enzyme may be substrate-homospecific with trypsin, since all naphthyl substrates for trypsin (but not for other characterized hydrolytic enzymes) have been found to be hydrolyzed in this tissue site (9), though a distinct difference in the relative substrate hydrolysis rates by trypsin and the mast cell enzyme is noted.

The excellent inhibition of the human mast cell enzyme(s) obtained using ethyl ϵ-aminocaproate and the failure of this compound to inhibit plasmin or tissue plasminogen activator indicates that the trypsin-like enzyme in the human mast cell is not identical with plasmin (fibrinolysin) or tissue plasminogen activator (3, 4). The possibility exists that this enzyme in human mast cells may be more closely related to trypsin than plasmin in its relative substrate specificity (15, 21).

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