BBA 66358

INHIBITORY EFFECTS OF ω-AMINO ACID ESTERS ON TRYPSIN, PLASMIN, PLASMA KALLIKREIN AND THROMBIN

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(Received February 23rd, 1971)

SUMMARY

The inhibitory effects of various esters of ε-aminocaproic acid and *trans*-4-aminomethylcyclohexanecarboxylic acid on trypsin, plasmin, plasma kallikrein and thrombin were examined. Among the various inhibitors tested, phenyl and 4′-(2″-carboxy)ethylphenyl *trans*-4-aminomethylcyclohexanecarboxylates were the most effective inhibitors of trypsin and plasmin, and they strongly inhibited the esterolytic activities of plasma kallikrein and thrombin.

INTRODUCTION

If effective synthetic inhibitors of trypsin, plasmin, plasma kallikrein and thrombin could be found they would be very useful for enzyme research and medical use. MURAMATU *et al.*[1,2] reported that hexyl ε-aminocaproate extensively inhibits the fibrinolytic and caseinolytic activities of plasmin. Moreover, MURAMATU *et al.*[3–6] found that among the various ω-amino acid and ω-guanidino acid esters tested, hexyl ε-aminocaproate and hexyl δ-guanidinovalerate were the strongest competitive inhibitors of trypsin and plasmin, and also prevented inactivation of trypsin by DFP (refs. 5, 6). Hexyl δ-guanidinovalerate is a strong, competitive inhibitor of plasma kallikrein[5], while hexyl ε-aminocaproate has no inhibitory effect[6]. However, neither compound inhibits thrombin activity[5,6]. This paper reports that aromatic esters of ω-amino acid are stronger inhibitors of trypsin, plasmin, plasma kallikrein and thrombin than aliphatic esters. MARES-GUIA AND SHAW[7] reported that benzamidine strongly inhibits tryptic activity. Therefore, in the present report the inhibitory effects of these esters were compared with those of benzamidine.

Abbreviations: TAME, methyl N^{α}-tosyl-L-argininate; BAPA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; BAEE, ethyl N^{α}-benzoyl-L-argininate; ε-ACA-Hex, hexyl ε-aminocaproate; ε-ACA-Bzl, benzyl ε-aminocaproate hydrochloride; AMCHA-Hex, *trans*-4-aminomethylcyclohexane carboxylate; AMCHA-Bzl, AMCHA-Phe and AMCHA-CEP, benzyl, phenyl and 4′-(2″-carboxy)ethylphenyl *trans*-4-aminoethylcyclohexane carboxylate hydrochlorides; respectively.

MATERIALS AND METHODS

Bovine trypsin, twice crystallized, salt-free, Lot 118B-0880, was purchased from Sigma Chemical Co. Thrombin, for topical use, was purchased from Mochida Pharmaceutical Co., Tokyo. "Varidase" was purchased from American Cyanamide Co., Pearl River, N.Y., and was used as streptokinase (human plasminogen activator). Human plasminogen was purified as described by MURAMATU *et al.*[8], and converted to plasmin by addition of streptokinase. Human plasmin preparation obtained from 1 mg of plasminogen hydrolyzed 280 μmoles of methyl N^{α}-tosyl-L-argininate in 0.1 M Tris–HCl buffer, pH 8.5, in 30 min at 37°. Human plasma kallikrein was obtained as follows: the euglobulin fraction was treated with 0.03 M disodium EDTA, at pH 4.8, as described previously[8]. After stirring the mixture overnight, the insoluble protein was removed by centrifugation at 4000 rev./min for 15 min. The supernatant was treated with acetone by the method of WEBSTER AND PIERCE[9]. 1 mg of the plasma kallikrein obtained hydrolyzed 7 μmoles of methyl N^{α}-tosyl-L-argininate (TAME) in 0.1 M Tris–HCl buffer, pH 8.5, in 30 min at 37°.

The protein contents of human plasminogen and human kallikreinogen preparations were determined by the method of LOWRY *et al.*[10]. The amount of protein was calculated from a calibration curve obtained with bovine serum albumin (Armour Pharmaceutical Co.).

Methyl N^{α}-tosyl-L-argininate (TAME), N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA) and ethyl N^{α}-benzoyl-L-argininate (BAEE) were purchased from the Foundation for Promotion of Protein Research, Institute for Protein Research, Osaka University, Osaka. The preparations of hexyl ε-aminocaproate (ε-ACA-Hex) and *trans*-4-aminomethylcyclohexanecarboxylate (AMCHA-Hex) were as described previously[6]. Benzyl ε-aminocaproate hydrochloride (ε-ACA-Bzl) and benzyl, phenyl and 4′-(2″-carboxy)ethylphenyl *trans*-4-aminomethylcyclohexanecarboxylate hydrochlorides (AMCHA-Bzl, AMCHA-Ph and AMCHA-CEP, respectively) were prepared in the Research Laboratories, Daiichi Seiyaku Co., Tokyo*. Benzamidine hydrochloride was purchased from Tokyo Chemical Industry Co., Tokyo.

The rate of hydrolysis of TAME by trypsin, plasmin, plasma kallikrein and thrombin were determined as described previously[5,6] at a substrate concentration of 10 mM. The rates of hydrolysis of BAEE by these enzymes were measured at 253 nm (ref. 11) at 20° in 0.05 M Tris–HCl, pH 8.0, at various concentrations of the substrate, ranging from 0.1 to 0.8 mM, in the presence and absence of various inhibitors. The reaction mixtures (3.2 ml) contained 10 μg of trypsin, 40 μg of plasminogen which was activated with 2400 units of streptokinase, 1 mg of kallikrein fraction described above and 300 μg of thrombin, respectively. The K_i values were determined from the LINEWEAVER–BURK[13] plot of the results. BAPA-hydrolytic activity of trypsin was determined as described by ERLANGER *et al.*[12] at 410 nm. The K_i values were determined as described by DIXON[14], at two concentrations of BAPA, $3 \cdot 10^{-4}$ M and $2 \cdot 10^{-4}$ M. The reaction mixture (3 ml) contained 20 μg of trypsin. With trypsin, the buffer contained 0.01 M $CaCl_2$. The caseinolytic and fibrinogenolytic activities of trypsin and plasmin were determined as described previously[6,8]. Final concentrations of both casein and fibrinogen were 2%.

* Medicochemical studies on these compounds will be reported elsewhere by scientists in the Research Laboratories, Daiichi Seiyaku Co., Ltd.

RESULTS AND DISCUSSION

The effects of various compounds on the caseinolytic activities of trypsin and plasmin, and the fibrinogenolytic activity of plasmin were examined. The concentrations required for 50% inhibition of caseinolysis and fibrinogenolysis are shown in Tables I and II, respectively. Table III shows the K_i values of various inhibitors on the hydrolysis of BAEE by trypsin, plasmin, plasma kallikrein and thrombin.

TABLE I

CONCENTRATIONS OF INHIBITORS FOR 50% INHIBITIONS OF CASEINOLYSIS

Incubations were carried out in 0.1 M borate buffer, pH 7.4, at 37°. The experimental procedure is described in the text.

Inhibitor	*Inhibitor concn. for 50% inhibition (mM)*	
	Trypsin	*Plasmin*
ε-ACA-Bzl	0.23	0.3
ε-ACA-Hex*	5	1.5
AMCHA-Bzl	1.4	0.09
AMCHA-Phe	0.0125	0.005
AMCHA-CEP	0.008	0.003
AMCHA-Hex*	No inhibition	1.5

* Cited from a previous report[6].

TABLE II

CONCENTRATIONS OF INHIBITORS FOR 50% INHIBITION OF FIBRINOGENOLYSIS BY PLASMIN

Incubations were carried out in 0.1 M borate buffer, pH 7.4, at 37°. For details see text.

Inhibitor	*Inhibitor concn. for 50% inhibition (mM)*
AMCHA-Phe	0.009
AMCHA-CEP	0.005
AMCHA-Bzl	0.05
ε-ACA-Bzl	1.1
ε-ACA-Hex	2

TABLE III

K_i VALUES FOR TRYPSIN, PLASMIN, PLASMA KALLIKREIN AND THROMBIN

K_i values were estimated in 0.05 M Tris–HCl, pH 8.0, at 20°. With trypsin, the buffer contained 0.01 M $CaCl_2$. BAEE was used as substrate.

Inhibitor	*K_i (M)*			
	Trypsin	*Plasmin*	*Kallikrein*	*Thrombin*
ε-ACA-Hex	$5.87 \cdot 10^{-5}$	—	—	—
ε-ACA-Bzl	$1.99 \cdot 10^{-5}$	$2.5 \cdot 10^{-5}$	$2.2 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$
AMCHA-Bzl	$5.0 \cdot 10^{-5}$	$7.6 \cdot 10^{-6}$	$6.4 \cdot 10^{-4}$	$5.6 \cdot 10^{-4}$
AMCHA-Phe	$1.7 \cdot 10^{-6}$	$4.7 \cdot 10^{-7}$	$3.7 \cdot 10^{-5}$	$4.2 \cdot 10^{-5}$
AMCHA-CEP			$2 \cdot 10^{-5}$	$2.6 \cdot 10^{-5}$
Benzamidine	$4.65 \cdot 10^{-5}$		—	—

TABLE IV

CONCENTRATIONS OF INHIBITORS FOR 50% INHIBITION OF ESTEROLYSIS

Incubations were carried out in 0.15 M KCl containing 0.01 M $CaCl_2$, at 25°. TAME was used as substrate (10 mM).

Inhibitor	*Inhibitor concn. for 50% inhibition (mM)*			
	Trypsin	*Plasmin*	*Kallikrein*	*Thrombin*
ε-ACA-Hex*	10	2	No inhibition	No inhibition
ε-ACA-Bzl	1.1	0.7	>8	No inhibition
AMCHA-Hex*	No inhibition	4	No inhibition	No inhibition
AMCHA-Bzl	4.6	0.25	2.75	No inhibition
AMCHA-Phe	0.06	0.01	0.13	0.34
AMCHA-CEP	0.03	0.01	0.125	0.9
Benzamidine	10	2		

* Cited from a previous report[6].

Table IV shows the concentrations required for 50% inhibition on the hydrolysis of TAME by these four enzymes. The concentrations for 50% inhibition and K_i values for the hydrolysis of BAPA by trypsin are shown in Table V.

Previously it was reported[3,6] that among the various saturated aliphatic esters of ω-amino acid tested ε-ACA-Hex was the strongest inhibitor of trypsin. It was also suggested that this compound combines with the active site of trypsin from (1) the results of kinetic experiments, (2) the fact that it is slowly hydrolyzed by trypsin, and (3) the finding that it protected trypsin from inactivation by diisopropylphosphofluoridate. Although ε-ACA-Bzl was slowly hydrolyzed like ε-ACA-Hex (their K_m or

TABLE V

I_{50} AND K_i VALUES FOR TRYPTIC HYDROLYSIS OF BAPA

Incubations were carried out in 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2$, pH 8.2, at 25°. For details see text. In experiments to determine I_{50} values, the final substrate concentration was 1 mM.

Inhibitor	I_{50} *(M)*	K_i *(M)*
ε-ACA-Hex	$6.0 \cdot 10^{-5}$	$2.0 \cdot 10^{-5}$
AMCHA-Bzl	$3.5 \cdot 10^{-5}$	$9.0 \cdot 10^{-6}$
AMCHA-Phe	$1.8 \cdot 10^{-6}$	$8.0 \cdot 10^{-7}$
AMCHA-CEP	$1.7 \cdot 10^{-6}$	
Benzamidine	$5.8 \cdot 10^{-5}$	$1.6 \cdot 10^{-5}$

k_{cat} values were not determined), they could be used in inhibition experiments and their K_i values could be estimated. As shown in Tables I–V, ε-ACA-Hex inhibited trypsin and plasmin activities, the K_i values for hydrolysis of BAEE and BAPA by trypsin being $5.87 \cdot 10^{-5}$ M and $2.0 \cdot 10^{-5}$ M, respectively. The inhibitory effects of ε-ACA-Bzl on trypsin and plasmin activities were greater than those of ε-ACA-Hex, and the K_i value for hydrolysis of BAEE by trypsin was $1.99 \cdot 10^{-5}$ M. MARES-GUIA AND SHAW[7] reported that benzamidine is a potent synthetic inhibitor of trypsin. As shown in Tables III and IV, this compound strongly inhibited the hydrolysis of

TAME by trypsin and plasmin, and its K_i for the hydrolysis of BAEE by trypsin was $4.65 \cdot 10^{-5}$ M. Its K_i value with plasmin could not be estimated, because the Lineweaver–Burk plot for this compound was not linear. The K_i value of benzamidine on tryptic hydrolysis of BAPA was determined as $1.84 \cdot 10^{-5}$ M by MARES-GUIA AND SHAW[7], and in the present experiments as $1.6 \cdot 10^{-5}$ M (see Table V). These results indicate that the inhibitory effect of ε-ACA-Hex is similar to that of benzamidine, while that of ε-ACA-Bzl is greater.

As described above, ε-ACA-Hex inhibits trypsin and plasmin activities. However, it has no effect on the hydrolysis of TAME by kallikrein, and it enhances the hydrolysis by thrombin. In the presence of 10 mM ε-ACA-Hex, thrombin activity increased to 270% of the control activity[6]. Similar results were obtained with ε-ACA-Bzl, in which thrombin activity increased to 200% of the control activity in the presence of 10 mM. However, these stimulatory effects of ε-ACA-Hex and ε-ACA-Bzl seem to be specific for the hydrolysis of TAME, since these compounds had no effect on the clotting time of fibrinogen by thrombin, and ε-ACA-Bzl slightly inhibited hydrolysis of BAEE by kallikrein and thrombin. As shown in Tables III and IV, ε-ACA-Bzl inhibited trypsin and plasmin activities more than ε-ACA-Hex, and its K_i values for trypsin, plasmin, kallikrein and thrombin were $1.99 \cdot 10^{-5}$ M, $2.5 \cdot 10^{-5}$ M, $2.2 \cdot 10^{-3}$ M and $2.5 \cdot 10^{-3}$ M, respectively.

AMCHA-Hex strongly inhibits plasmin, but has no effect on trypsin and thrombin. However, as shown in Table I, AMCHA-Bzl inhibited the caseinolytic activity of trypsin and plasmin more strongly than AMCHA-Hex. Moreover, Tables III and IV show that AMCHA-Bzl inhibited the hydrolysis of TAME by trypsin, plasmin and kallikrein, and the hydrolysis of BAEE by trypsin, plasmin, kallikrein and thrombin. AMCHA-Hex at a concentration of 5 mM did not inhibit the hydrolysis of TAME by thrombin.

The strong inhibitory effects of AMCHA-Phe and AMCHA-CEP on trypsin, plasmin, kallikrein and thrombin are shown in Tables I–V. K_i values of AMCHA-Phe for the hydrolysis of BAEE by trypsin and plasmin, and for the hydrolysis of BAPA by trypsin were $1.7 \cdot 10^{-6}$ M, $4.7 \cdot 10^{-7}$ M and $8.0 \cdot 10^{-7}$ M, respectively (see Tables III and V). Lineweaver–Burk and Dixon plots of AMCHA-CEP for trypsin and plasmin activity were not linear so that the K_i values of this compound were not obtained. However, these values must be similar to those of AMCHA-Phe judging from the structural similarity of these compounds and their I_{50} values shown in Tables I, II, IV and V. MARES-GUIA AND SHAW[7] reported that benzamidine strongly inhibits the hydrolysis of BAPA by trypsin and its K_i was determined as $1.84 \cdot 10^{-5}$ M. In the present experiments, the K_i was estimated to be $1.6 \cdot 10^{-5}$ M for the same substrate. Its K_i with plasmin was not determined (Table III) because a Lineweaver–Burk plot for this compound was not linear. These results indicate that AMCHA-Phe and AMCHA-CEP are stronger inhibitors of trypsin and plasmin than benzamidine.

Different K_i values for trypsin inhibitors are observed when BAEE and BAPA are used as substrate, as shown in Tables III and V. However, the reason why these results were obtained is unknown.

ACKNOWLEDGMENT

We are greatly indebted to Miss Kazuko Okamura for her skillful technical assistance.

REFERENCES

1 M. MURAMATU, T. ONISHI, S. MAKINO, S. FUJII AND Y. YAMAMURA, *J. Biochem. Tokyo*, 57 (1965) 402.
2 M. MURAMATU, T. ONISHI, S. MAKINO, S. FUJII AND Y. YAMAMURA, *J. Biochem. Tokyo*, 57 (1965) 450.
3 M. MARAMATU, T. ONISHI, S. MAKINO, Y. HAYAKUMO AND S. FUJII, *J. Biochem. Tokyo*, 58 (1965) 214.
4 M. MURAMATU, Y. HAYAKUMO AND S. FUJII, *J. Biochem. Tokyo*, 62 (1967) 408.
5 M. MURAMATU AND S. FUJII, *J. Biochem. Tokyo*, 64 (1968) 807.
6 M. MURAMATU AND S. FUJII, *J. Biochem. Tokyo*, 65 (1969) 17.
7 M. MARES-GUIA AND E. SHAW, *J. Biol. Chem.*, 240 (1965) 1597.
8 M. MURAMATU, Y. HAYAKUMO, T. ONISHI, T. SATO AND S. FUJII, *J. Biochem. Tokyo*, 65 (1969) 329.
9 M. E. WEBSTER AND J. V. PIERCE, *J. Pharmacol. Exptl. Therap.*, 130 (1960) 484.
10 O. H. LOWRY, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 G. W. SCHWERT AND Y. TAKENAKA, *Biochim. Biophys. Acta*, 16 (1955) 570.
12 B. F. ERLANGER, N. KOSOWSKY AND W. COHEN, *Arch. Biochem. Biophys.*, 95 (1961) 271.
13 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
14 M. DIXON, *Biochem. J.*, 55 (1953) 170.