BBA 65762

THE PLASMIN INHIBITION BY SYNTHETIC ANTIFIBRINOLYTIC AGENTS IN RELATION TO THE TYPE OF SUBSTRATE

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(Received January 15th, 1968)
(Revised manuscript received March 25th, 1968)

SUMMARY

The inhibitory effect of synthetic antifibrinolytic agents: ε-aminocaproic acid, *p*-aminomethyl benzoic acid and the active isomer of 1,4-aminomethyl cyclohexane carboxylic acid on plasmin (EC 3.4.4.14), acting on various substrates, was studied. All the inhibitors had a very weak effect, if any at all, on casein and fibrinogen proteolysis by plasmin. On the other hand, they effectively inhibited fibrin clot lysis by plasmin. This effect was even greater when stabilized fibrin was used as a substrate for plasmin action, as measured by clot-lysis time.

It is postulated that the mechanism of the inhibitory effect of synthetic antifibrinolytic agents consists in the formation of ineffective acceptors for plasmin binding sites on the peptide chains of the fibrin molecule.

INTRODUCTION

ε-Aminocaproic acid (EACA), the active isomer of 1,4-aminomethyl cyclohexane carboxylic acid (AMCA) and *p*-aminomethyl benzoic acid (PAMBA) are potent antifibrinolytic agents. Though many works have been published on the subject, the mechanism of action of these compounds is not clear. OKAMOTO[1] stated that they act as antiplasmins. According to ABLONDI *et al.*[2] and ALKJAERSIG, FLETCHER AND SHERRY[3], EACA acts as an inhibitor of the plasminogen–plasmin conversion. A similar mechanism is also indicated with respect to AMCA and PAMBA[4–6]. More recently, evidence has been presented that EACA may be involved in the alteration of the fibrin structure[7–9] and in the modification of the action of plasmin on fibrinogen[10,11].

It has recently been found that EACA inhibits the activation of bovine plasminogen into plasmin only at high concentrations ($5 \cdot 10^{-2}$ M), but it does not affect the activation of human plasminogen[12]. EACA inhibits fibrinolysis at considerably lower concentrations (10^{-4} M). Moreover, it has been demonstrated that the antifibrinolytic effect of EACA is more pronounced if a stabilized clot is used as a substrate for plasmin.

Abbreviations: EACA, ε-aminocaproic acid; PAMBA, *p*-aminomethyl benzoic acid; AMCA, active isomer of 1,4-aminomethyl cyclohexane carboxylic acid; FSF, fibrin stabilizing factor.

The purpose of this work was to compare the inhibitory effects of EACA, AMCA and PAMBA on plasmin acting on the following substrates: fibrinogen, non-stabilized fibrin, stabilized fibrin and casein. Visual fibrinolytic and proteolytic methods were both used for studying the effects of plasmin.

MATERIALS AND METHODS

The materials used were: EACA, "Ziołolek", Poznań; PAMBA, VEB Dresden, East Germany; AMCA, "Amikapron", manufactured by Kabi, Stockholm; plasmin (EC 3.4.4.14): pig plasmin–lysofibrin, Novo Industri A/S, Copenhagen[13]. Bovine, plasminogen-freefibrinogen was purified by the method of KEKWICK *et al.*[14]. Thrombin, Lubelska Wytwórnia Surowic i Szczepionek, Lublin, Poland (the preparation of thrombin in the concentration used did not show any significant fibrinolytic or antifibrinolytic activity). Fibrin-stabilizing factor (FSF; factor XIII) was prepared according to the method of LOEWY *et al.*[15] as modified by LORAND AND KONISHI[16]. The purified FSF preparation contained 0.8% protein and 270 threshold units per ml. The preparation was free of any proteolytic and antiplasmin activity as tested using casein as a substrate. Casein was obtained according to the JACOBSEN[17] method. Cysteine, 0.1 M (cysteine·HCl was adjusted to pH 7.4 with 0.1 M NaOH). Buffer Tris–HCl, pH 7.4 (0.05 M Tris in 0.1 M NaCl). All experiments were performed at 37° and at pH 7.4. The antifibrinolytic agents were always added to the system prior to clot formation.

Fibrinolysis time was measured using the test-tube method. A complete disappearance of the clot was taken to be the end point of fibrinolysis. Clots were considered stabilized when insoluble in 1% monochloroacetic acid during 24 h. Non-stabilized clots usually dissolved within 15 min. Fibrinolysis was measured by adding concentrated thrombin (40 units/ml) to the digested fibrinogen. The absence of the clot in the test tube was taken to be the end point of fibrinogenolysis.

Proteolysis was determined by measuring trichloroacetic acid-soluble tyrosine using the Folin–Ciocalteau reagent.

RESULTS

Fig. 1 shows that EACA does not inhibit proteolysis of casein by plasmin over a wide range of concentrations (10^{-1}–10^{-5} M). A slight enhancement of proteolysis is seen at a concentration of 10^{-2}–10^{-5} M. Proteolysis of fibrinogen and fibrin is slightly inhibited at an EACA concentration of 10^{-1}–10^{-2} M.

Figs. 2 and 3 present the effects of AMCA and PAMBA on the proteolytic activity of plasmin. Casein proteolysis is inhibited by high concentrations of inhibitors (10^{-1} M). Fibrinogen and fibrin proteolysis is inhibited by PAMBA and AMCA at concentrations of 10^{-1}–10^{-4} M.

In further experiments, fibrinogenolysis and fibrinolysis of stabilized and non-stabilized clots were compared (Figs. 4, 5 and 6). Relatively high concentrations of all compounds (10^{-1}–10^{-2} M EACA; 10^{-1}–10^{-3} M PAMBA; 10^{-1}–10^{-4} M AMCA) were necessary to inhibit fibrinogenolysis. Fibrinolysis of non-stabilized clots was inhibited in lower concentrations of these substances (10^{-3} M EACA; 10^{-6}–10^{-7} M AMCA and PAMBA).

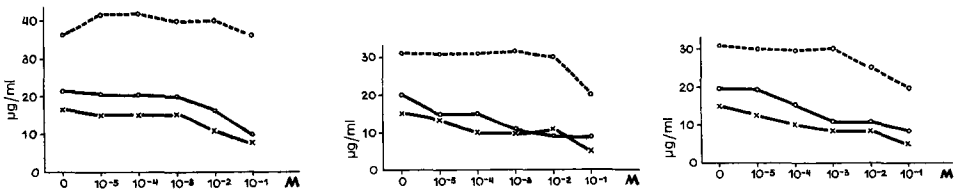

Fig. 1. Effect of EACA on the proteolytic activity of plasmin tested on casein (○— — —○), fibrinogen (○—○) and fibrin (×—×). Experiments were performed in the following system: 0.5 ml plasmin (125 µg/ml) + 0.5 ml EACA + 1 ml of 1% casein (or 1 ml of 1% fibrinogen or 1 ml of 1% fibrinogen + 0.1 ml thrombin). 5 ml 10% trichloroacetic acid were added after a 60-min incubation. Abscissa: EACA concentration. Ordinate: increase in trichloroacetic acid-soluble tyrosine (µg/ml).

Fig. 2. Effect of PAMBA on the proteolytic activity of plasmin tested on casein (○— — —○), fibrinogen (○—○) and fibrin (×—×). The system is similar to that described in the legend of Fig. 1. Abscissa: PAMBA concentration. Ordinate: increase in trichloroacetic acid-soluble tyrosine, (µg/ml).

Fig. 3. Effect of AMCA on the proteolytic activity of plasmin tested on casein (○ — — —○), fibrinogen (○—○) and fibrin (×—×). The system is similar to that of the experiment presented in Fig. 1. Abscissa: AMCA concentration. Ordinate: increase in trichloroacetic acid-soluble tyrosine (µg/ml).

On the other hand, very low concentrations of EACA (10^{-5} M) AMCA and PAMBA (10^{-7} M) were needed to produce pronounced inhibition of plasmin acting on stabilized clots. In all the concentrations of EACA, AMCA and PAMBA tested, the most effective inhibition was that of fibrinolysis of stabilized clots. Inhibition of non-stabilized clots was less effective whereas fibrinogenolysis was only partially inhibited even at high concentrations of these compounds.

It should be noted that the inhibitory effect of all compounds on fibrinolysis

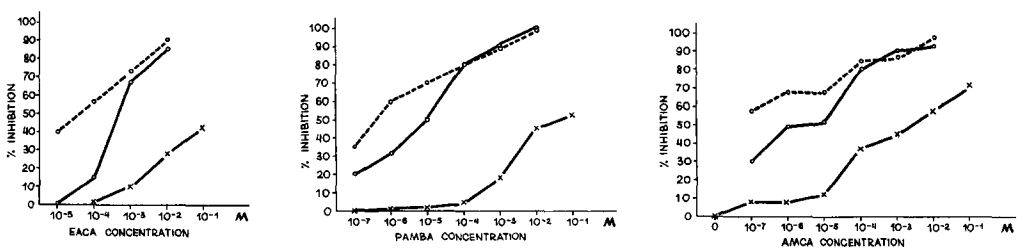

Fig. 4. Effect of EACA on the plasmin activity tested on fibrinogen (×—×), on non-stabilized (○—○) and on stabilized fibrin (○- - -○). Fibrinolysis and fibrinogenolysis time was evaluated on the basis of visual observation. The following experimental system was used to study the lysis of stabilized fibrin: 0.4 ml of 0.2% fibrinogen + 0.1 ml of 0.1 M cysteine + 0.1 ml of 0.25 M $CaCl_2$ + 0.1 ml of FSF + 0.1 ml of EACA + 0.1 ml of plasmin + 0.1 ml of thrombin (20 µ/ml). In the non-stabilized system, 0.1 ml of Tris buffer was added instead of FSF. In the experiments with fibrinogenolysis, Tris buffer was added instead of cysteine, calcium, FSF and thrombin solution. 0.2 ml of incubation mixture was added to 0.2 ml of thrombin every 2 min.

Fig. 5. Effect of PAMBA on the plasmin activity tested on fibrinogen (×—×), on non-stabilized (○—○) and on stabilized fibrin (○- - -○). For further details, see Fig. 4.

Fig. 6. Effect of AMCA on the plasmin activity tested on fibrinogen (×—×), on non-stabilized (○—○) and on stabilized fibrin (○ - - - ○). For further details, see Fig. 4.

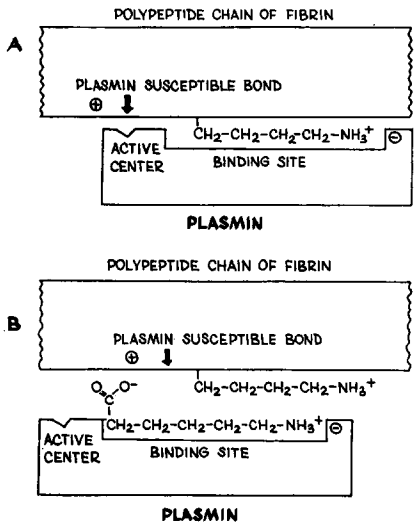

Fig. 7. The postulated mechanism of the antifibrinolytic action of EACA. A. Proteolysis of fibrin by plasmin. B. Inhibition of the plasmin proteolysis of fibrin by EACA.

was more pronounced when investigated by visual observation of clot lysis then by determination of trichloroacetic acid-soluble tyrosine.

Control experiments demonstrated that the inhibitory effect of EACA on plasmin fibrinolysis is not influenced by calcium ions and that inactive FSF does not inhibit fibrinolysis.

DISCUSSION

The results of the present work indicate that the inhibitory effect of EACA, AMCA and PAMBA on plasmin depends significantly on the nature of the substrate. These substances are not specific plasmin inhibitors in terms of classical enzymology. Their inhibitory effect is rather weak with regard to casein or fibrinogen proteolysis by plasmin. On the other hand, their effect seems to be more specific when fibrin clots are used as plasmin substrates. Therefore, it is concluded that the inhibitors studied do not block active centers of plasmin.

It is known that stabilization of fibrin clots make them more resistant to the plasmin action[12,18,19]. This can be explained by the appearance of new covalent bonds between ε-amino groups and γ-glutamyl residues of adjacent fibrin monomers[20,21] which may protect some of the peptide bonds susceptible to the plasmin action. Similar mechanisms may be responsible for the synergistic effect of fibrin stabilization and synthetic antifibrinolytic agents. The synergistic effect is particularly pronounced when EACA is used as an inhibitor. This may be due to the fact that ε-aminocaproic acid and lysine side chains are identical. The inhibition of fibrin clot lysis by synthetic antifibrinolytic agents can be explained as follows. It is postulated that the lysine side chains of fibrin represent groups which react with binding sites of plasmin. EACA and related compounds may form, with the polypeptide chain of the fibrin monomer,

potential acceptor sites for the binding of plasmin, possibly through the ionic bonding between their carboxyl groups and positive charges of the fibrin peptide moiety. In such a modified substrate, proteolysis would be impaired because of a shift of the active center of plasmin with respect to the susceptible peptide bond of a fibrin chain. Such a view is compatible with the observation of MAXWELL AND ALLEN[9] that EACA alters the structure of fibrin in the presence of plasmin. This hypothesis is also supported by the fact that the presence of amino and carboxyl groups and the characteristic distance between them in all synthetic antifibrinolytic agents is essential for their activities[6]. The postulated mechanism of the antifibrinolytic action of EACA is presented in Fig. 7.

The antifibrinolytic effect of EACA, AMCA and PAMBA may also be explained in terms of KOSHLAND'S[22] concept of the flexible active sites of the enzyme, according to which the proper alignment of catalytic groups depends on the substrate structure and dimensions.

No explanation can be offered, however, as to why synthetic antifibrinolytic agents are more potent inhibitors of fibrinolysis than fibrinogenolysis. It is possible that this may be due to the difference in the tertiary structure between fibrin and fibrinogen.

It has been reported that there are only a few specific peptide bonds split by plasmin, which are responsible for the maintenance of the coagulability of fibrinogen[23]. At this stage of digestion, very little trichloroacetic acid-soluble tyrosine is released. Since EACA, AMCA and PAMBA do not inhibit plasmin proteolytic action, it may be concluded that they effectively protect these particular peptide bonds at the early stage of proteolysis of fibrinogen and fibrin by plasmin.

Recently AMBRUS *et al.*[24] found that EACA and AMCA inhibit the fibrinolytic effect of plasmin but not its caseinolytic activity. These authors advanced the hypothesis that EACA and AMCA interact with fibrin, protecting it against proteolysis.

ACKNOWLEDGMENT

This work was aided in part by the grant from the Biochemical and Biophysical Committee, Polish Academy of Sciences.

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