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The blockage of the high-affinity lysine binding sites of plasminogen by EACA significantly inhibits prourokinase-induced plasminogen activation

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Abstract

Prourokinase-induced plasminogen activation is complex and involves three distinct reactions: (1) plasminogen activation by the intrinsic activity of prourokinase; (2) prourokinase activation by plasmin; (3) plasminogen activation by urokinase. To further understand some of the mechanisms involved, the effects of epsilon-aminocaproic acid (EACA), a lysine analogue, on these reactions were studied. At a low range of concentrations (10–50 μM), EACA significantly inhibited prourokinase-induced (Glu-/Lys-) plasminogen activation, prourokinase activation by Lys-plasmin, and (Glu-/Lys-) plasminogen activation by urokinase. However, no inhibition of plasminogen activation by Ala^{158}-prourokinase (a plasmin-resistant mutant) occurred. Therefore, the overall inhibition of EACA on prourokinase-induced plasminogen activation was mainly due to inhibition of reactions 2 and 3, by blocking the high-affinity lysine binding interaction between plasmin and prourokinase, as well as between plasminogen and urokinase. These findings were consistent with kinetic studies which suggested that binding of kringle 1–4 of plasmin to the N-terminal region of prourokinase significantly promotes prourokinase activation, and that binding of kringle 1–4 of plasminogen to the C-terminal $lysine^{158}$ of urokinase significantly promotes plasminogen activation. In conclusion, EACA was found to inhibit, rather than promote, prourokinase-induced plasminogen activation due to its blocking of the high-affinity lysine binding sites on plasmin(ogen).

Keywords: Plasminogen activation; Prourokinase activation; Lysine binding site; ε-Aminocaproic acid

1. Introduction

The zymogen plasminogen is a multi-domain glycoprotein consisting of an amino terminal peptide, five homologous kringle domains (kringle 1–5) and a trypsin like serine protease domain [1,2]. Plasminogen is activated to plasmin by specific cleavage of the Arg^{561}–Val^{562} peptide bond by plasminogen activators [3] including tissue plasminogen activator

Abbreviations: UK, urokinase or two-chain urokinase-type plasminogen activator; pro-UK, prourokinase or single-chain urokinase-type plasminogen activator; t-PA, tissue plasminogen activator; EACA, ε-aminocaproic acid; DFP, diisopropyl fluorophosphate; OD, optical density; CPB, carboxypeptidase B; A, absorbance

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(t-PA) [4–6], urokinase (UK) [7–9] and prourokinase (pro-UK) [10,11]. Whereas the enzymology of t-PA- or UK-induced plasminogen activation is well established, that of pro-UK is complex and less well understood.

Pro-UK is a single chain zymogen plasminogen activator which has an intrinsic activity against plasminogen and is itself activated to UK by plasmin by cleavage of the Lys^{158}–Ile^{159} peptide bond [12]. This feedback activation means that plasmin generation by pro-UK is the integrated consequence of three distinct reactions: (1) activation of plasminogen to plasmin by the intrinsic activity of prourokinase; (2) activation of prourokinase to urokinase by plasmin; (3) activation of plasminogen to plasmin by urokinase. The molecular interactions among these proteins, particularly the non-catalytic interactions, are not well understood. Therefore, in the present study, these reactions were investigated using mini-plasmin(ogen), a low-molecular-weight mutant of pro-UK/UK, a plasmin resistant mutant of pro-UK (Ala^{158}-pro-UK) and EACA to evaluate the roles of these interactions in pro-UK-induced plasminogen activation.

There are two classes of EACA binding sites on Glu-plasminogen, the native form of the molecule, a single strong lysine binding site with a dissociation constant (K_d) of 9 μM and five weaker sites with a K_d of 5 mM [13]. On Lys-plasminogen, probably also on Lys-plasmin, there is one strong EACA binding site of K_d 18–35 μM, a slightly weaker site of K_d 260 μM, and four much weaker sites of average K_d 10 mM [13,14]. Saturating the weak binding sites of Glu-plasminogen by EACA induces a conformation transition to an open or Lys-plasminogen-like shape [15–19]. This results in an acceleration of Glu-plasminogen activation by t-PA and UK. Occupation by EACA of the strong binding site on Glu-plasminogen does not have this effect [16–18]. For this reason, the function of the strong lysine-binding site on Glu-plasminogen is readily overlooked.

It was previously reported that in the presence of Cl^-, pro-UK-induced Glu-plasminogen activation was significantly promoted by EACA [20]. However, in that study, only one high EACA concentration (2.0 mM) was tested. In the present study, a range of concentrations of EACA (0–10 mM) in pro-UK-induced plasminogen activation was studied to evaluate the roles of both the higher and lower affinity binding sites. We found that the net effect of EACA on pro-UK-induced Glu-plasminogen activation was inhibition rather than promotion. When the mechanism of this inhibition was studied, it was found to be due mainly to the effect of EACA on pro-UK activation by plasmin and on plasminogen activation by UK. In addition, the binding of plasmin kringle 1–4 to the N-terminal region (Ser^1–Lys^{135}) of pro-UK, containing an EGF and kringle domain, significantly promoted pro-UK activation by plasmin, and the binding of plasminogen kringle 1–4 to the C-terminal lysine of UK promoted plasminogen activation by UK. These binding iterations were also prevented by low concentrations of EACA and presumably mediated by the high-affinity lysine binding site located in kringle 1–4 of plasmin and plasminogen.

2. Materials and methods

Recombinant pro-UK and a low-molecular-mass mutant, Δ125-pro-UK missing the EGF and kringle domains, were obtained from Farmitalia Carlo Erba (Milan, Italy). Two-chain UK and Δ125-UK were made by plasmin treatment of their single chain precursors as described previously [21]. A plasmin-resistant mutant of recombinant pro-UK constructed by site-directed mutagenesis with a substitution of Lys^{158} to Ala^{158} (Ala^{158}-pro-UK), was a gift from Collaborative Research Inc. (Bedford, MA). Human Glu-plasminogen and Lys-plasminogen were purified from diisopropyl fluorophosphate (DFP) treated bank plasma essentially by the method of Castellino and Powell [16]. Mini-plasminogen and kringle 1–3 fragment of plasminogen were obtained by digesting plasminogen with a catalytic amount of porcine pancreatic elastase and purified as described previously [22]. Mini-plasminogen and kringle 1–3 fragment were treated with DFP before use. Lys-plasmin and mini-plasmin were produced from plasminogen and mini-plasminogen respectively by using immobilized UK. Chromogenic substrates, S2444 and S2251, were obtained from Kabi (Franklin, OH). Immobilized carboxypeptidase B (CPB) was purchased from Calbiochem (San Diego, CA). EACA was obtained from Sigma (St. Louis, MO).

2.1. Effects of EACA on pro-UK, Δ125-pro-UK and Ala^{158}-pro-UK-induced plasminogen activation

In the absence or presence of EACA, the time-absorbance curves of pro-UK, Δ125-pro-UK and Ala^{158}-pro-UK-induced plasminogen activation were determined by measuring the optical density (OD) increase in the reaction mixture with time at 405 nm on a microtiter plate reader as previously described [10]. The reaction mixture contained S2251 (1.5 mM), EACA (0–10 mM), plasminogen (1.0 μM for Glu-plasminogen, 0.2 μM for Lys-plasminogen and mini-plasminogen), and pro-UK (0.5 nM), Δ125-pro-UK (2.0 nM) or Ala^{158}-pro-UK (10 nM) in the assay buffer (50 mM Tris–HCl, 0.1 M NaCl, 0.1% BSA and 0.01% Tween 80, pH 7.4) at room temperature.

2.2. Effects of EACA on amidolytic activities of Lys-plasmin, mini-plasmin, UK, and Δ125-UK

To evaluate a direct inhibition by EACA on the catalytic activity of plasmin, the amidolytic activities of Lys-plasmin (5.0 nM) and mini-plasmin (5.0 nM) against S2251 (1.2 mM) were assayed in the presence and absence of EACA (0–50 mM).

The effect of EACA on the amidolytic activities of UK and Δ125-UK were also studied. Briefly, a range of concentrations of S2444 (0.18, 0.3, 0.6, 1.2, 1.8, 2.4 mM) was incubated with UK or Δ125-UK (4.0 nM) in the absence or presence of EACA (10, 25, 50, 100 mM) in assay buffer at room temperature. The reaction rate was measured by the linear OD increase with time at 405 nm on a microtiter plate reader. The Michaelis constant (K_m), catalytic rate constant (k_{cat}) and inhibition constant (K_i) were determined with a computerized program (Enzfitter; Elsevier Biosoft, Cambridge, UK).

2.3. Effects of EACA and kringle 1–3 fragment on plasminogen activation by UK, Δ125-UK, CPB-pretreated UK and Δ125-UK

Glu-plasminogen, Lys-Plasminogen or mini-plasminogen (0.2 μM) was incubated with UK, Δ125-UK, CPB-pretreated UK or CPB-pretreated Δ125-UK (0.1 nM) and S2251 (1.2 mM) in the presence of EACA (0–10 mM) or kringle 1–3 fragment (0–8.0 μM). The OD increase in the reaction mixture with time at 405 nm was measured with a microtiter plate reader.

2.4. Effects of EACA and kringle 1–3 fragment on pro-UK and Δ125-pro-UK activation by Lys-plasmin and mini-plasmin

Pro-UK or Δ125-pro-UK (0.2 μM) was incubated with Lys-plasmin or mini-plasmin (0.1 nM) and S2444 (1.2 mM) in the presence of EACA (0–10 mM) or kringle 1–3 fragment (0–2.0 μM). The OD increase in the reaction mixture with time at 405 nm was measured with a microtiter plate reader.

2.5. Kinetic studies of plasminogen (Glu-, Lys- and mini-) activation by UK, Δ125-UK and CPB-pretreated UK in the absence or presence of EACA

In the absence of EACA, a range of concentrations of (Glu-, Lys- and mini-) plasminogen (1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 μM) was incubated with UK, Δ125-UK or CPB-pretreated UK (0.05 nM) and S2251 (1.2 mM) in the assay buffer at room temperature. Plasminogen activation rate was calculated by the OD increase with the time square (t^2) at 405 nm [10]. The Michaelis constant (K_m) and catalytic rate constant (k_{cat}) were determined from Lineweaver–Burk plots with Enzfitter program.

In the presence of EACA, time-absorbance curves of plasminogen activation at room temperature were obtained by measuring the OD increase in the reaction mixture with time at 405 nm. The reaction mixture contained S2251 (1.2 mM), Glu- or Lys-plasminogen (4.0 μM and 8.0 μM), UK (0.05 nM), and EACA (0–10 mM). The K_m and k_{cat} were calculated as previously described [10].

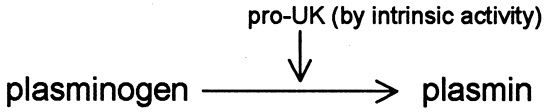
2.6. Kinetic studies of pro-UK and Δ125-pro-UK activation by Lys-plasmin and mini-plasmin in the absence or presence of EACA

A range of concentrations of pro-UK or Δ125-pro-UK (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 μM) was incubated with Lys-plasmin or mini-plasmin (0.1 nM) and S2444 (1.2 mM) in the absence or presence of EACA (0.05–5.0 mM) in assay buffer at room tem-

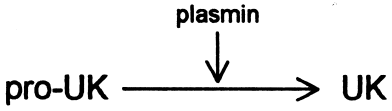
perature. The same range of concentrations of pro-UK or Δ125-pro-UK without plasmin was incubated with S2444 as background. The apparent OD (OD_{app}) increase in the reaction mixture was measured at 405 nm. The actual OD (OD_{act}) was calibrated with OD_{app} divided by $(K_{m,S2444}+[S2444])/\{K_{m,S2444}(1+[I]/K_i)+[S2444]\}$, where $K_{m,S2444}$ is Michaelis constant of UK against S2444, [S2444] is the concentration of S2444, [I] is the concentration of EACA, K_i is the competitive inhibition constant of EACA against amidolytic activity of UK. The pro-UK activation rate was calculated by the ODact increase with the time square (t^2) as previously described [10].

3. Results and discussion

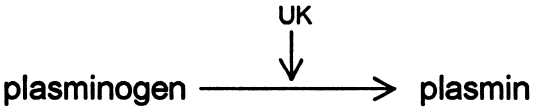
Three distinct reactions are involved in the reciprocal activation of plasminogen and pro-UK:



(Reaction 1)



(Reaction 2)



(Reaction 3)

The chain reactions are triggered by the intrinsic activity of pro-UK which is substantially higher (0.1–0.4% that of UK) than those of other protease zymogens [21,23]. After plasminogen activation has been initiated by pro-UK, plasmin induces the conversion of pro-UK to UK, and then UK catalyzes more rapid plasmin generation. Factors which change the rate of any one of the three reactions will affect net plasmin generation. Plasminogen activation by pro-UK and UK is affected by certain fibrin fragments [10,11,24], synthetic peptides [25,26], and plasmin activation of pro-UK is also modulated by uPAR [27,28].

By inducing an open conformation in Glu-plasminogen, EACA can promote plasminogen activation by plasminogen activators, including UK or plasmin resistant pro-UK [10]. EACA also has an antifibrinolytic effect which is related to its inhibition of the binding of plasminogen and plasmin with fibrin [29,30]. This property is used clinically for the treatment of certain hemorrhagic conditions [30,31].

3.1. Effects of EACA on pro-UK, Δ125-pro-UK and Ala^{158}-pro-UK-induced plasminogen activation (total effect and Reaction 1)

A significant inhibition of pro-UK-induced Glu-plasminogen (Fig. 1A) or Lys-plasminogen (Fig. 1B) activation occurred in the presence of EACA. In the case of Lys-plasminogen, inhibition by EACA occurred in a single-phase dose-dependent manner. By contrast, the inhibition of Glu-plasminogen activation by EACA was separable into three phases depending on EACA concentration. From 0.01 mM to 0.25 mM, EACA inhibited pro-UK-induced Glu-plasminogen activation in a dose-dependent manner. From 0.25 mM to 5.0 mM, EACA promoted pro-UK-induced Glu-plasminogen activation, consistent with its induction of an open conformation in Glu-plasminogen. However, the overall activation rate of Glu-plasminogen in the presence of 0.25–5.0 mM EACA was still much lower than that in the absence of EACA. The third phase was found when EACA concentration was above 5.0 mM, and the generation of plasmin was inhibited in an EACA dose-dependent manner, probably due to a direct inhibition of the active site of UK by EACA (see below).

By contrast, EACA caused only slight inhibition of pro-UK-induced mini-plasminogen activation (data not shown), suggesting that the kringle 1–4 of plasminogen was involved in the inhibitory effect of EACA.

Ala^{158}-pro-UK, A plasmin resistant mutant (which has the same intrinsic activity as wild-type pro-UK [11]), was used to investigate the effect of EACA on the intrinsic activity of pro-UK against plasminogen (Reaction 1). EACA had little effect on Lys-plasmin

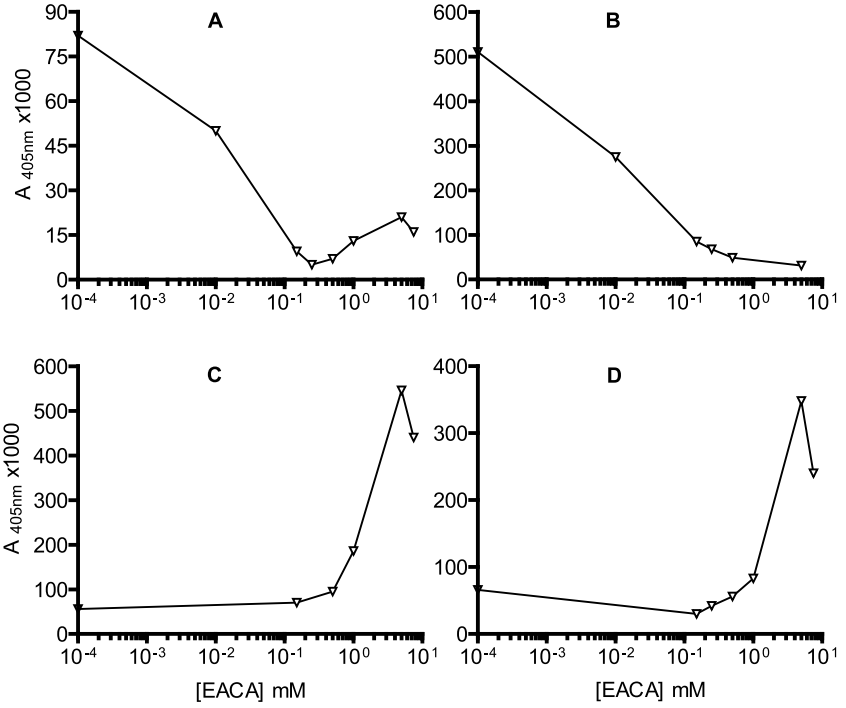


Fig. 1. Influence of EACA on activation of (A) Glu-plasminogen (1.0 μM) by pro-UK (0.5 nM), (B) Lys-plasminogen (0.2 μM) by pro-UK (0.5 nM), (C) Glu-plasminogen (1.0 μM) by Ala^{158}-pro-UK (10 nM), (D) Glu-plasminogen (1.0 μM) by Δ125-pro-UK (2.0 nM). The OD increase was measured after the reaction mixture was incubated at room temperature for 50 min.

ogen activation by Ala^{158}-pro-UK [10]. The effect on Glu-plasminogen activation was separable into two phases (Fig. 1C). When the concentration of EACA was below 5.0 mM, EACA promoted Ala^{158}-pro-UK activation of Glu-plasminogen in a dose-dependent manner, but above 5.0 mM, the promoting effect of EACA decreased as the EACA concentration increased. These results indicated that the overall inhibition of EACA on (wild-type) pro-UK-induced plasminogen activation was not related to its effect on Reaction 1.

To investigate the roles of the EGF and kringle domain of pro-UK, Δ125-pro-UK was used. When the EACA concentration was between 1.0 mM to 5.0 mM, Δ125-pro-UK-induced Glu-plasminogen activation was promoted by EACA in a dose-dependent manner (Fig. 1D). However, when the EACA concentration was below 0.5 mM, a slight inhibition was seen. These results suggested that the N-terminal region of pro-UK was involved in the inhibitory effect of EACA.

In summary, EACA caused no inhibition of Reaction 1. Both the kringle 1–4 of plasmin(ogen) and N-terminal region of pro-UK were involved in the inhibitory effect of EACA on pro-UK-induced Glu-plasminogen activation.

3.2. *Effects of EACA on amidolytic activities of Lys-plasmin, mini-plasmin, UK and Δ125-UK*

It was previously reported that the amidolytic activity of plasmin was inhibited by EACA with a K_i of 98 mM [32]. However, in the present study, no measurable effect of EACA on Lys-plasmin or mini-plasmin activity against S2251 was observed when the concentration of EACA was below 50 mM (data not shown). In contrast, the amidolytic activities of both UK and Δ125-UK were competitively inhibited by EACA with a similar inhibition constant (K_i) of 10.5 ± 0.9 mM.

3.3. *Effects of EACA and kringle 1–3 fragment on plasminogen activation by UK, Δ125-UK, CPB-pretreated UK and Δ125-UK (Reaction 3)*

Glu-plasminogen activation by UK and by Δ125-UK were comparable (Fig. 2A,B), and the generation of plasmin was decreased when the EACA concen-

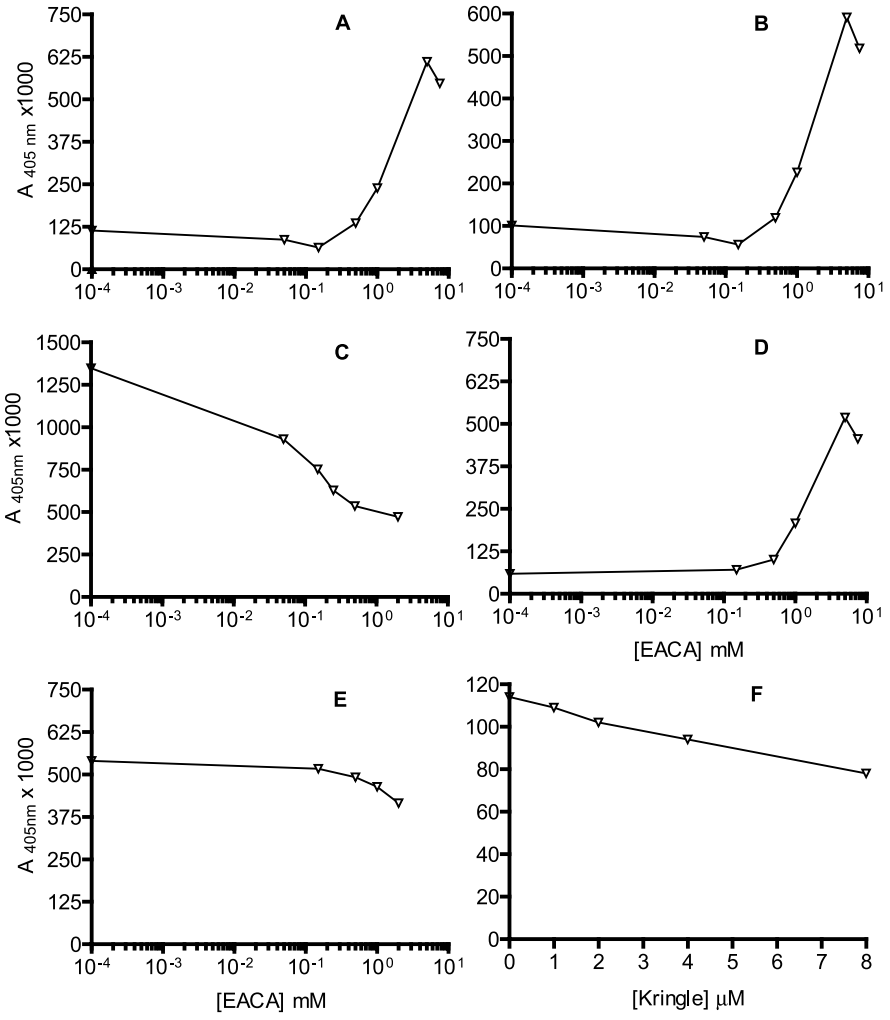


Fig. 2. Influence of EACA (A–E) and kringle 1–3 fragment (F) on activation of (A) Glu-plasminogen (0.2 μM) by UK (0.1 nM), (B) Glu-plasminogen (0.2 μM) by Δ125-UK (0.1 nM), (C) Lys-plasminogen (0.2 μM) by UK (0.1 nM), (D) Glu-plasminogen (0.2 μM) by CPB-pretreated UK (0.1 nM), (E) mini-plasminogen (0.2 μM) by UK (0.1 nM), (F) Glu-plasminogen (0.2 μM) by UK (0.1 nM). The OD increase was measured after the reaction mixture was incubated at room temperature for 50 min.

tration was below 0.5 mM. While an EACA does-dependent inhibition of Glu-plasminogen activation was observed in the presence of low EACA concentrations (<0.15 mM), an EACA dose-dependent promotion was found at higher EACA concentrations (0.5–5.0 mM), reaching a limit at an EACA concentration of 5.0 mM (Fig. 2A,B).

Both UK (Fig. 2C) and Δ125-UK (data not shown) induced Lys-plasminogen activation were significantly and comparably inhibited by EACA in a dose-dependent manner.

The finding that the EACA effect on UK and Δ125-UK-induced plasminogen activation was comparable indicated that the EGF and kringle domains of UK were not involved in the effect of EACA on Reaction 3. The different inhibitory behavior of EACA on Lys-, Glu-, and mini-plasminogen (see below) activation suggested that more than a direct effect by EACA on the active site of UK was involved.

UK-induced plasminogen activation was previously shown to be promoted by the C-terminal lysine (Lys^{158}) on the A-chain of UK [33]. Recently, the $UK^{149-158}$ region was proposed to be an important functional motif that could act as a ligand for the kringles of plasminogen [34]. The role of Lys^{158} in the EACA effect was studied by CPB treatment of UK (Fig. 2D) and Δ125-UK (data not shown). When

EACA concentration was below 5.0 mM, Glu-plasminogen activation by CPB-pretreated UK was promoted by EACA in a dose-dependant manner, and no inhibition was found at low concentrations of EACA. These results suggested that the Lys^{158} of UK was involved in the inhibitory effect of EACA. Since it seems unlikely that the C-terminal Lys^{158} on the A-chain (of UK) could affect the catalytic site of UK, we postulate that the interaction between plasminogen and Lys^{158} of UK, which facilitates plasminogen activation, might be blocked in the presence of EACA. This inhibitory effect of EACA was previously overlooked, probably because the UK used [6–8,10] was purified from urine and therefore, was missing the C-terminal Lys^{158} [35].

Based on the above results, the dose-dependent effect of EACA on Glu-plasminogen activation could be explained. At low EACA concentrations (<0.15 mM), inhibition predominated, probably due to blocking the interaction of Glu-plasminogen with the C-terminal lysine of UK. At higher EACA concentrations, promotion predominated due to inducing an open conformation of Glu-plasminogen.

Neither EACA (Fig. 2E) nor CPB pretreatment of UK (data not shown) had a significant effect on mini-plasminogen activation, indicating that the kringle1–4 of (Glu- or Lys-) plasminogen was involved in the inhibitory effect of EACA on plasminogen activation by UK (Reaction 3). This was further confirmed by the inhibition of kringle 1–3 fragment on UK-induced plasminogen activation (Fig. 2F).

In summary, these findings suggested that the interaction between (Glu or Lys-) plasminogen and UK is mediated by the kringle 1–4 of plasminogen and the C-terminal Lys^{158} of UK, and that this interaction (which facilitated plasminogen activation) might be blocked by low concentrations of EACA, thereby causing inhibition of Reaction 3.

3.4. *Effects of EACA and kringle 1–3 fragment on pro-UK and Δ125-pro-UK activation by Lys-plasmin and mini-plasmin (Reaction 2)*

Pro-UK activation by Lys-plasmin was significantly inhibited by EACA in a dose-dependent manner (Fig. 3A), whereas EACA only slightly inhibited Δ125-pro-UK activation by Lys-plasmin (Fig. 3B) and pro-UK activation by mini-plasmin (Fig. 3C). These findings suggested that the kringle 1–4 of Lys-plasmin as well as the EGF and/or kringle domain of pro-UK were involved in the inhibitory effect of EACA on pro-UK activation by Lys-plasmin (Reaction 2). A dose-dependent inhibition of kringle 1–3 fragment on pro-UK activation by Lys-plasmin was also observed (Fig. 3D). By contrast, kringle 1–3 fragment had little effect on plasmin activation of Δ125-pro-UK (data not shown). These results suggested that the interaction between Lys-plasmin and pro-UK is mediated by the kringle 1–4 of plasmin and the N-terminal region of pro-UK, and that this interaction (which facilitated pro-UK activation) could be blocked by EACA, thereby causing inhibition of Reaction 2.

3.5. *Kinetic studies of plasminogen (Glu-, Lys- and mini-) activation by UK, Δ125-UK and CPB-pretreated UK in the absence or presence of EACA*

The catalytic efficiencies (k_{cat}/K_m) of plasminogen (Glu-, Lys- or mini-) activation by UK and Δ125-UK were comparable (Table 1), indicating that the N-terminal region of UK were not significantly involved in this reaction.

The catalytic efficiencies of mini-plasminogen activation by UK, CPB-pretreated UK or Δ125-UK were comparable, but were only about half as efficient as that of Lys-plasminogen activation (Table 1). These data were consistent with earlier findings (Fig. 2), and indicated that the binding of kringle 1–4 of plasminogen with the C-terminal Lys^{158} of UK facilitated plasminogen activation. This was further supported by the finding that Lys-plasminogen and mini-plasminogen activation by CPB-pretreated UK were also comparable (Table 1).

Both the K_m and k_{cat} of UK-induced Glu-plasminogen activation were increased by EACA in a dose-dependent manner (Table 2). At low EACA concentrations (<0.5 mM), the increase in K_m was predominant. When the concentration of EACA was more than 0.5 mM, the increase in k_{cat} exceeded K_m, and plasmin generation was promoted.

Since the K_d for the weak EACA binding site of Glu-plasminogen was 5.0 mM [13], less than 1% of the weak binding site was occupied at 0.05 mM

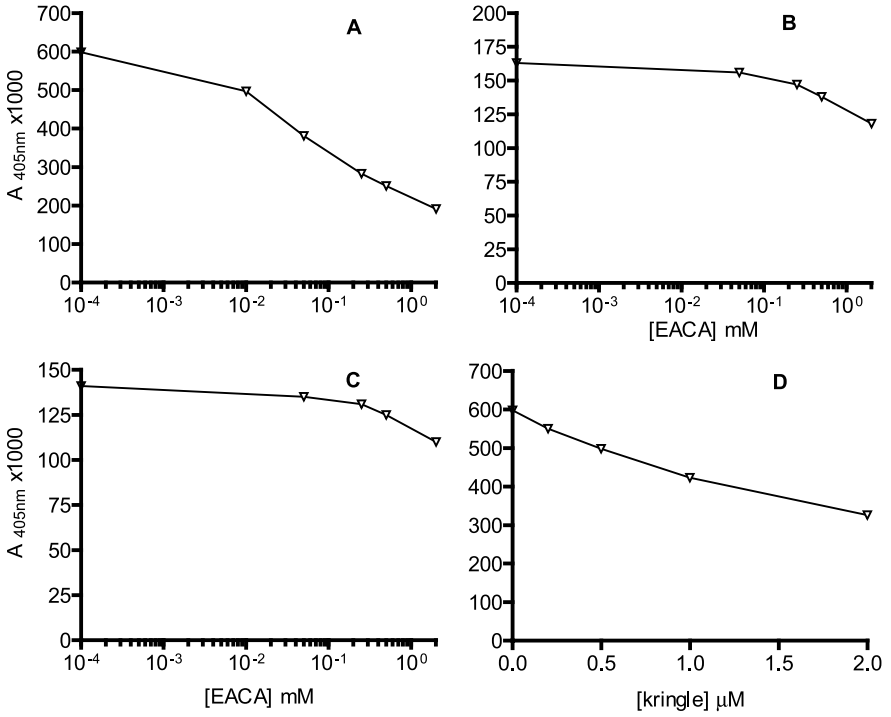


Fig. 3. Influence of EACA (A–C) and kringle 1–3 fragments (D) on activation of (A) pro-UK (0.2 μM) by Lys-plasmin (0.1 nM), (B) Δ125-pro-UK (0.2 μM) by Lys-plasmin (0.1 nM), (C) pro-UK (0.2 μM) by mini-plasmin (0.1 nM), (D) pro-UK (0.2 μM) by Lys-plasmin (0.1 nM). The OD increase was measured after the reaction mixture was incubated at room temperature for 50 min.

EACA. Based on the results shown in Fig. 2 and Table 2, it was postulated that low concentrations of EACA inhibited UK activation of Glu-plasminogen by occupying the high-affinity lysine binding site located in kringle 1–4 of plasminogen and preventing plasminogen binding to the C-terminal Lys^{158} of UK.

3.6. *Kinetic studies of pro-UK and Δ125-pro-UK activation by Lys-plasmin and mini-plasmin in the absence or presence of EACA*

The catalytic efficiency of pro-UK activation by Lys-plasmin was 4.2-fold that by mini-plasmin, and

Table 1
Catalytic activity of UK, CPB-UK, Δ125-UK against Glu-, Lys-, and mini-plasminogen

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)
UK			
Glu-plasminogen	3.4 ± 0.52	8.1 ± 0.72	2.38
Lys-plasminogen	4.8 ± 0.66	101 ± 9.5	21.0
Mini-plasminogen	24.5 ± 3.4	252 ± 18.7	10.3
CPB-pretreated UK			
Glu-plasminogen	6.4 ± 0.83	7.9 ± 0.52	1.23
Lys-plasminogen	11.5 ± 1.68	117 ± 11.2	10.2
Mini-plasminogen	26.2 ± 4.6	248 ± 15.3	9.47
Δ125-UK			
Glu-plasminogen	3.8 ± 0.54	8.7 ± 0.73	2.29
Lys-plasminogen	5.6 ± 0.75	110 ± 14.3	19.6
Mini-plasminogen	28.7 ± 4.7	264 ± 21.8	9.2

Table 2
The effect of EACA on catalytic activity of UK against Glu-plasminogen

EACA (μM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	Effect of EACA
0	3.4 ± 0.52	8.1 ± 0.72	2.38	1.0
50	4.6 ± 1.1	8.2 ± 1.3	1.79	0.75
150	5.4 ± 1.5	8.4 ± 1.5	1.56	0.65
250	5.8 ± 1.7	12.1 ± 2.4	2.08	0.87
500	6.5 ± 2.2	16.5 ± 3.2	2.54	1.07
1000	7.4 ± 2.5	24.7 ± 6.9	3.33	1.4
5000	8.7 ± 3.5	74.5 ± 25.8	8.57	3.6
7500	9.1 ± 3.8	67.1 ± 24.9	7.34	3.08

3.8-fold greater than that of Δ125-pro-UK activation (Table 3). By contrast, the catalytic efficiencies of Δ125-pro-UK activation by Lys-plasmin and mini-plasmin were comparable. These data supported previous speculation that the interaction between pro-UK and the kringles of plasminogen affected the binding and activation of pro-UK at the active site of plasmin [36], and further confirmed that the N-terminal region of pro-UK and kringle 1–4 of plasmin were both required to facilitate pro-UK activation.

In the presence of EACA, the K_m increased in a dose-dependent manner and the catalytic efficiency (k_{cat}/K_m) of pro-UK activation by Lys-plasmin was reduced. By contrast, EACA only had slight inhibition of Δ125-pro-UK activation by Lys-plasmin (Table 4), which was consistent with previous finding that the N-terminal region of pro-UK was involved in the inhibitory effect of EACA on pro-UK activation.

Since Pro-UK activation by Lys-plasmin was significantly inhibited when the EACA concentration was only 0.05 mM (Fig. 3 and Table 4), far less than the Kd for the weak EACA binding site of Lys-plasmin [14], we postulated that this inhibition was related to EACA occupying the high-affinity lysine binding site located in kringle 1–4 of plasmin, which prevented the binding interaction between plasmin and the N-terminal region of pro-UK.

The different effects of EACA on plasmin generation induced by Ala^{158}-pro-UK, pro-UK and Δ125-pro-UK could be explained by the influence of EACA on Reactions 1–3. For Ala^{158}-pro-UK activation of Glu-plasminogen, only Reaction 1 was involved, since Ala^{158}-pro-UK cannot be activated by plasmin. EACA promoted Reaction 1 by inducing an open conformation of Glu-plasminogen.

For pro-UK-induced Glu-plasminogen activation in the presence of low concentrations of EACA (< 0.5 mM), Reaction 1 was slightly promoted by EACA, while Reactions 2 and 3 were significantly inhibited, and net plasmin generation was decreased. At higher EACA concentrations (0.5–5.0 mM), Reaction 3 became promoted by EACA in a dose-dependent manner, resulting in a decline in overall inhibition.

For Δ125-pro-UK-induced Glu-plasminogen activation at low concentrations of EACA (< 0.5 mM), both Reactions 2 and 3 were inhibited, which accounted for a net decrease of plasmin generation. At higher concentrations of EACA (1.0–5.0 mM), the promoting effect of EACA on Reaction 3 overcame its weak inhibitory effect on Reaction 2 (Fig. 3 and Table 4), and overall plasminogen activation was enhanced.

Our results differed from those in a previous report [20] in which 2.0 mM EACA was found to promote pro-UK-induced Glu-plasminogen activation in the presence of Cl^-. The reason for this difference is

Table 3
Catalytic activity of Lys-plasmin and mini-plasmin against pro-UK and Δ125-pro-UK

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)
Lys-plasmin			
Pro-UK	3.2 ± 0.4	29.5 ± 3.5	9.22
Δ125-Pro-UK	14.1 ± 1.5	34.3 ± 4.6	2.43
Mini-plasmin			
Pro-UK	16.5 ± 1.8	36.5 ± 3.9	2.21
Δ125-Pro-UK	19.7 ± 2.5	40.6 ± 4.8	2.06

Table 4
The effect of EACA on catalytic activity of Lys-plasmin against pro-UK and Δ125-pro-UK

EACA (μM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	Effect of EACA
Pro-UK				
0	3.2 ± 0.4	29.5 ± 1.0	9.22	1.0
50	5.9 ± 1.6	39.2 ± 2.8	6.65	0.72
150	7.8 ± 2.2	40.5 ± 3.1	5.20	0.56
250	10.4 ± 2.7	42.2 ± 3.3	4.06	0.44
500	12.1 ± 3.4	43.5 ± 4.6	3.60	0.39
1000	13.8 ± 4.8	43.3 ± 4.5	3.13	0.34
5000	16.4 ± 5.4	40.2 ± 3.4	2.45	0.27
Δ125-Pro-UK				
0	14.1 ± 0.7	34.3 ± 1.2	2.43	1.0
50	14.8 ± 1.7	34.5 ± 1.9	2.33	0.96
150	15.1 ± 2.1	34.9 ± 2.5	2.31	0.95
500	15.7 ± 3.2	34.0 ± 2.4	2.16	0.89
1000	16.3 ± 3.5	33.5 ± 3.8	2.05	0.85
5000	18.6 ± 4.8	34.6 ± 4.3	1.86	0.77

unknown. Within a wide range of concentrations of pro-UK (0.1–10 nM) and Glu-plasminogen (0.2–8.0 μM) tested, we found a similar inhibitory effect of EACA on pro-UK-induced plasminogen activation.

In conclusion, EACA is an inhibitor of pro-UK-induced plasminogen activation. This effect was due to its inhibition of two reactions, UK activation of plasminogen and plasmin activation of pro-UK. Analysis of the effects of EACA showed that the binding of kringle 1–4 of plasmin to the N-terminal region of pro-UK promoted pro-UK activation, and that the binding of kringle 1–4 of plasminogen to the C-terminal Lys^{158} of UK promoted plasminogen activation. These interactions were interrupted by EACA, accounting for its inhibitory effect.

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