Brazilian Journal of Medical and Biological Research (1999) 32: 39-43
ISSN 0100-879X Short Communication

Modulation of staphylokinase-dependent plasminogen activation by mono- and divalent ions

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Abstract

The effect of several ions (Cl^-, Na^+, K^+, Ca^{2+}) on the rate of plasminogen (Pg) activation by recombinant staphylokinase (rSTA) is reported. Both monovalent and divalent ions affect the rate at which Pg is activated by rSTA, in a concentration-dependent manner (range 0-100 mM). In almost all cases, a decrease of the initial velocity of activation was observed. Cl^- showed the most striking inhibitory effect at low concentrations (64% at 10 mM). However, in the presence of a fibrin surface, this inhibition was attenuated to 38%. Surprisingly, 10 mM Ca^{2+} enhanced the Pg activation rate 21% when a polymerized fibrin matrix was present. These data support the idea that ions can modulate the rate of Pg activation through a mechanism that may be associated with changes in the molecular conformation of the zymogen. This effect is strongly dependent on the presence of a fibrin clot.

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Research supported by CDCHT/ULA (No. C-678-94).

Received March 17, 1998
Accepted October 7, 1998

Key words
- Staphylokinase
- Plasminogen activation
- Fibrinolysis

Staphylokinase (STA), a bacterial plasminogen activator produced by some strains of *Staphylococcus aureus*, is considered at present as the most promising fibrinolytic agent for the treatment of patients with acute myocardial infarction (1). Human plasminogen (Pg) activation by STA is being studied to elucidate the mechanism of activation since STA is not an enzyme. It has been established that STA forms a stoichiometric complex with Pg (STA.Pg) in which a plasmin (Pm) activity is rapidly detected. The STA.Pm complex is the active enzyme which specifically activates other Pg molecules on the surface of a fibrin clot (2,3). This preferential activation of fibrin-bound Pg has been explained by the rapid inhibition of circulating complexes by α-2-antiplasmin in plasma but not on the fibrin surface, where the molecular domains involved in the inhibition process closely associate with the clot (4).

Kinetic studies of Pg activation by STA have focused mainly upon the activation mechanism, fibrin specificity, the correlation among various inhibitors and the effect of STA concentration on plasminogen activation (5). It is well known that ions could act as negative or positive effectors of Pg activation by streptokinase and urokinase, two well-known Pg activators currently in therapeutic use (6,7). However, to date no results concerning these effects on Pg activation by STA have been described. We report here, for the first time, the effect of Ca^{2+} and Cl^-, as well as other ions, on the rate of Pg activation by recombinant STA (rSTA).

rSTA and human Glu-Pg were purified and characterized as described elsewhere (8,9). Lys-Pg was produced by plasmin digestion of Glu-Pg at 37ºC in 40 mM L-lysine for 1 h (10). rSTA.Pm complexes were obtained by incubation of Pg with rSTA (3 µM each, with a 5% molar excess of rSTA) at 37°C for 10 min in 50 mM HEPES, pH 7.4, containing 25% glycerol (11). Solid phase fibrin plates were prepared to activate plasminogen in the presence of a polymerized fibrin surface. Briefly, after treatment of polyvinyl chloride (PVC) plates with 2.5% glutaraldehyde, purified human fibrinogen (0.1 mg/ml in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM $CaCl_2$) was covalently fixed and converted into a fibrin network by thrombin treatment (12). Activation assays were performed in microtiter and fibrin plates and were based on the development of amidolytic activity of Pm generated by Pg activation, towards a chromogenic substrate specific for plasmin, MM-Hyp-Arg-pNA (CBS 00.65, Diagnostica STAGO, Asnieres, France) (13). The assays were performed at 37ºC in a buffer containing 50 mM HEPES, pH 7.4, the substrate CBS 00.65 (1 mM), rSTA (50 nM) or rSTA.Pm complexes (10 nM) and different levels of ions ranging from 0 to 100 mM. Glu-Pg (1 µM) was added to start the reaction and the absorbance at 405 nm of the mixtures was continuously monitored. Initial reaction rates were determined from the slopes of plots of A_{405} versus t^2, according to the method of Wohl (14).

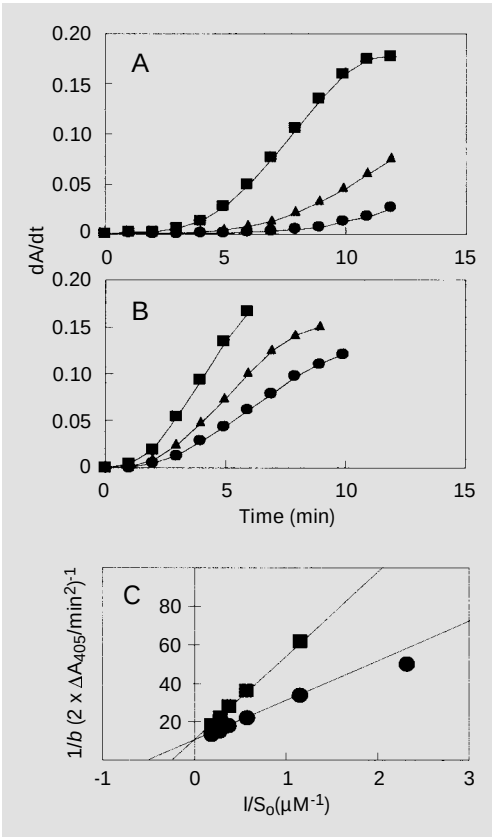

Figure 1 - Kinetics of plasminogen activation by rSTA at 37ºC. Glu-Pg (A) and Lys-Pg (B) at different concentrations were activated with 50 nM rSTA in the presence of a chromogenic substrate (CBS 00.65). Absorbance at 405 nm was monitored every minute and dA/dt was plotted against time. Squares (4.5 µM), triangles (1.7 µM), circles (0.85 µM). *C*, Double reciprocal plot of the rate of plasminogen activation by rSTA, as a function of plasminogen concentration. Glu-Pg (squares), Lys-Pg (circles).

As expected, rSTA activated human Glu-Pg with a lag period of at least 5 min which almost disappeared when Lys-Pg was used (Figure 1A and B). Activation of Pg by rSTA obeyed Michaelis-Menten kinetics as revealed by linear double-reciprocal plots of the initial activation rates versus the Pg concentration (Figure 1C). When preformed rSTA.Pm complexes were used as the activator species, the lag period also diminished. This was also observed when activation assays were performed in the presence of a fibrin surface on fibrin plates (results not shown).

Taken together, these results support the view that during the activation process of Glu-Pg by STA there is a rate-limiting step during which the activator must bind to the zymogen causing a molecular conversion of Glu-Pg to Lys-Pg to take place. It has been shown that Lys-Pg possesses a more open conformation and interacts more easily with activators (15). In fibrin plates, the binding of Glu-Pg to fibrin through kringle domains induces a similar conformational change, from a "closed" to an "open" form that accelerates the activation rate (16).

These observations were confirmed when the activation experiments were carried out in the presence of different ions (Figure 2). The effect of Cl^-, which stabilizes the "closed" form of the zymogen (17), is markedly inhibitory even at 1/10 of its physiological concentration. SDS-PAGE analysis of

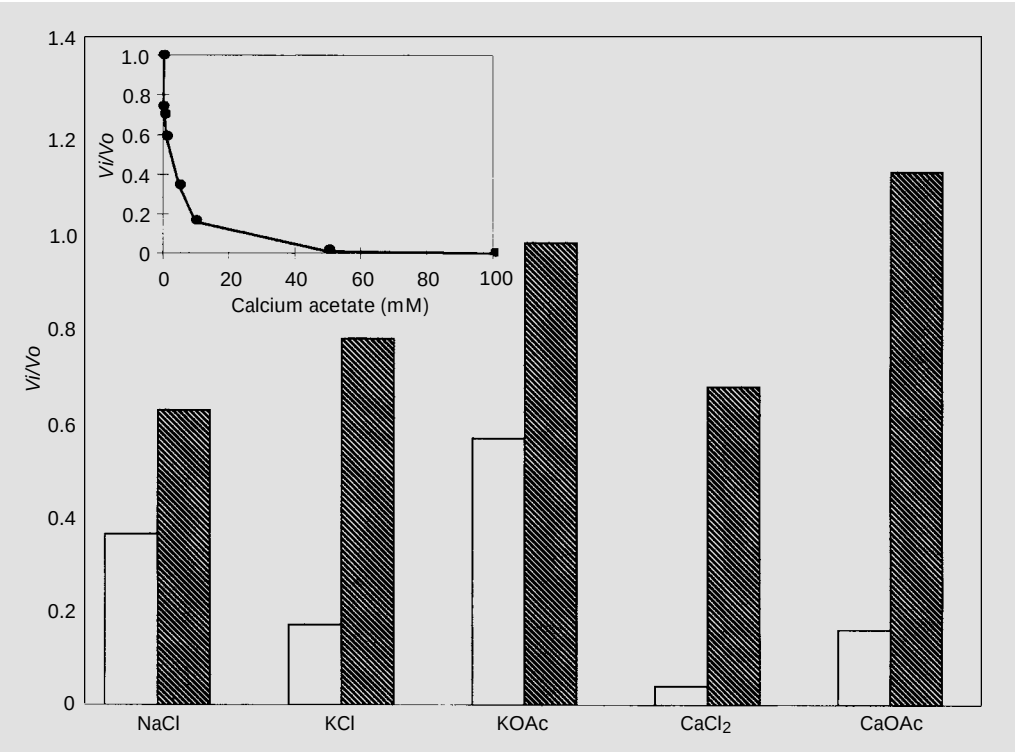

Figure 2 - Effect of ions on the rate of plasminogen activation by rSTA. Pg was activated by rSTA in 50 mM HEPES, pH 7.4, in the presence of a chromogenic substrate for plasmin (MM-Hyp-Arg-pNA, CBS 00.65) and different ions (10 mM each). *Vi* and *Vo* are the initial velocities of hydrolysis of CBS 00.65 by plasmin in the presence and absence of ions, respectively. Data are reported as the mean of three experiments. Experiments were performed on PVC plates in the absence (open bars) and in the presence of a fibrin surface (striped bars). *Inset*, Concentration-dependent decrease of the initial plasminogen activation rate in the presence of increasing concentrations of calcium acetate and in the absence of a fibrin surface.

Pg activation products revealed that in the presence of 20 mM NaCl the one-chain Glu-Pg form did not undergo the molecular transition to the enzymatically active two-chain Pm (not shown). Cl^--induced inhibition has been reported previously in studies where Pg was activated with streptokinase and urokinase, two fibrinolytic agents used worldwide for the treatment of acute myocardial infarction (5). However, in the presence of a fibrin surface, a significant reduction of the inhibitory effect of Cl^- was observed. Once again, Pg binding to fibrin appears to reverse the inhibition, probably due to Cl^- exclusion from the domains involved in such interaction (17).

The inhibitory effect of other mono- and divalent ions has also been established. Their effect was not so striking as the Cl^- inhibition, but a concentration-dependent decrease of the Pg activation rate was observed in almost all cases (Figure 2, inset). The anion acetate does not inhibit Glu-Pg activation to the same extent as Cl^-. As already stated, acetate does not bind as strongly to the anion-binding sites on Glu-Pg kringles as does Cl^- and therefore does not modulate as effectively the production of the "closed" state (17). Surprisingly, when calcium in the acetate salt form was added to activation mixtures in fibrin plates, an enhancement of the rate of Pg activation by rSTA was observed. This effect attained its maximum at a concentration of 5 mM, which is close to the physiological concentration.

The role of Ca^{2+} in fibrinolysis is unknown and has been investigated by several authors. Stack et al. (7) observed that divalent cations inhibited the activation of Glu-Pg but stimulated the activation of Lys-Pg, confirming that the molecular conformation of the substrate is critical for the inhibitory/enhancer effect. Markus et al. (18) observed that casein, a Ca^{2+}-rich phosphoprotein, is a potent accelerator of the rate of Pg activation by both urokinase and tissue Pg-activator. Their findings showed that partial removal of Ca^{2+} by chelating agents such as EDTA or

EGTA reduced the enhancing effect by an average of 38% of the control. Recently, Kojima et al. (19) reported that a physiological concentration of Ca^{2+} significantly shortened the euglobulin clot lysis time, an assay used to assess systemic fibrinolytic activity. Furthermore, tetranectin, a plasminogen-binding protein which possesses intact calcium binding sites, has been shown to interact specifically with kringle 4. The function of tetranectin is unknown, but the protein has been reported to enhance Pg activation by t-PA, a mechanism that could be closely associated with calcium binding (20).

Our findings add to these results, showing that Ca^{2+} enhances the rate at which Pg is activated by rSTA in the presence of polymerized fibrin. Even though the role of Ca^{2+} requires further investigation, it is clear that Ca^{2+} may modulate the rate at which Pg is converted to Pm by physiological and therapeutical activators. Such modulation of fibrinolytic activity should be very important in determining the rate of activation in the microenvironment of this zymogen, particularly in the vicinity of a fibrin clot.

Nevertheless, since the mechanism of Pg activation is triggered and further amplified by specific interactions of fibrin with the Pg kringle domains, it is clear that these studies should be developed at least in the presence of a fibrin surface in order to obtain experimental conditions as close as possible to the physiological plasma/fibrin interphase. If not, some effects could be over- or underestimated, as shown in the present study.

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