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Induction of Catalytic Activity of Plasminogen by Monoclonal Antibody IV-Ic in the Presence of Divalent Metal Cations and α_2-Antiplasmin

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Abstract—Investigation of the influence of divalent metal cations on the induction of plasminogen catalytic activity by monoclonal antibody IV-Ic showed that the presence of metal cations in the reaction medium changes the induction by slowing down or accelerating the process. Ions of Zn^{2+}, Mn^{2+}, and Cu^{2+} completely inhibit activation. Ions of Co^{2+} and Ni^{2+} decrease the rate of the first and second phases of the reaction more than 2 times. Ca^{2+} ions do not have any effect on the activation rate. Ions of Mg^{2+}, Ba^{2+}, and Sr^{2+} increase the rate of the first phase of the reaction by 1.5, 2.0, and 2.0 times and the rate of the second phase by 2.0, 3.8, and 4.7 times, correspondingly. Sr^{2+} ions have the strongest stimulating effect on plasminogen activation by monoclonal antibody IV-Ic. Investigation of the dose dependent effect of Sr^{2+} on the rate of plasminogen activation by monoclonal antibody IV-Ic showed stimulating effect of Sr^{2+} at concentrations from 0.1 to 1.0 mM with half maximum at 0.6 mM. However, Sr^{2+} ions do not affect amidolytic activity of plasmin and activation of plasminogen by streptokinase. Sr^{2+} ions also do not affect monoclonal antibody IV-Ic binding to plasminogen. The effect of Sr^{2+} is specific and mediated by the IV-Ic component. The presence of metal cations affects conformational changes in the process of active site formation. Metal cations also affect structure of the plasminogen molecule active site in the complex with monoclonal antibody IV-Ic and enzyme–substrate interaction. The effect of α_2-antiplasmin on the induction of plasminogen catalytic activity by monoclonal antibody IV-Ic in range of concentrations from 5 to 30 nM has been studied. α_2-Antiplasmin at concentration 30 nM almost completely inhibits induction of plasminogen catalytic activity by monoclonal antibody IV-Ic at the ratio plasminogen/α_2-antiplasmin of 3 : 1. This can be explained by competition of α_2-antiplasmin and monoclonal antibody IV-Ic for the lysine-binding sites of plasminogen and inhibition of the active center in activated complex plasminogen*–mAB IV-Ic. Divalent metal cations and α_2-antiplasmin are important factors in induction of plasminogen catalytic activity by monoclonal antibody IV-Ic.

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Key words: plasminogen, monoclonal antibody, divalent metal cations, α_2-antiplasmin

The plasminogen (Pg)–plasmin (Pm) system is one of the important proteolytic systems in mammals. It participates in the regulation of many different physiological and pathological processes, such as maintenance of the liquid state of the blood, wound healing, tissue restructuring, infectious and allergic inflammation, and tumor invasion [1-3].

The key reaction of this fibrinolytic system is the reaction of activation Pg into Pm. *In vivo* Pg activation occurs by specific enzymes such as urokinase and tissue plasminogen activator (tPA). Also there are non-enzymatic activators of Pg that induce catalytic activity in the protease domain of Pg without cleavage of the activation peptide bond. These activators include proteins streptokinase (SK) and staphylokinase produced by streptococci and staphylococci respectively [4]. For the invasion of a host tissue, these microorganisms use the Pg–Pm system of a host and exogenously produced SK or staphylokinase [5]. Streptococcus and staphylococcus infections cause many widely spread diseases among populations. SK and staphylokinase in the blood form very strong complexes with Pg. These complexes are very powerful antigens [6]. The ability to induce catalytic activity of Pg was also found

Abbreviations: α_2-AP) α_2-antiplasmin; mAB) monoclonal antibody; Pm) plasmin; Pg) plasminogen; Pg*) activated plasminogen; SK) streptokinase; tPA) tissue plasminogen activator.

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in anti-Pg monoclonal antibody (mAB) IV-Ic [7]. IgG fraction isolated from plasma of patients with acute myocardial infarction contains high titer of anti-SK antibodies, and 2% of the fraction are anti-Pg autoantibodies, which appear in plasma a month after treatment of the patients with SK. These data suggest the possibility of appearance the autoantibodies similar to IV-Ic in patients with streptococcus infections. Pg–IV-Ic-like complexes are potentially dangerous for an organism because they might create background proteolytic and activating level, and play a part in pathogenesis and complications associated with streptococcus and staphylococcus infections [8].

There are some data on inhibition of the process of Glu-Pg activation by physiological activators, and on inhibition of the induction of Pg catalytic activity by SK in the presence of high concentration (50 mM) of bivalent cations of Ca^{2+}, Mg^{2+}, or Mn^{2+} [9]. The $[I]_{50}$ for Ca^{2+}, Mg^{2+}, and Mn^{2+} are 32, 330, and 7 mM, correspondingly. At the same time, a contrary effect of Ca^{2+} and Mg^{2} was shown upon activation of Lys-Pg by tPA. Mn^{2+} ions inhibited activation of Glu-Pg as well as Lys-Pg. The authors explained this inhibition by conformational changes in the molecule of the zymogen, which led to decrease in the Stokes' radius and increase in the molecular compactness.

Cations Cu^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} (10^{-5}-10^{-2} M) inhibit esterase activity of urokinase and its Pg activation, while Ca^{2+} and Mg^{2+} ions are not effective [10].

Blood plasma contains Ca^{2+} in concentrations 1.0-1.2 mM [11], and Mg^{2+} and Li^{+} in 1.5 mM or less. Metal cations of Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} are contained in the plasma in microquantities [12].

α_2-Antiplasmin (α_2-AP), the primary fast-acting inhibitor of Pm, has blood concentration 1 μM and participates in the regulation of fibrinolysis in the human body [13]. Therefore, to study the process of induction of Pg catalytic activity by mAB VI-Ic in the presence of bivalent metal cations and α_2-AP is very important.

The goal of this research was to investigate the effect of bivalent metal cations on induction of Pg catalytic activity by mAB VI-Ic, and the influence of α_2-AP on this process.

MATERIALS AND METHODS

Glu-Pg was isolated from blood donors' citrate plasma by affinity chromatography on lysine-Sepharose (Amersham Biosciences, USA) [14] followed by gel filtration on Sephacryl S-200 (Amersham Biosciences).

Pm was produced by activation of Glu-Pg using urokinase (Sanofi Winthrop, France) immobilized on BrCN-Sepharose-4B (Amersham Biosciences) [15]. The percentage of active centers in Pm and Pm derivatives was determined by titration as described in [16].

α_2-AP was isolated from blood donors' citrate plasma using consecutively connected columns with lysine-Sepharose and K1-3-Sepharose [17].

SK (Kabikinase; Kabi, Sweden) was purified by affinity chromatography on Blue Sepharose CL-6B (Amersham Biosciences) [18]. The purity of the protein was monitored electrophoretically by SDS-PAGE [19].

Anti-Pg mAB IV-Ic was produced in the Department of Molecular Immunology, Palladin Institute of Biochemistry, the National Academy of Sciences of Ukraine, according to the common procedure using electrophoretically homogeneous human Glu-Pg as antigen described in [20]. Efficiency of IV-Ic clones was 4.9 ± 0.051 μg/ml. IV-Ic was isolated from culture medium after removal of cells by two-step affinity chromatography on protein A-Sepharose (Amersham Biosciences) and Glu-Pg-Sepharose [21]. The IV-Ic fraction was electrophoretically homogeneous. Immunochemical analysis showed that immunoglobulin IV-Ic belongs to IgG1 isotype.

Degree of IV-Ic binding to Pg was evaluated by ELISA [22].

Amidolytic activity in the process of Pg activation by IV-Ic or SK was determined by the rate of *p*-nitroaniline release during hydrolysis of S-2251 substrate (H-D-Val-L-Leu-L-Lys-*p*-nitroanilide) (Chromogenix, Sweden). *p*-Nitroaniline concentration was determined spectrophotometrically at 405 nm. The reaction volume was 0.25 ml. Glu-Pg and mAB IV-Ic were taken at equimolar concentrations of 100 nM or Glu-Pg, Pm, and SK at 10 nM; S-2251 substrate concentration was 0.3 mM. Concentration of metal chlorides was 1.0 mM in 0.1 M Tris-HCl buffer, pH 7.4 at 37°C.

Bidistilled water purified with Milli-Q (Millipore, USA) was used in experiments.

The rate of the first phase of the reaction of Pg activation was calculated using kinetic curves of Pg activation by mAB IV-Ic (dependence of absorption at 405 nm (A_{405}) on time) as the value inverse to the lag-period ($1/\tau$), and the rate of the second phase was calculated as the rate of formation of Pm active centers per minute: $V = ([Pm_2] - [Pm_1])/(t_2 - t_1)$, nM Pm/min. Concentration of Pm active centers at time point t was quantitatively determined using a calibration curve in coordinates: [Pm], nM versus tanα, where tanα is the tangent of the calibration curve slope of S-2251 hydrolysis by Pm, or the tangential angle to the kinetic curve of Pg activation at time point t.

Data is presented as mean result of three to five experiments with standard error not exceeding 3%.

RESULTS AND DISCUSSION

The described mechanism of the induction of Pg catalytic activity by mAB IV-Ic includes two phases: the first long lag-phase where the catalytic activity of Pg is not

manifested, and the second phase of rapid activation. The first phase of the reaction involves the formation of Glu-Pg–IV-Ic complex and conformational changes in the Pg molecule which lead to the formation of the active center in the protease domain of Pg. The second phase of the reaction is manifestation of amidolytic activity by Pg*–IV-Ic complex and rapid increase in the number of active centers. This is reflected in the rapid increase in amidolytic activity until a constant value is achieved, and, therefore, to the constant rate of the substrate hydrolysis [23].

Induction of the Pg catalytic activity by mAB IV-Ic is characterized by a long lag-period (90 min on average) and rapid accumulation of active centers. The mechanism of active center formation in Pg–IV-Ic is a complicated process with the rate constant of active complex formation equal to $0.02 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$, and the activation energy (E_a) of the interaction of activated complex with substrate S-2251 equal to 18.7 kcal/mol. The activation energy at the stage of conformational changes in Pg and formation of active center is 38 kcal/mol. For the Pg*–SK complex, these values are 10^6 $M^{-1} \cdot sec^{-1}$ and 7.2 and 10.5 kcal/mol, correspondingly [24].

Analysis of the effect of bivalent metal ions at concentration 1 mM on Pg activation by mAB IV-Ic has shown that presence of the metal ions in the media changed the rate of the reaction, increasing or decreasing it (Fig. 1). The presence of metal ions in the blood might alter the equilibrium in the fibrinolytic system when the blood also has anti-Pg antibodies.

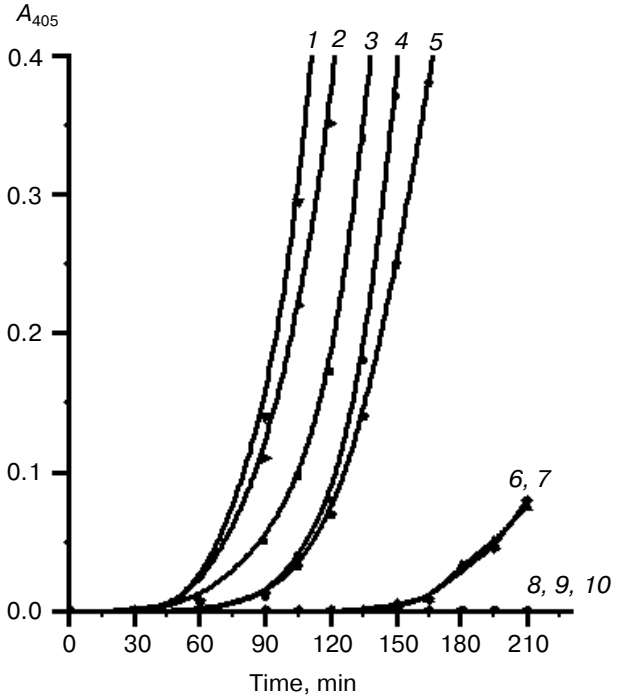

Fig. 1. Kinetic curves of the induction of Glu-Pg catalytic activity by mAB IV-Ic in the presence of 1.0 mM metal cations: *1*) Sr^{2+}; *2*) Ba^{2+}; *3*) Mg^{2+}; *4*) Ca^{2+}; *5*) without cations; *6*) Ni^{2+}; *7*) Co^{2+}; *8*) Cu^{2+}; *9*) Mn^{2+}; *10*) Zn^{2+} (A_{405} is absorbance at 405 nm). Concentration of the components: Glu-Pg and mAB IV-Ic, 100 nM; S-2251, 0.3 mM; buffer 0.1 M Tris-HCl, pH 7.4, 37°C.

Cations of heavy metals slow down the process of activation. Zn^{2+}, Mn^{2+}, and Cu^{2+} ions completely inhibit the activation. Ions of Co^{2+} and Ni^{2+} decrease the rate of the first and second phases of the reaction by more than 2-fold. Ions of the alkaline earth metals Mg^{2+}, Ba^{2+}, and Sr^{2+} stimulate the process of activation. Ca^{2+} ions in examined concentrations do not influence the rate of the activation, although Mg^{2+}, Ba^{2+}, and Sr^{2+} increase the rate of the first phase of the reaction by 1.5, 2.0, and 2.0 times, and rate of the second phase by 2.0, 3.8, and 4.7 times, correspondingly (Fig. 2).

According to [10, 25], cations of the heavy metals Cu^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} have an inhibiting effect on the activation and catalytic activity of proteinases, while Ca^{2+} and Mg^{2+} ions are not effective. It was shown that amidolytic activity of Pm was inhibited by Zn^{2+}, Cu^{2+}, Cd^{2+}, and Au^{+} at concentrations 10^{-5}-10^{-3} M, while Ca^{2+} and Mg^{2+} ions at the same concentrations do not change it. The effect of similar cations on Pm catalytic activity also depends on the nature of the substrate. Cations of Zn^{2+} and Cu^{2+} at concentration $5 \cdot 10^{-4}$ M completely inhibit Pm proteolytic action on fibrinogen, while Cd^{2+} and Au^{+} inhibit it only partially [25].

The interaction of metal cations with residues of histidine in the catalytic triad of serine proteinases was shown in [9]. However, in the authors' opinion, effective inhibition occurs during interaction of the cations with enzyme–substrate complex. Conformational features of a substrate are also important for the inhibition effect.

The inhibition mechanism of the catalytic activity of serine proteinases in the presence of metal cations was studied in more detail with mutant trypsin using X-ray analysis [26]. Metal cations were shown to bind histidine of the active center of trypsin forming chelate compounds. As a result of reorganization of the active center, a water molecule comes into the active center where hydrogen bonds Asp102–O-delta-2 and His57–N-delta occur. This specific reorganization of the active center leads to the inhibition of the catalytic activity. It can be considered using all these data, that the main factor of inhibition of the catalytic activity is structural changes of the active center of serine proteinases under the influence of metal cations.

Sr^{2+} cations effectively accelerate the induction of Pg catalytic activity by mAB IV-Ic. The dose dependent effect of Sr^{2+} is shown on Fig. 3. The increase in the rate of the first phase of the reaction with the increase in Sr^{2+} concentration has graded character, increasing by 1.3-fold with increase in Sr^{2+} concentration from 0.4 to 0.6 mM and then staying constant until 1.2 mM (Fig. 3a). The functional dependence of the rate of the second

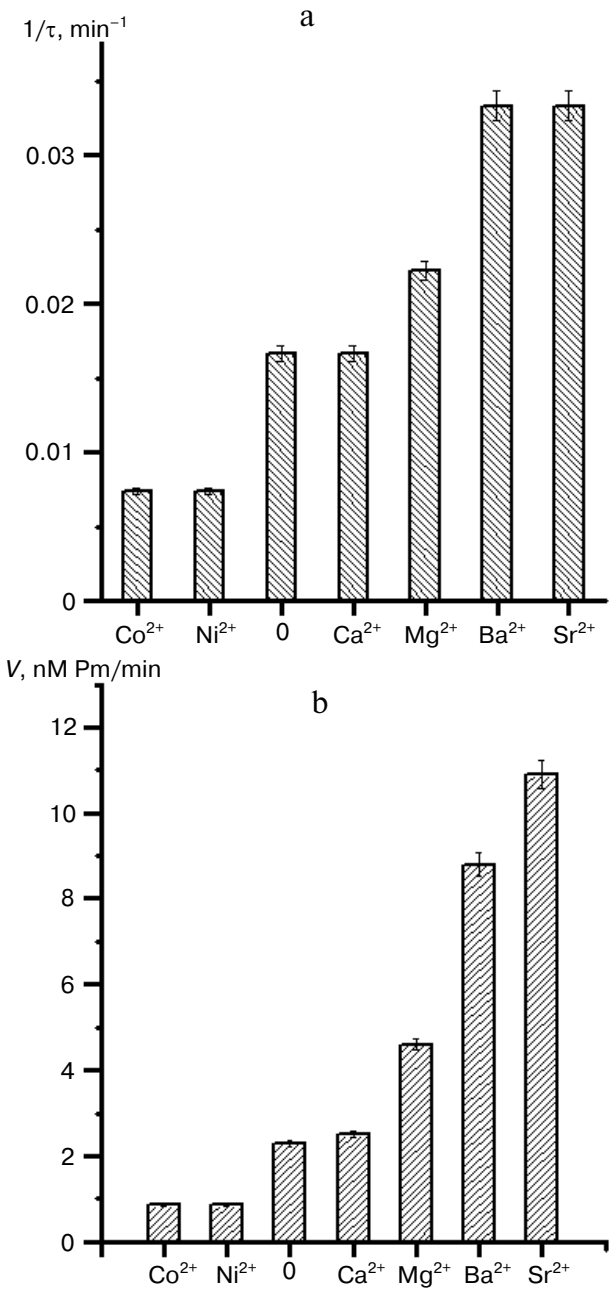

Fig. 2. Effect of bivalent metal cations (1.0 mM) on the rates of the first ($1/\tau$) (a) and the second (V) (b) phases of the reaction of Pg activation by mAB IV-Ic; 0, metal cations are absent.

phase of the reaction on Sr^{2+} concentration in the incubation medium has typical sigmoid character with half-maximum 0.6 mM and with plateau starting in the range 0.8-1.0 mM (Fig. 3b).

The first phase of mAB IV-Ic interaction with Pg is binding. This is a fast process (K_d = 0.5 nM) [23]. Sr^{2+} cations in the range of studied concentrations have almost no affect on the binding of Pg with mAB IV-Ic (table).

The presence of Sr^{2+} in the incubation medium (0.2-1.2 mM) does not change the amidolytic activity of Pm and does not affect activation of Pg by SK (table). We also did not find an effect of Ca^{2+} and Sr^{2+} at concentrations from 10^{-7} to 10^{-2} M on amidolytic activity of Pm and on the rate of activation of Glu-Pg by SK (table). Apparently, the Ca^{2+}-binding loop of Pg [27] is not directly related to the effect of Sr^{2+}. The influence of Sr^{2+} on the induction of catalytic activity in the Pg–IV-Ic complex is specific and mediated by the presence of the IV-Ic component.

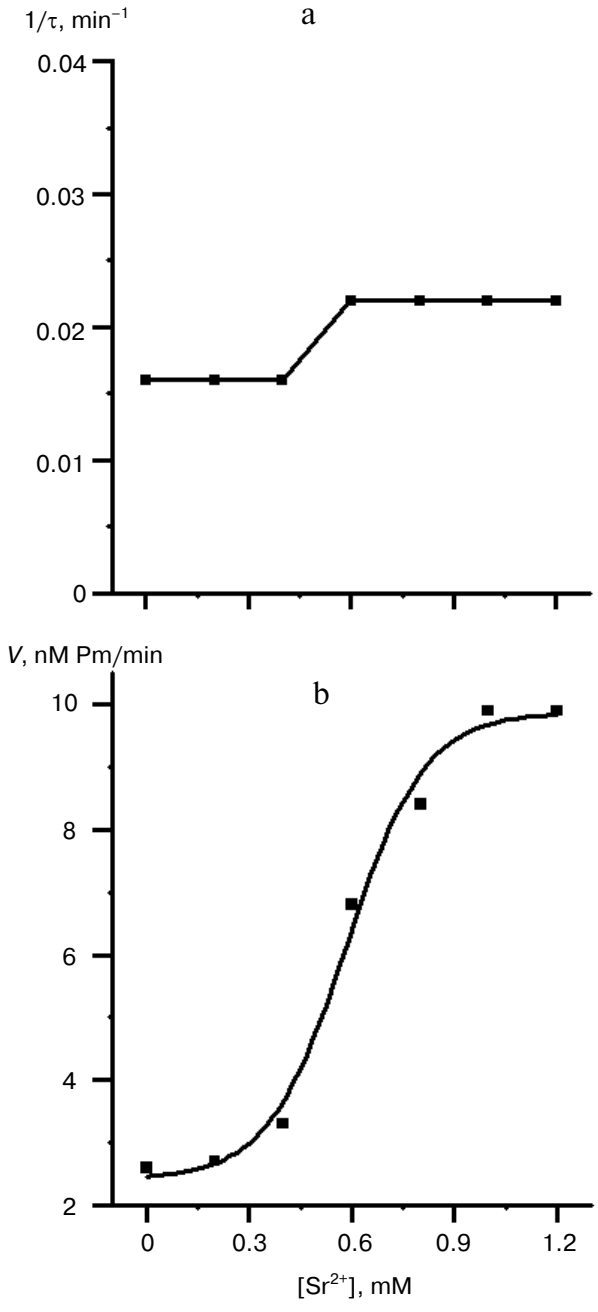

Fig. 3. Effect of Sr^{2+} cations on the rates of the first ($1/\tau$) (a) and the second (V) (b) phases of the reaction of Pg activation by mAB IV-Ic.

Effect of Sr^{2+} on the rate of S-2251 substrate hydrolysis by Pm, on the rate of the reaction of Pg activation by SK, and on the degree of binding of Pg with mAB IV-Ic

$[Sr^{2+}]$, mM	V_{Pm}, o.u./min	V_{PgSK}, o.u./min	Degree of Pg binding with mAB IV-Ic, %
0	0.053	0.050	100
0.2	0.052	0.049	105
0.4	0.053	0.051	105
0.6	0.054	0.050	105
0.8	0.052	0.051	107
1.0	0.052	0.050	110
1.2	0.053	0.049	110

Note: o.u., optical units.

It was shown that antibodies of IgG type can be metal-dependent. The presence of Sr^{2+}, Mn^{2+}, Ba^{2+}, or metal ions similar to Ca^{2+} has negative clinical effect in some diseases accompanied by accumulation of Ca^{2+}-dependent anti-prothrombin antibodies [28].

For catalytic antibodies, metal cations can play the role of cofactors. Different methods and procedures were used for the formation of the metal-binding sites in catalytic antibodies in order to increase their activity. However, binding of Zn^{2+}, Cu^{2+} [29] and Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+} did not enhance formation of the catalytic conformation [30].

Monoclonal antibodies of IgG type can have metal-binding sites [28, 31, 32]. It was shown that human polyclonal IgG antibodies and F′c fragments of these antibodies have higher affinity to bivalent metal ions (Cu, Ni, Zn, Co) than Fab′2. This can be explained by the presence of a histidine cluster in F′c antibody fragment of antibodies, most possibly in the sequence His433-x-His435 in the CH3 domain of the heavy chain [33]. Therefore, the metal-binding site for bivalent metal cations can also exist in mAB IV-Ic, and as a result of this binding, the role of this antibody in induction of Pg catalytic activity can change.

The influence of bivalent cations on the mechanism of active center formation in the Pg molecule and manifestation of amidolytic activity in Pg*–mAB IV-Ic can be examined in two directions. On one side, heavy metal cations react either directly with histidine in the active center or with the neighboring to the active center parts of the Pg molecule. The reorganization of the active center and alteration of the catalytic activity of activated complex occur as a result of this process. On the other side, the effect of the cations can be explained by their interaction with binding sites of antibodies. In the case of Sr^{2+}, stimulation of catalytic activity is mediated by antibody presence since Sr^{2+} does not affect the amidolytic activity of Pm and activation of Pg by SK. The presence of metal cations changes the mechanism of interaction of Pg with mAB IV-Ic. Thus the cations influence conformational changes in the process of active center formation and structure of the active center of Pg molecule in the complex with mAB IV-Ic. As a result of this influence we observed the change in rate of the reaction during all steps of the process of induction of Pg catalytic activity by mAB IV-Ic. The effect of bivalent metal cations on induction of Pg catalytic activity by mAB IV-Ic also depends on the nature of the metals.

It has been shown earlier that α_2-AP in equimolar ratio with Pm did not inhibit amidolytic activity of Pm–SK complex, but inhibited amidolytic activity of Pm–mAB IV-Ic by 80% [34]. The effect of α_2-AP within the range of concentrations from 5 to 30 nM on activation of Pg by mAB VI-Ic is shown in Fig. 4. The presence of α_2-AP in the incubation medium at concentration 5 nM extends the lag-period by 1.2-fold and reduces the rate of the active center formation by 3.6-fold. At concentration 30 nM the activation was almost completely suppressed, and the ratio Pg/α_2-AP equals 3 : 1. The dependence of the rate of the first and second phases of the reaction of Pg activation by mAB IV-Ic on concentration of α_2-AP is shown in Fig. 5.

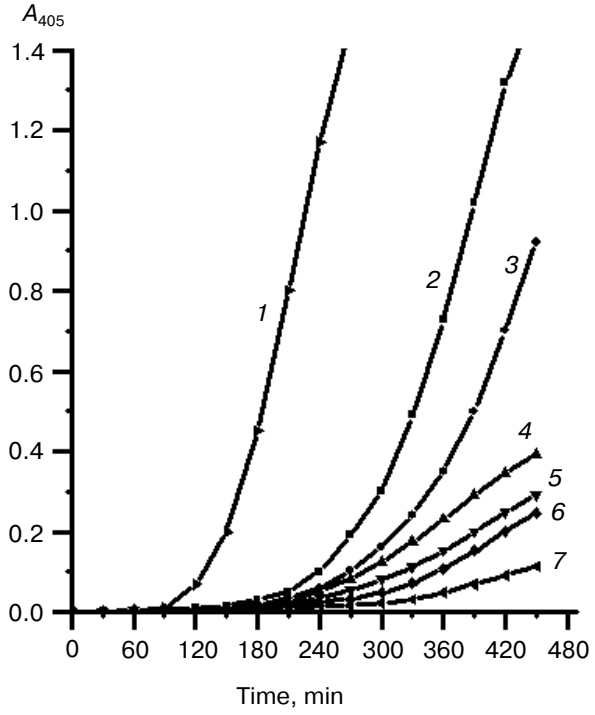

Fig. 4. Kinetic curves of the induction of Pg catalytic activity by mAB IV-Ic in the presence of α_2-AP (nM): *1*) 0; *2*) 5; *3*) 10; *4*) 15; *5*) 20; *6*) 25; *7*) 30 (A_{405} is absorbance at 405 nm). Concentration of the components: Glu-Pg and mAB IV-Ic, 100 nM; S-2251, 0.3 mM; buffer 0.1 M Tris-HCl, pH 7.4, 37°C.

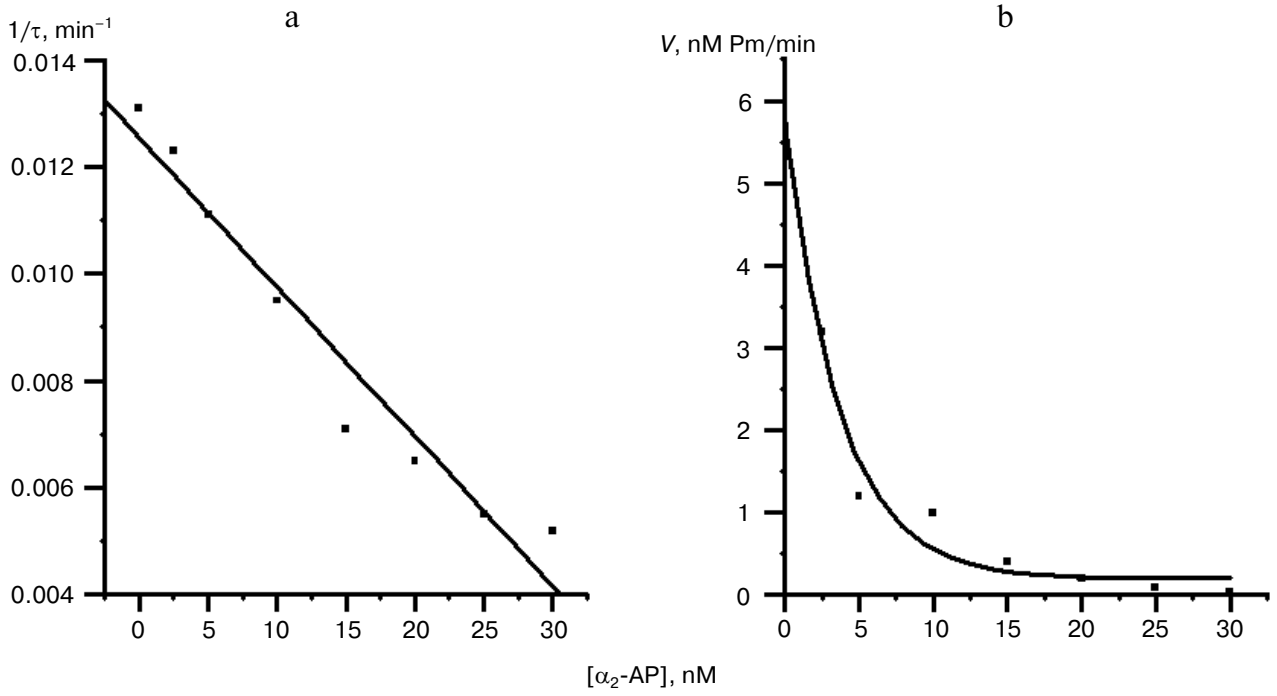

Fig. 5. Effect of α_2-AP on the rates of the first ($1/\tau$) (a) and the second (V) (b) phases of the reaction of Pg activation by mAB IV-Ic.

α_2-AP inhibits the rate of both phases of the activation with different affinity. $[I]_{50}$ for inhibition of the lag-phase was 15 nM, that is by an order of magnitude less than the value of the inhibition constant of Pm by α_2-AP (0.2 μM) [35]. α_2-AP inhibit the second phase of the reaction with higher affinity ($[I]_{50} \approx 3.5$ nM).

It was determined that the manifestation of the catalytic activity of Pg–IV-Ic complex requires the double-center interaction of IV-Ic paratope with serine proteinase domain of Glu-Pg and C-terminal lysine of the antibody with one of the lysine-binding sites of Pg. The equimolar complex of antibody IV-Ic with antigen (V709-G718) forms at first. Then Glu-Pg transforms into the conformation of the open Lys-form of Pg. Due to this transformation the interaction of lysine-binding sites of the first and fourth cringles with C-terminal lysine of one of the chains of IV-Ic becomes possible and causes the exposition of the active center in the protease domain [23].

Since α_2-AP also binds with Pg/Pm by lysine-binding sites [36], the competition for these sites is possible. As a result of absence of such a binding between Pg and mAB IV-Ic, the required conformational transformation and exposition of the active center do not occur, the lag-period prolongs, and the concentration of active centers decreases. These phenomena lead to deceleration of the rates of the first as well as the second phases of the reaction (Fig. 4). In the second phase of the reaction the active center in Pg*–mAB IV-Ic is formed, and interaction with α_2-AP by active center is very likely. The affinity of α_2-AP to Pg*–mAB complex increases, and $[I]_{50}$ decreases by 4-fold. The presence of α_2-AP in the incubation medium at the stage of active center formation in the Pg molecule impairs the interaction of Pg with monoclonal antibody, inhibits the active center in Pg*–mAB IV-Ic activated complex, and leads to decrease in the reaction rate of the individual phases and to the inhibition of the process as a whole.

Cations of bivalent metals and α_2-AP are important factors in the process of induction of Pg catalytic activity by mAB IV-Ic. These factors in the blood significantly contribute to the fibrinolytic system in the presence of anti-Pg antibodies.

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