Vol. 90, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 27, 1979 Pages 641-647

THE EFFECTS OF PHOSPHOLIPID AND FACTOR Va ON THE INHIBITION OF FACTOR Xa BY ANTITHROMBIN III

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Received July 17,1979

SUMMARY: The rate of inhibition of Factor Xa by antithrombin III was found to be influenced by either phospholipid or Factor Va combined with phospholipid. Our results confirmed that Factor Va and phospholipid could protect Factor Xa from inhibition. However, when antithrombin III levels were extrapolated to infinity, the protective effect of lipid and Factor Va were eliminated and the rate of inhibition was accelerated. This indicated that the protective effect that was observed at low antithrombin III concentrations in the presence of phospholipid and Factor Va was due to inhibition of binding of the inhibitor to Factor Xa.

INTRODUCTION

The formation of a clot in blood is mediated through the activation of a series of proteases which make up the "clotting cascade" (1). The final steps of the cascade are the conversion of prothrombin to thrombin and the thrombin catalyzed conversion of fibrinogen into the fibrin clot (1). The activation of prothrombin is a key step in the cascade because thrombin is believed to be a product activator of the cascade in addition to its role in clot formation (1). Prothrombin activation is catalyzed by the serine protease, Factor Xa, with the co-factors Factor Va, calcium ion and phospholipid (2,3).

The activity of the clotting serine proteases is regulated, in part, by plasma protease inhibitors (4) of which antithrombin III appears to be the most important (5,6). Antithrombin III inhibits proteases by forming a 1:1 covalent complex with the active site of the enzyme (7,8). It is effective against a wide range of the coagulation factors including thrombin, Factor Xa (9), IXa (9), XIa (10), XIIa (11), kallikrein (12), and one lytic factor, plasmin (10). Antithrombin III is also known as heparin co-factor as the rate of inhibition of serine proteases by antithrombin is enhanced by heparin (7).

The regulation of the antithrombin-protease interaction is a subject that has not received much attention. We have previously demonstrated that prothrombin fragment 2 can reduce the rate of antithrombin inhibition of thrombin (13). We found that the fragment interacted with thrombin and inhibited the binding of antithrombin to thrombin but had no effect on the rate of formation of the covalent complex.

0006-291X/79/180641-07$01.00/0

Several factors have been suggested to be involved in the regulation of the antithrombin-Factor Xa interaction. Yin (14) has suggested that phospholipid can protect Factor Xa from antithrombin III inhibition. However, contrasting this, Marciniak (15) has reported that Factor Va must be present in addition to calcium ions and phospholipid for Factor Xa to be protected. This apparent contradiction has existed in the literature for sometime. We have, therefore, reevaluated the effects of phospholipid and Factor Va as regulators of the Factor Xa-antithrombin interaction and assessed the importance of the Factor Va-phospholipid protection as a control mechanism in blood.

MATERIALS AND METHODS

Materials: Bovine blood was the generous gift of the Wilson Foods Corporation. The Factor Xa chromogenic substrate, N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-paranitroanilide hydrochloride, (S2222) was purchased from Ortho. All other reagents were the highest grade commercially available.

Proteins: Bovine Factor X was purified from oxalated bovine plasma exactly as described previously (16). Activated Factor X was prepared by activating Factor X with the Factor X activator from Russell's viper venom as described earlier (17). Factor V was purified as previously described (18) and converted to activated Factor V by incubation with thrombin (18). Factor Va was purified from the activation products and thrombin by chromatography on QAE Sephadex Q50 (18). Bovine antithrombin III was prepared by a modification of previous methodology (19). This isolation from barium sulfate absorbed plasma involves ammonium sulfate fractionation (40-70%), chromatography on insolubilized heparin (20), and ion exchange chromatography on DEAE Sephadex. The column was developed at room temperature with a linear gradient of 0.1 to 0.3 M NaCl in 0.02 M Tris-HCl, Benzamidine HCl, 0.2% sodium azide, pH 7.5 at room temperature. The rate of inactivation of thrombin could be increased more than 100-fold by the addition of heparin.

All proteins were greater than 95% homogeneous as judged by both the dodecyl sulfate electrophoresis and alkaline disc gel electrophoresis. Before use, benzamidine-HCl was removed from the protein preparations by dialysis.

The values for the molecular weight and $E^{1\%}_{280}$ used for calculating protein concentrations were: Factor Xa, 40,000, 10.0 (21); antithrombin III, 56,000, 6.0 (9); Factor Va, 180,000, 10.0 (18).

Phospholipid preparation: Phospholipid was prepared from acetone dried bovine brain by the method of Bligh and Dyer (22). Phospholipid vesicles were prepared by mixing the phospholipid in chloroform and then drying under nitrogen onto the walls of a glass tube. The lipid was dispersed into buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5) by sonicating the tube with a Branson bath sonicator for four hours at room temperature.

Determination of second-order rate constants: The second order rate constants were determined as previously described (13) by measuring the residual concentrations of Factor Xa. The data was plotted in the form A/(A-i) versus times where A is the concentration of antithrombin III and i stands for A_0-B_0 where A_0 is the initial inhibitor concentration and B_0 is the initial concentration of Factor Xa. The slopes of the lines plotted in this fashion is ki where k is the second order rate constant.

Residual Factor Xa was determined by a modification of the method of Bachman (24). At the indicated times samples were removed from the reaction mixture and diluted into 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 and 1 mg/ml

bovine serum albumin. The diluted Factor Xa (0.1 ml) was mixed with 0.1 ml phospholipid (0.5 mg/ml), 0.1 ml $CaCl_2$ (0.025 M). Clotting was initiated by the addition of bovine plasma. Activity was determined by comparing the clotting time to a standard curve prepared daily with the Factor Xa stock.

Determination of k_3: The interaction between irreversible enzyme inhibitors in general (25) and Factor Xa and antithrombin III, in particular (26), can be considered as a two step process. The first step is the formation of a weak complex which is dissociable followed by the formation of the tight or covalent (in the case of Factor Xa-antithrombin) complex. This series of reactions can be written in the stepwise reaction sequence:

$$Xa + ATIII \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Xa \cdots ATIII \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Xa — ATIII$$

where Xa is Factor Xa, ATIII is antithrombin III, Xa ··· ATIII is the reversible interaction between thrombin and antithrombin III and Xa — ATIII is the covalent complex. For this reaction the thrombin-antithrombin complex is covalent making k_4 negligible. The reaction sequence, therefore, simplified to:

$$Xa + ATIII \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Xa \cdots ATIII \xrightarrow{k_3} Xa — ATIII$$

where

$$Xa + ATIII \xrightarrow{k_{app}} Xa — ATIII.$$

ATIII Factor Xa then

$$k_{app} = \frac{k_3}{1 + K_I/ATIII}$$

then

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_I}{k_3} \cdot \frac{1}{ATIII}$$

When $1/K_{app}$ is plotted as a function of 1/ATIII the intercept of the vertical axis is $1/k_3$.

Determination of k_{app}. The inhibition of Factor Xa by antithrombin III was determined by incubating Factor Xa (0.02 μM) with the indicated concentrations of Factor Va, phospholipid and antithrombin III in 0.01 M $CaCl_2$, 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, and 0.2 mM S2222. The final volume of the reaction mixture was 0.600 ml and the reaction was carried out at room temperature. The reaction was initiated by the addition of Factor Xa and the course of the reaction was followed by measuring the appearance of the chromaphore, p-nitroanailide, at 405 nm with a Beckman model 25 double beam spectrophotometer. The pseudo first-order rate constant for inactivation was determined by plotting the slope of the reaction time course versus time. The slopes of these semi-log plots are $-k_{app}$. The constants obtained by this method are only apparent constants since the substrate, S2222 is an inhibitor of the Factor Xa-antithrombin interaction.

RESULTS

The inactivation of Factor Xa by antithrombin III was a second-order process. The second order rate constant for the inactivation of Factor Xa was observed to be 6.3×10^4 $M^{-1}min.^{-1}$. When phospholipid was added the rate of inhibition decreased. The inhibition of Factor Xa could be further slowed by the addition of Factor Va (Fig. 1). The effect of the phospholipid was

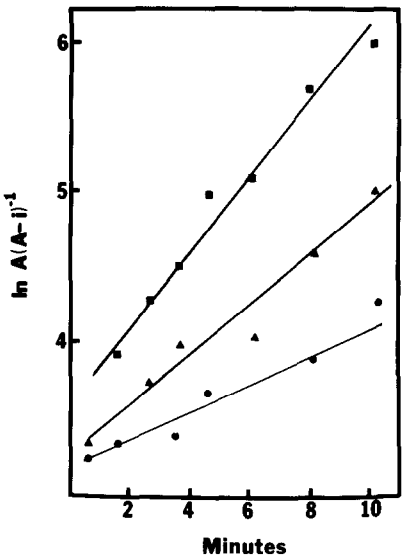
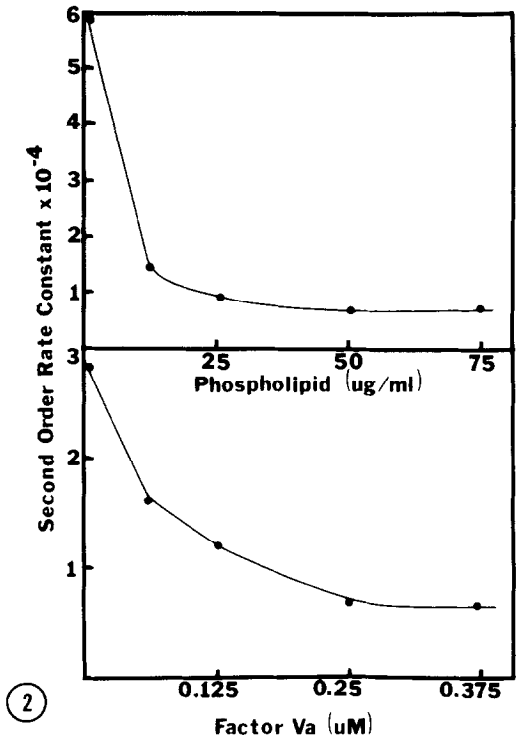


Figure 1. The effect of phospholipid or phospholipid and Factor Va on the inhibition of Factor Xa by antithrombin III. Factor Xa (0.2 μM) was incubated alone (■—■) or with phospholipid (10 μg/ml) (▲—▲) or with phospholipid (10 μg/ml) and Factor Va (0.25 μM) (●—●) in 0.01 M $CaCl_2$, 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, and bovine serum albumin (1 mg/ml) at 37°. The reaction was initiated by the addition of antithrombin III (4.3 μM). At the times indicated samples were removed and assayed as indicated in "Methods." The ln $(A/(A-i)^{-1})$ portion of the second-order rate expression is plotted versus time.

saturable. The maximum effect was observed with lipid concentrations of approximately 50 ug/ml. Under these conditions the second-order rate constant was reduced to 0.7×10^4 $M^{-1}min.^{-1}$ (Fig. 2). The effect of Factor Va on the rate of inhibition was also saturable (Fig. 2). In the presence of a limiting concentration of phospholipid the effect of Factor Va saturated approximately at 0.25 uM which is close to the Factor Xa concentration in the reaction mixture (0.2 uM).

The effect of phospholipid and Factor Va on the Ki of antithrombin for Factor Xa could be due either to an alteration of the binding properties (a reversible interaction between antithrombin and Factor Xa) between the inhibitor and enzyme or to an inhibition of the formation of the covalent complex. These two effects can be separated by measuring the effects of phospholipid or phospholipid and Factor Va on k_3, the rate of formation of the covalent complex. With this analysis we found that at low antithrombin III concentrations both phospholipid and phospholipid and Factor Va protected Factor Xa from inhibition by antithrombin. However, at high antithrombin concentrations the rate of inhibition was accelerated by both of these effectors (Fig. 3). Extrapolation to infinite antithrombin concentrations



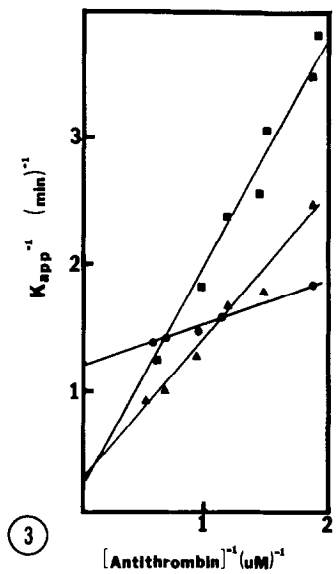


Figure 2. The effect of increasing concentrations of phospholipid or Factor Va on the second order rate constant of inhibition of Factor Xa by phospholipid. A. Factor Xa was incubated at 37° with the concentrations of phospholipid indicated the figure in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 with 1 mg/ml bovine serum albumin and 0.01 M $CaCl_2$. The reaction was initiated by the addition of antithrombin III (4.3 uM). The first order rate constant was determined as described in "Materials and Methods." B. The inhibition of Factor Xa was exactly as above except that the phospholipid concentration was fixed at 10 ug/ml and the Factor Va concentration was varied. The concentrations of the Factor Va are indicated in the figure.

Figure 3. The effect of phospholipid and Factor Va on the K_{app} of antithrombin III inhibition of Factor Xa. Factor Xa (0.02 uM) was incubated alone (●—●) or with phospholipid (83 μg/ml) (▲—▲) or with phospholipid (83 μg/ml) and Factor Va (0.03 μM) (■—■) in 0.01 M $CaCl_2$, 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, with 0.2 mM S2222 and the indicated contractions of antithrombin III. The reaction was initiated by the addition of Factor Xa and the hydrolysis of S2222 was followed for four minutes. K_{app} was determined as described under "Methods."

indicated that with either phospholipid or phospholipid and Factor Va the rate of complex formation was accelerated. Since k_3 was increased by both effectors the protective effect at low antithrombin concentrations must be due to a decrease in Factor Xa-antithrombin binding (k_1/k_2).

DISCUSSION

Confirming the results of Yin (14), we have observed that phospholipid can protect Factor Xa from inhibition by antithrombin. We also have confirmed

the observation that Factor Va and phospholipid can protect Factor Xa (15). Our results indicate that in the presence of phospholipid the maximal protective effect of Factor Va is at a concentration equal to that of Factor Xa. This indicates that the stoichiometry between Factor Va and Factor Xa may be 1:1.

It is also possible that the conflict between the results reported by Yin and those reported by Marciniak can be resolved by some of our observations. Marciniak reported that phospholipid alone could not protect Factor Xa from antithrombin III (15). We have observed that, at high concentrations of antithrombin III, the protection by phospholipid was abolished. It is possible that the observation that phospholipid did not protect could be due to the use of conditions in which the antithrombin III concentration was high enough that the lipid effects was abolished while the Factor Va-lipid effect remained.

The inhibition of Factor Xa by antithrombin III followed second-order kinetics. The second-order rate constant that we report is smaller than the value reported by Jesty. However, the value reported by Jesty (27) was determined under pesudo-first order reaction conditions. The value that we report is also smaller than the value that we (13) and others (6) have reported for the inhibition of thrombin (8.5×10^5 M^{-1} $min.^{-1}$). These results would indicate that Factor Xa is inhibited more slowly than is thrombin by antithrombin III.

Our results indicate that the inhibition of the rate of the antithrombin III-Factor Xa interaction by either phospholipid or phospholipid and Factor Va is due to a reduction of the affinity between the inhibitor and the enzyme. It may be that when Factor Xa is bound to lipid that antithrombin III is sterically hindered from binding to the correct site on the enzyme. The problem would be magnified when Factor Va is bound to Factor Xa since Factor Va is an extremely large protein ($Mr \approx 200,000$). It is interesting to note that once the inhibitor binds in a reversible manner to the enzyme, the rate of the covalent complex formation is enhanced by phospholipid. Factor Va does not appear to contribute to the covalent complex formation since in the presence of lipid, k_3 was not increased by the addition of Factor Va. This is an interesting result in the light of the observation that phospholipid enhances the rate of prothrombin activation (28) and the addition of Factor Va gives a further rate enhancement (29). Besides prothrombin, antithrombin III is one of the few known protein substrates for Factor Xa. The observation that phospholipid enhances the rate of the covalent complex formation between Factor Xa and antithrombin may be due to a process that is similar to lipid enhancement of prothrombin activation.

ACKNOWLEDGEMENTS

This study was supported by a grant from the National Heart, Lung and Blood Institute, grant #HL-17812 and a grant-in-aid from the American Heart Association and with funds contributed in part by the Oklahoma Heart

Association. This work was done during the tenure of the John L. Dickson Memorial of the American Heart Association. F. J. W. was supported by a postdoctoral fellowship from the Oklahoma Division of the American Heart Association.

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