Effect of Surfaces on Fluid-Phase Prekallikrein Activation

By Cheryl F. Scott, Edward P. Kirby, Paul K. Schick, and Robert W. Colman

The activation of prekallikrein by factor XII fragments (XII), during incubation in plastic tubes was previously noted to be increased by high molecular weight (HMW) kininogen as well as other plasma proteins. In this report, we investigated the mechanism responsible for this increase. Although we confirmed that HMW kininogen, bovine serum albumin, fibrinogen, cold insoluble globulin, and mixed phospholipids apparently increased prekallikrein activation, we found that the product of prekallikrein activation (kallikrein) lost substantial activity in less than 0.5 min after exposure to a variety of fresh surfaces. This loss was partially prevented by the presence of various protective substances in the assay system. Thus, the surface could be decreased by including "protective" substances in the assay system. Therefore, we concluded that various substances apparently affect prekallikrein activation in a purified system by preventing the enzyme and product in the reaction mixture from losing activity due to adsorption to a surface.

Materials and Methods

Deficient Plasma

Prekallikrein-deficient plasma was kindly supplied by Dr. C. Abildgaard, University of California, Davis, Calif. Factor-XI-deficient plasma, with less than 0.05 U/ml factor XI, was donated directly to us. Kinonogen-deficient plasma was donated to us by Mrs. M. Williams.

Fibrinogen

Grade I (95% clottable) fibrinogen was purchased from Kabi, Stockholm, Sweden. Fibrinogen demonstrated no detectable amidolytic activity. Fibrinogen incubated with prekallikrein failed to generate amidolytic activity.

Fresh Frozen Plasma

Fresh frozen plasma for protein purification was generously supplied by the American Red Cross, 23rd and Chestnut Street, Philadelphia, Pa.

Factor VIII Concentrate

Hemofil was obtained from Hyland, Division of Travalen Laboratories, Inc., Costa Mesa, Calif.

Other Reagents

Mixed soybean phospholipids (Inosithin) were purchased from American Concentrates, New York, N.Y. Bovine serum albumin...
High Molecular Weight Kininogen

This was prepared by the method of Kerbiriou and Griffin.12 The specific activity was 12 coagulant units1 per milligram protein.13 A single band of Mr = 110,000 on reduced SDS polyacrylamide gel electrophoresis was observed. It had no endogenous amidolytic activity and did not generate amidolytic activity when incubated with prekallikrein.

Bovine Cold Insoluble Globulin

This was prepared by the method of Engvall and Ruoslahti.14 No endogenous amidolytic or prekallikrein activator activity was present. The protein concentration was determined by the method of Lowry et al.15

Prekallikrein Activity

Prekallikrein (40 μg/mL containing IgG 1 mg/mL) in 0.1 M Na phosphate, pH 7.6, containing 0.15 M NaCl, and 50 μg/mL BSA was used as a stock substrate. XII, (25 μg/mL), plus 50 μg/mL BSA in the same buffer was used as the stock enzyme. Five microliters of the stock prekallikrein was added to 20 μL buffer or sample to be tested and preincubated for 1 min at 25°C. Then, 5 μL of stock XII, were added and incubated at 25°C. At precisely 2 min, 20 μL were then removed and assayed for kallikrein activity (see below).

Kallikrein Assay

Twenty microliters of the activation mixture (prekallikrein and XII,) or preformed kallikrein were transferred to a prewarmed glass cuvette (37°C) containing 330 μL of H-D-Pro-Phe-Arg-pNA (0.09 mM) in the same buffer, as described above. The change in absorbance per minute at 405 nm was measured over the first 4 min and was always linear during that time period. The absorbance per minute was converted to μmol hydrolysed/min/mL using an extinction coefficient of 10,600 for p-nitroaniline at this pH and wavelength. At H-D-Pro-Phe-Arg-pNA = 0.09 mM, the XII,, 4 μg/mL used to activate prekallikrein had no detectable amidolytic activity under these conditions. XII,, was assayed directly on H-D-Pro-Phe-Arg-pNA (0.5 mM) in the same buffer described above. The concentration of the chromogenic substrate in the direct assay of XII,, was 5.5 times greater than that used in the kallikrein assay, and much higher concentrations of XII,, (25–50 μg/mL) were employed in the direct assay of XII, than were required for prekallikrein activation. Purified kallikrein and XII, did not lose any activity in the cuvette during this period, as evidenced by the linearity of the substrate hydrolysis. All enzyme assays were performed on a Gilford Model 240 recording spectrophotometer.

Radial Immunodiffusion of Kallikrein

Rabbit antiserum against kallikrein, which has been shown to be monospecific,8 was employed at a final concentration of 2.8% in a radial immunodiffusion assay.8 A standard curve was prepared from HMW kininogen-deficient plasma in order to quantitate uncomplexed kallikrein.13

Statistical Methods

The data in Fig. 2 and Table 1 were analyzed by the Gauss-Newton method18 of nonlinear regression using a computer program. Other data were calculated by linear regression, using a computer program, and then the lines were extrapolated to time zero in order to determine initial loss of activity. This method demonstrated similar results to the nonlinear regression method for calculating initial loss.

RESULTS

Effect of Proteins and Phospholipids on the Activation of Prekallikrein

We studied the effect of HMW kininogen, components of cryoprecipitate, BSA, and phospholipids on the "fluid-phase" activation of prekallikrein. HMW
kininogen and BSA (100 μg/ml) apparently increased prekallikrein activation twofold under our assay conditions (Fig. 1A). Fibrinogen, mixed phospholipids (inosithin), and cold insoluble globulin were also found to increase the measurable kallikrein activity in the assay (Fig. 1B). These findings confirm previous observations that HMW kininogen and other substances could apparently increase XII-catalyzed prekallikrein activation.

**Effect of Proteins and Phospholipids on Kallikrein and XII Activity**

To investigate whether these substances were affecting the enzyme (XII), substrate (prekallikrein), or product (kallikrein) in the prekallikrein activation assay, we studied the influence of each of the potentiating substances on the activity of kallikrein or XII. One-hundred microliters of kallikrein (8 μg/ml), containing IgG (200 μg/ml) (Fig. 2) or 100 μl XII (25 μg/ml) were incubated in a polypropylene microcentrifuge tube (0.75-ml capacity) in the presence and absence of substances found to affect prekallikrein activation (see Fig. 1) at a final concentration of 100 μg/ml. At various times, aliquots were removed and assayed for amidolytic activity. XII showed an immediate initial activity loss, after which the activity remained stable (data not shown). For kallikrein, a biphasic semilogarithmic loss of activity was observed in all cases. The initial loss was so rapid (T₁/₂ < 0.5 min) that insufficient data points were available for direct comparison of the rate constants. Addition of phospholipid or various proteins greatly affected the initial loss of kallikrein activity (Fig. 2 and Table 1). The T₁/₂ for kallikrein loss did not differ greatly in the experiments containing an additional protein or phospholipid (Table 1). Thus, various proteins and phospholipids can decrease the initial loss of kallikrein and XII activity, but not the secondary loss of kallikrein activity.
Effect of IgG on Kallikrein Activity

The effect of IgG on kallikrein activity was also studied. One-hundred microliters of kallikrein (16 \( \mu g/ml \)) containing 400 \( \mu g/ml \) IgG, as well as IgG-free kallikrein, were incubated in a 0.75-ml polypropylene microcentrifuge tube in the presence and absence of BSA (100 \( \mu g/ml \)). A biphasic semilogarithmic activity loss was also observed in all cases. The initial activity loss was 50% for the IgG-free kallikrein and was 30% for kallikrein containing IgG. In the presence of BSA, however, the IgG-free kallikrein lost 10.5% of its original activity and the kallikrein containing IgG lost 7.1%. Since IgG, even at 400 \( \mu g/ml \), had little effect on the initial loss of kallikrein activity as compared to proteins such as BSA, we chose to use the kallikrein containing IgG for all subsequent experiments.

Effect of Multiple Exposures of XII, to Polypropylene

When XII, (Fig. 3) was exposed to a polypropylene surface and then subsequently reexposed to a fresh polypropylene surface, a loss of activity occurred upon each exposure, after which the activity remained constant. This indicates that exposure to a polypropylene surface is directly causing a loss of XII, activity.

Effect of Other Surfaces on Kallikrein Activity

One-hundred microliters of kallikrein (8 \( \mu g/ml \)) were incubated in either a 10 \( \times \) 75 mm glass or 10 \( \times \) 75 mm polystyrene test tube in the presence and absence of BSA (100 \( \mu g/ml \)). The initial activity loss for the sample incubated in glass with buffer was 42%, and polystyrene with buffer was 40%. The initial activity loss for the sample incubated with BSA in glass was 13%, and for BSA in polystyrene was 11%. Therefore, glass and polystyrene inactivate kallikrein similarly to polypropylene.

Effect of Precoating Polypropylene Tubes on the Loss of Kallikrein Activity

Polypropylene microcentrifuge tubes (0.75-ml capacity) were filled with either BSA (10 mg/ml), fibrinogen (3.5 mg/ml), or plasma (1:2 dilution). After incubation for 18 hr, the tubes were rinsed inverted, drained, and then air dried. Fibrinogen was also incubated in a tube for 2 hr, rinsed, and transferred to an oven at 100°C where it was maintained until dry. One-hundred microliters of kallikrein (8 \( \mu g/ml \)) were then incubated in the tubes under the conditions outlined in Fig. 2. The initial loss of activity for fibrinogen, plasma, and BSA ranged from 32% to 39%, similar to the buffer control (41%). The heated fibrinogen gave the best protection (22% loss), although it was not as effective as including proteins in the incubation mixture (see Table 1).

Table 1. The Effect of Proteins and Phospholipids on the Loss of Kallikrein and XII, Activity

<table>
<thead>
<tr>
<th>Protecting Substance ±</th>
<th>Kallikrein</th>
<th>XII,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Loss of Activity (%) ± SEM</td>
<td>Secondary Loss (min ± SEM)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>9.2 ± 1.9</td>
<td>55.4 ± 6.5</td>
</tr>
<tr>
<td>Mixed phospholipids</td>
<td>11.3 ± 1.8</td>
<td>50.1 ± 6.5</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>15.6 ± 3.6</td>
<td>48.0 ± 9.0</td>
</tr>
<tr>
<td>HMW kininogen</td>
<td>18.4 ± 1.0</td>
<td>44.7 ± 4.8</td>
</tr>
<tr>
<td>Cold insoluble globulin</td>
<td>32.0 ± 6.3</td>
<td>64.4 ± 27.6</td>
</tr>
<tr>
<td>None (buffer control)</td>
<td>40.6 ± 1.0</td>
<td>25.6 ± 1.4</td>
</tr>
</tbody>
</table>

*Data derived from nonlinear regression (Fig. 2).

Kallikrein (8 \( \mu g/ml \)) containing IgG (200 \( \mu g/ml \)) or XII, (25 \( \mu g/ml \)) was incubated in a final volume of 100 \( \mu l \) in a 0.75-ml polypropylene microcentrifuge tube in the presence or absence of additional protein or phospholipid (100 \( \mu g/ml \)).
Phe-Arg-pNA was added to each tube (0.09 mM to the former and 0.5 mM to the latter). The substrate was incubated in each tube at 37°C for 10 min and the absorbance at 405 nm was read against substrates that had been incubated in clean tubes under the same conditions. No measurable hydrolysis of the substrate occurred in either case. This observation indicates that when kallikrein or XIIa binds to the polypropylene, it loses the ability to hydrolyse H-D-Pro-Phe-Arg-pNA.

Kallikrein (40 µg/ml) was also subjected to a series of successive transfers into 8 polystyrene tubes (10 x 75 mm; Fig 4, curve A) in order to quantify the surface adsorption. The supernatant was assayed for amidolytic activity in various tubes following a 2-min incubation. A progressive decrease in activity was observed coincident with the increasing number of transfers. Following 8 transfers, the activity in the supernatant was only 16% of the original activity. Kallikrein antigen in the supernatant was then quantified by radial immunodiffusion. The residual antigen, after 8 transfers, was 18.5%, in good agreement with the residual activity. To explore the effect of adsorption on further loss of kallikrein, an additional 100 µl of kallikrein was added to the first tube, which had been previously exposed to kallikrein (tube 1, curve A) and successive transfers carried out again. At each point measured (curve B), the loss of activity was considerably less than what was observed with clean tubes from curve A. A similar experiment (curve C) showed even less activity loss. The loss of activity was greater in the later tubes than in the earlier tubes since kallikrein was adsorbed onto the surfaces of the earlier tubes and therefore, was not available for further surface adsorption.

**Influence of Experimental Vessel Surface Area on Kallikrein Activity**

To further demonstrate that the loss of activity was a direct result of surface adsorption leading to inactivation, we incubated various concentrations of kallikrein (containing IgG) in polypropylene vessels, varying the ratio of surface area to sample volume. The initial activity loss was then calculated and plotted against the surface area contacted by each sample (Fig. 5). For any concentration of kallikrein tested, the absolute loss of activity increased as the surface area increased. As the concentration of kallikrein was increased at each surface area tested, a greater absolute loss of kallikrein activity resulted. At a given surface area to volume ratio, the proportion of kallikrein lost was fairly constant at kallikrein concentrations between 4 and 16 µg/ml (Table 2). Above 16 µg/ml, however, this relationship did not hold, possibly due to saturation of many sites on the plastic surface.

**Influence of Surface Area on the Prekallikrein Activation Assay**

The observation that the activity loss of XIIa and kallikrein on polypropylene was decreased by the addition of certain proteins and phospholipids suggested that the apparent increase in prekallikrein activation produced by the substances in Fig. 1 may be due to their ability to prevent the loss of both the enzyme (XIIa) and product (kallikrein) from the reaction mixture. This was supported by the observation (Fig. 6) that the enhancement observed upon adding BSA to the incubation mixture was most pronounced under
Effect of BSA on XII-Catalyzed Prekallikrein Activation in the Presence of HMW Kininogen

The question then remained whether or not HMW kininogen had an additional enhancing effect on purified prekallikrein activation in the "fluid-phase," other than preventing the loss of activity on the surface of the incubation vessel. To answer this, we tested the effect of added BSA on the apparent ability of HMW kininogen to increase prekallikrein activation (Fig. 7). As the concentration of BSA was increased, the apparent increase in prekallikrein activation, caused by addition of HMW kininogen, was diminished until the effect of HMW kininogen became negligible. Nevertheless, it should be noted that 360 µg/ml of BSA were required to partially counteract the activity increase produced by 80 µg/ml HMW kininogen, and even higher concentrations of BSA were needed to completely obliterate the effect of HMW kininogen.

Table 2. The Relative Loss of Kallikrein Activity as a Function of Surface Area

<table>
<thead>
<tr>
<th>Surface Area</th>
<th>Initial Loss of Activity (%)</th>
<th>Kallikrein µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(sq mm)</td>
<td>Volume (µl)</td>
<td>Volume (sq mm/µl)</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>1.00</td>
</tr>
<tr>
<td>122</td>
<td>100</td>
<td>1.22</td>
</tr>
<tr>
<td>75</td>
<td>50</td>
<td>1.50</td>
</tr>
<tr>
<td>49</td>
<td>30</td>
<td>1.63</td>
</tr>
</tbody>
</table>
| *Data calculated from Fig. 5.*
Thus, HMW kininogen does not appear to be a specific potentiator of "fluid-phase" prekallikrein activation in a purified system.

**DISCUSSION**

In this study we have examined the determinants of "fluid-phase" activation of prekallikrein. In particular, we have delineated factors affecting the effective concentration of the substrate, enzyme, and product in the vessels used in experiments where prekallikrein was activated by XII. To evaluate the role of surface, we have considered critical variables such as surface area, concentration of reactants, and the effects of various proteins alone as well as in combination. The ability of various substances to apparently increase XII-catalyzed prekallikrein activation in test tubes (Fig 1) in a purified system was explained by the observation that each substance tested could diminish the extent of activity loss of kallikrein and XII experienced upon contacting the surface (Fig. 2, Table 1). The observed activity loss was clearly due to exposure to fresh surface (Figs. 3 and 4). Activity loss was not due to low protein concentrations in the incubation mixture, since when IgG was present at a final concentration of 200 μg/ml in the kallikrein samples, we still observed rapid activity loss when the sample was transferred to a clean vessel. Kallikrein lost appreciable activity upon contacting a surface even in the presence of 400 μg/ml IgG.

By preventing a decrease in the concentration of reactants and/or product in the reaction mixture, both the rate of activation as well as the yield of the product were apparently increased (Fig. 1). Since we observed the greatest loss of activity where the ratio of surface area to volume was the largest (Fig. 5), we concluded that HMW kininogen, as well as the other substances tested, were protecting against surface adsorption and inactivation (Fig. 6) of kallikrein and XII rather than potentiating the activity of XII as previously reported. However, in order to mask the protective effect of HMW kininogen, it was necessary to include concentrations of BSA in the incubation mixture that were more than tenfold greater than the HMW kininogen utilized (Fig. 7). Furthermore, the "enhancement effect" of HMW kininogen on kallikrein activity is only observed if the HMW kininogen is added to the experimental vessel before the kallikrein. Thus, when kallikrein was added to the incubation mixture before HMW kininogen, the HMW kininogen did not affect the kallikrein activity. In those experiments involving highly purified reagents, the order of addition of those reagents, surface area, and competition of HMW kininogen for binding sites at low albumin concentrations probably influenced the apparent rate of prekallikrein activation that was observed. Our previous data also showed that the greatest "enhancement" of XII-catalyzed prekallikrein activation occurred at prekallikrein concentrations between 4 and 16 μg/ml, which is the range of concentrations where adsorption effects would be evident.

In contrast to what was seen with kallikrein, we observed that prekallikrein failed to lose activity after exposure to polypropylene, suggesting that it does not bind to hydrophobic surfaces. This behavior contrasts with the binding of prekallikrein to negatively charged surfaces. However, plastic, glass, and kaolin have a similar effect on kallikrein activity. Ratnoff and Saito observed inactivation of kallikrein by kaolin, which was prevented by pretreatment of the kaolin with HMW kininogen, and to a lesser extent, by IgG and cytochrome-C. As was observed on kaolin, the activity loss that we found could be prevented by the prior presence of other proteins or phospholipids. This study suggests that potentiators of "fluid-phase" prekallikrein activation that have been previously described were most likely proteins and/or phospholipids that prevented inactivation of components in the reaction mixture. Lee et al. observed that in a mixture of albumin, IgG, and fibrinogen, fibrinogen was preferentially adsorbed to hydrophobic polymers when the proteins were present at the same concentrations. It is therefore not surprising that fibrinogen protected kallikrein against surface adsorption more efficiently than the other proteins tested. Furthermore, since saturation of a plastic surface occurs with a monolayer of molecules, a point would be reached in a vessel where there would be no further activity loss as a result of adsorption. This phenomenon would allow kallikrein or XII to be efficiently stored. In the absence of other "protecting substances," some kallikrein or XII would initially be lost on the tube until saturation was achieved, but subsequently, there would be no loss. It would be advantageous, therefore, to reuse the container for future storage of kallikrein or XII or to store very concentrated material.

It is of interest that HMW kininogen, in the presence of BSA, was able to prevent activity loss on a hydrophobic surface at lower concentrations than either HMW kininogen or BSA alone (Fig. 7). Therefore, low concentrations of HMW kininogen would certainly influence the study of a purified "fluid-phase" prekallikrein activation system by providing protection from activity loss. This protection, however, is not limited to this cofactor. However, a specific effect of HMW kininogen on XII-catalyzed prekallikrein activation in plasma does exist. We have demon-
strated that HMW kininogen can protect kallikrein from inactivation by protease inhibitors, such as C1-inhibitor, in plasma. Therefore, in plasma, HMW kininogen would appear to accelerate "fluid-phase" prekallikrein activation by protecting the measurable product in the incubation mixture.

This study provides a methodological approach to evaluating the influence of experimental vessel surfaces on the reactants under investigation. Clearly, it is important to prevent this type of effect when working with these proteins, or possibly other proteins, before interpreting experimental results.

Finally, these results may have implications for the understanding of in vivo prekallikrein activation that may occur in disease states. Biologic surfaces may either increase or decrease the formation of kallikrein from prekallikrein via activated factor XII. Furthermore, HMW kininogen may not only serve to facilitate the activation of zymogens to active enzymes, but also to protect these enzymes, once formed, from inactivation by naturally occurring protease inhibitors or possibly from inactivation on biologic surfaces.

ACKNOWLEDGMENT

We would like to acknowledge stimulating discussions by Dr. Leo Vroman. We also thank Lee D. Silver for preparing high molecular weight kininogen, Dr. Marek Kloczewiak for performing statistical analyses of the data, and Terry Cruice for typing this manuscript.

REFERENCES

Effect of surfaces on fluid-phase prekallikrein activation

CF Scott, EP Kirby, PK Schick and RW Colman