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Autoactivation of Human Plasma Prekallikrein*

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Incubation of purified human plasma prekallikrein with sulfatides or dextran sulfate resulted in spontaneous activation of prekallikrein as judged by the appearance of amidolytic activity toward the chromogenic substrate H-p-Pro-Phe-Arg-p-nitroanilide. The time course of generation of amidolytic activity was sigmoidal with an apparent lag phase that was followed by a relatively rapid activation until finally a plateau was reached. Soybean trypsin inhibitor completely blocked prekallikrein activation whereas corn, lima bean, and ovomucoid trypsin inhibitors did not. The $K_i$ of the reversible inhibitor benzamidine for autoactivation (240 $\mu$M) was identical to the $K_i$ of benzamidine for kallikrein. Thus, spontaneous prekallikrein activation and kallikrein showed the same specificity for a number of serine protease inhibitors. This indicates that prekallikrein is activated by its own enzymatically active form, kallikrein. Immunoblotting analysis of the time course of activation showed that, concomitant with the appearance of amidolytic activity, prekallikrein was cleaved. However, prekallikrein was not quantitatively converted into two-chain kallikrein since other polypeptide products were visible on the gels. This accounts for the observation that in amidolytic assays not all prekallikrein present in the reaction mixture was measured as active kallikrein. Kinetic analysis showed that prekallikrein activation can be described by a second-order reaction mechanism in which prekallikrein is activated by kallikrein. The apparent second-order rate constant was 2.7 X 10^4 M^{-1} s^{-1} (pH 7.2, 50 $\mu$M sulfatides, ionic strength $I$ = 0.06, at 37°C). Autocatalytic prekallikrein activation was strongly dependent on the ionic strength, since there was a considerable decrease in the second-order rate constant of the reaction at high salt concentrations. In support of the autoactivation mechanism it was found that increasing the amount of kallikrein initially present in the reaction mixture resulted in a significant reduction of the lag period and a rapid completion of the reaction while the second-order rate constant was not influenced. Our data support a prekallikrein autoactivation mechanism in which surface-bound kallikrein activates surface-bound prekallikrein.

Human plasma prekallikrein is the zymogen of a serine protease which circulates in the blood. Incubation of plasma with negatively charged surfaces such as glass, kaolin, sulfatides, or dextran sulfate results in contact activation, a process during which the zymogen prekallikrein becomes activated to kallikrein through limited proteolysis (for recent reviews see Refs. 1 and 2). The enzyme primarily responsible for the activation of prekallikrein during contact activation is activated blood coagulation Factor XII. Kallikrein in its turn catalyzes the activation of Factor XII, thus amplifying its own formation. Kallikrein is also responsible for the liberation of bradykinin from high molecular weight kininogen. Recently, it was reported that kallikrein can also activate prourokinase (3) and plasminogen (4) and that it can function as an activator of neutrophils (5, 6). Thus, plasma kallikrein exhibits a broad spectrum of activities and can exert many functions.

During the course of our studies on the function of negatively charged surfaces in the activation of prekallikrein, we observed that under certain experimental conditions incubation of purified prekallikrein with surfaces such as sulfatides or dextran sulfate resulted in spontaneous activation of the zymogen. The present report concerns a kinetic evaluation of this process and a description of the experimental conditions under which it occurs. It is shown that prekallikrein is autocatalytically cleaved by its own enzymatically active form, kallikrein, in a reaction that is dependent on the presence of a suitable negatively charged surface.

EXPERIMENTAL PROCEDURES

Materials

Materials for the purification of prekallikrein, kallikrein, and its isolated heavy and light chains were obtained from sources described previously (7). H-D-Pro-Phe-Arg-p-nitroanilide (S 23022) was obtained from AB Kabi Diagnostica. Benzamidine, bovine serum albumin, soybean trypsin inhibitor, ovomucoid trypsin inhibitor, and lima bean trypsin inhibitor were from Sigma. Bovine brain sulfatides were purchased from Supelco. Dextran sulfate (M, 500,000 average) was from Pharmacia P-L Biochemicals. H-D-Phe-Phe-Arg-chloromethyl ketone was obtained from Behring Diagnostica. Materials necessary for immunoblotting were obtained from sources described previously (8, 9). All other materials were of the highest grade commercially available.

Methods

Protein Preparations—Prekallikrein, kallikrein, and its isolated light chains were purified as described previously (7). The protein preparations were homogeneous as judged by gel electrophoresis on 10% polyacrylamide gels (with 5% stacking gel) run in the presence of sodium dodecyl sulfate as described by Laemmli (10). Protein concentrations were determined according to Lowry et al. (11) using bovine serum albumin as a standard. The purified prekallikrein had a specific clotting activity of 21 units/mg protein. Corn trypsin

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1 The abbreviations used are: S 23022, H-D-Pro-Phe-Arg-p-nitroanilide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Prekallikrein Autoactivation

Inhibitor (single chain) was purified from sweet corn according to the method of Swartz et al. (12).

**Measurement of Prekallikrein Activation**—Prekallikrein activation was carried out in 1.6-ml Sarstedt polyethylene snap-cap centrifuge tubes. To prevent loss of protein to the tube walls, the tubes were preincubated overnight at 20°C with 50 mM Hepes (pH 7.2) containing 5 mg/ml ovalbumin. After 5 min, 50 μl of sulfatides in 50 mM Hepes (pH 7.2), 60 mM NaCl were added to start the reaction. After various time intervals, aliquots were transferred to a cuvette (500 μl total volume) containing 100 mM Tris (pH 8.3 at 37°C), 150 mM NaCl, 0.5 mg/ml ovalbumin, and 385 μM S 2302. The solution of S 2302 by prekallikrein was determined using a Cary 210 spectrophotometer (thermostatted at 37°C). From the absorbance change at 405 nm the amount of kallikrein present in the sample was calculated using a calibration curve made under the same conditions with known amounts of kallikrein.

**Kinetic Data Analysis**—The simplest mechanism to describe auto-activation of prekallikrein (PK) is given by the following second-order mechanism:

\[
\text{prekallikrein} + \text{kallikrein} \rightarrow \text{2 kallikrein}
\]  

(1)

The rate equation for kallikrein (K) formation is as follows.

\[
d[K]/dt = k_4[PK][K]
\]  

(2)

Defining \([PK]_{\text{total}}\) as the total amount of kallikrein plus prekallikrein present in the reaction mixture Equation 2 becomes the following.

\[
d[K]/dt = k_4([PK]_{\text{total}} - [K])[K]
\]  

(3)

Integration yields Equation 4.

\[
\ln([PK]/[K]) = -k_4([PK]_{\text{total}} - [K])t + C
\]  

(4)

\[([PK],_{\text{int}})\] and \([K]_{\text{int}}\) are the respective concentrations of prekallikrein and kallikrein at time t. The integration constant C equals \(\ln([PK]/[K])\) at time 0 and thus the final solution to Equation 3 is as follows.

\[
\ln([PK],/[K]_{\text{int}}) = -k_4([PK]_{\text{total}} - [K])t + \ln([PK]_{\text{total}}/[K]_{\text{int}})
\]  

(5)

From Equation 5 it can be seen that a plot of \(\ln([PK],/[K]_{\text{int}})\) versus time (t) should give a straight line of which the slope equals \(-k_4\) times the total amount of prekallikrein plus kallikrein present in the reaction mixture and of which the intercept at time 0 equals the natural logarithm of prekallikrein over kallikrein present at time 0. When the total amount of prekallikrein added to the reaction mixture is known, the plot can be constructed by measurement of the amount of prekallikrein present at each given time and calculating the remaining amount of prekallikrein. Alternatively, inspection of Equation 3 shows that the final plateau of kallikrein reached in the reaction mixture is \([PK]_{\text{bound}}\) since kallikrein formation stops when \([K]_{\text{int}}\) nears \([PK]_{\text{bound}}\). Thus, the second-order plot can also be constructed by using the final plateau value reached to calculate \([PK]_{\text{bound}} - [K]_{\text{int}}\) for each time point, thereby obviating the need to know exactly how much prekallikrein has been added.

Equations 1–5 apply to prekallikrein autoactivation in free solution. However, a "bound" form will be shown that: 1) prekallikrein autoactivation only occurs at appreciable rates in the presence of a negatively charged surface, and 2) that it is presumably surface-bound prekallikrein that is activated by surface-bound kallikrein. In case of an autoactivation with surface-bound reactants, Equation 2 becomes the following.

\[
d[K]/dt = k_2[PK]_{\text{bound}}[K]_{\text{bound}}
\]  

(6)

From the general expression for the adsorption isotherm the following can be calculated.

\[
[PK]_{\text{bound}} = (1 - K_2^b/[sites_{\text{bound}}])^{-1} [PK]
\]  

(7)

\[K_{\text{bound}} = (1 - K_2^b/[sites_{\text{bound}}])^{-1} [K]
\]  

(8)

\(K_2^b\) and \(K_2\) are the dissociation constants of the prekallikrein- and kallikrein surface complex, respectively, and \([sites_{\text{bound}}]\) is the concentration of nonoccupied surface binding sites. It is reasonable to assume that during the time course of the autoactivation reaction \([sites_{\text{bound}}]\) is constant since (a) kallikrein and prekallikrein bind with rather weak affinity to sulfatides (13) and (b) the experiments presented in this paper have been carried out at a high concentration of surface component \([sites_{\text{bound}}]\). This means that the amount of surface-bound kallikrein plus bound kallikrein will be a constant fraction of the total amount of prekallikrein and kallikrein present and Equations 7 and 8 reduce to the following.

\[
[PK]_{\text{bound}} = c_7\cdot[PK]
\]  

(9)

\[K_{\text{bound}} = c_8\cdot[K]
\]  

(10)

c_7 and c_8 are the fractions of, respectively, prekallikrein and kallikrein that are bound to the sulfatide surface. Substitution of Equations 9 and 10 in Equation 6 gives the following.

\[
d[K]/dt = k_2\cdot c_8\cdot [PK]_{\text{bound}}[K]_{\text{bound}} - k_2\cdot c_7\cdot [PK]_{\text{bound}}[K]_{\text{bound}}
\]  

(11)

\(h_1\) is the apparent second-order rate constant of surface-dependent autoactivation, which is a function of the binding parameters for surface-prekallikrein and surface-kallikrein interaction. The final equation for prekallikrein autoactivation in the presence of surface then becomes the following.

\[
\ln([PK],/[K]_{\text{int}}) = -k_2\cdot c_8\cdot [PK]_{\text{bound}}t + \ln([PK]_{\text{bound}}/[K]_{\text{int}})
\]  

(12)

This is the same mathematical form as Equation 5.

The inhibitory effect of the reversible inhibitor benzamidine on the apparent rate of autoactivation can be analyzed as described before for Factor XII autoactivation (14). Assuming (a) the complex of kallikrein with inhibitor does not participate in the reaction; (b) there is rapid binding equilibrium between kallikrein and benzamidine; and (c) there is a large excess of inhibitor over the enzyme, the amount of kallikrein (\(K_{\text{inact}}\)) available for proteolysis of prekallikrein can be written as follows.

\[
[K]_{\text{inact}} = (K/K_{\text{inact}} + 1)\cdot[K]
\]  

(13)

\(K_{\text{inact}}\) is the inhibitor constant of inhibition of kallikrein by benzamidine. \(I\) is the concentration of benzamidine, and \([K]\) is the total amount of kallikrein present at a given time. The rate of kallikrein formation in the presence of benzamidine can then be written as follows.

\[
d[K]/dt = k_2\cdot (K/K_{\text{inact}} + 1)\cdot [PK]_{\text{bound}}
\]  

(14)

Substituting Equation 13 into Equation 14 gives the following.

\[
d[K]/dt = k_2\cdot K_2\cdot [PK]_{\text{bound}}
\]  

(15)

This is recognized to be of exactly the same form as Equations 2 and 11 except that \(k_1\) is now reduced by a factor of \((K/K_{\text{inact}} + 1)\) due to the presence of the inhibitor. Thus the second-order logarithmic plots will again yield a straight line with a slope that will decline at increasing benzamidine concentrations with a factor equal to \((K/K_{\text{inact}} + 1)\).

\(K_{\text{inact}}\) can be accurately determined from second-order plots made at varying benzamidine concentrations.

**Immunoblotting**—Immunoblotting of prekallikrein and prekallikrein activation products formed during autoactivation was performed as follows. 30-μl aliquots of the reaction mixture were added to 70 μl of hot (>80°C) gel electrophoresis sample buffer (10) containing 1% sodium dodecyl sulfate and boiled for 5 min. Since a constant amount of carrier protein is essential for a proportional transfer of proteins from sodium dodecyl sulfate gels to nitrocellulose membranes (8, 9), 25 μl of the latter solution was added to 10 μl of prekallikrein-deficient plasma diluted 1:10 in 10 mM Tris, pH 7.4, containing 140 mM NaCl, 1 mg/ml of bovine serum albumin, and 10 μl of sodium dodecyl sulfate gel electrophoresis sample buffer, and subsequently was boiled for 5 min. The 45-μl samples (containing 150 ng of prekallikrein in 1 μl of prekallikrein-deficient plasma) were then electrophoresed on 7.5% polyacrylamide slab gels in the presence of sodium dodecyl sulfate at 5 mA/gel for 18 h according to Laemmli (10). Electrotoblotting of proteins onto nitrocellulose membranes and immunodetection of immobilized antigens (prekallikrein and its activation products) were subsequently performed as described in detail elsewhere (8, 9) using goat anti-human prekallikrein polyclonal antibodies and 125I-prekallikrein as detector. Apparent molecular weights were determined by running prestained standard proteins on the gel.

**Lipid Preparations**—An appropriate volume of a 10 mM stock solution of sulfatides in CHCl₃/MeOH (1/1, v/v) was dried under a
Prekallikrein Autoactivation

rate constant of autoactivation was some 5-fold higher in the presence of sulfatides than in the presence of dextran sulfate.

The Effect of Kallikrein and Prekallikrein Concentration on Autocatalytic Prekallikrein Activation—In Fig. 1B it is shown that the intercept of the second-order semilogarithmic plot at the ordinate equals the value for \( \ln([PK]/[K]) \) that can be calculated from a kallikrein determination at time 0 and from a determination of the plateau value of kallikrein reached. This also held true for other starting conditions as is illustrated in an experiment in which the initial amount of kallikrein present was varied but in which the total amount of prekallikrein plus kallikrein present in the reaction mixture was kept constant (Fig. 2). A set of parallel lines was obtained of which only the intercept at time 0 varied while the slope of the second-order plots remained constant (Fig. 2). In this experiment variation of the initial amount of kallikrein present was obtained by incubation of prekallikrein with a limited amount of the irreversible kallikrein inhibitor, H-D-Phe-Phe-Arg-CH₂Cl, or by adding a small amount of exogeneous kallikrein. Addition of the isolated light chains of kallikrein did not alter the time course of autoactivation (data not shown). This indicates that not only the catalytic region but also the heavy chain region of the kallikrein molecule was required for optimal autoactivation of prekallikrein.

For an autoactivation reaction in which prekallikrein is activated by its own enzymatically active form, kallikrein, the rate of autoactivation, as judged by the slope of the second-order logarithmic plot, should increase with increasing amounts of prekallikrein present in the reaction mixture (see mathematical model under "Experimental Procedures"). That such was the case is shown by the experiment presented in Fig. 3 in which the total amount of prekallikrein in the reaction mixture was varied. In all cases linear second-order

stream of nitrogen. The dried sulfatides were suspended in 2 ml of 50 mM Hepes (pH 7.2), 60 mM NaCl by mixing for 2 min at 70 °C. This suspension was sonicated for 5 min using a Model W 375 sonicator from Heat Systems. The sonicated preparation was centrifuged for 5 min in an Eppendorf centrifuge to remove residual large particles. Subsequently, the supernatant was centrifuged for 5 min in a Beckman Airfuge at 100,000 × g. The pellet that contained 70% of the original amount of sulfatides was resuspended in 2 ml of the same buffer. Sulfatide concentrations were determined according to Kean (15).

RESULTS

Surface-dependent Activation of Prekallikrein—In Fig. 1 it is shown that incubation of purified prekallikrein with 50 μM sulfatides or 0.5 μg/ml dextran sulfate at low ionic strength resulted in the generation of amidolytic activity toward the chromogenic substrate S 2302. No amidolytic activity was generated when prekallikrein was incubated in the absence of sulfatides or dextran sulfate. The time course of appearance of amidolytic activity in the presence of sulfatides or dextran sulfate showed a typical sigmoidal behavior in which an apparent lag period was followed by a relatively rapid activation until finally a plateau was reached. The characteristics of this process resembled that observed when Factor XII was incubated with sulfatides or dextran sulfate (14). In that case Factor XII, formation was due to activation of the Factor XII zymogen by its own enzymatically active form, α-Factor XII (14, 16). Therefore, we decided to investigate whether the spontaneous activation of prekallikrein could also be analyzed according a mechanism of autoactivation in which prekallikrein is activated by its own enzymatically active form, kallikrein. In that case a plot of the natural logarithm of the ratio of prekallikrein to kallikrein versus time should give a straight line of which the slope is equal to \(-k_2\) (the second-order rate constant) times the total concentration of prekallikrein plus kallikrein participating in the autoactivation process. The intercept at the ordinate should be equal to the natural logarithm of the ratio of prekallikrein to kallikrein present at time 0 (14) (also see "Experimental Procedures"). As seen in Fig. 1B, straight lines were obtained when the data were analyzed according to such a second-order mechanism. Based on the slope of the lines (Fig. 1B) the second-order

Fig. 1. Generation of kallikrein amidolytic activity from prekallikrein in the presence of sulfatides or dextran sulfate. Purified human plasma prekallikrein was preincubated at 37 °C in 250 μl of a buffer containing 50 mM Hepes (pH 7.2 at 37 °C), 27 mM NaCl, and 0.6 mg/ml ovalbumin. After 5 min, the reaction was started with the addition of 50 μl of a buffer containing 50 mM Hepes (pH 7.2 at 37 °C), 60 mM NaCl and 300 μM sulfatides or 3 μM dextran sulfate. Final concentrations were: 50 mM Hepes (pH 7.2 at 37 °C), 32.5 mM NaCl, 0.5 mg/ml ovalbumin, 234 nM prekallikrein, and 50 μM sulfatides. Variation of the initial amount of kallikrein present was achieved by: O—O, preincubation of the stock solution prekallikrein (3.8 μM) with H-D-Phe-Phe-Arg-CH₂Cl (80 μM final concentration) for 20 min at 37 °C to inhibit the major part of the kallikrein present in the prekallikrein preparation (usually approximately 3-4%); ▲—▲, no pretreatment of the prekallikrein stock solution; and ●—●, addition of kallikrein to the reaction mixture to result in an initial kallikrein concentration of 10.5 nM. The amount of kallikrein present in the reaction mixture was determined as described under "Experimental Procedures." The second-order semilogarithmic plots shown in panel B were constructed as described under kinetic data analysis under "Experimental Procedures," using the amounts of kallikrein (K) determined and the plateau value of kallikrein reached (P) to calculate \( \ln([P-K]/K) \).

Fig. 2. Sulfatide-dependent prekallikrein autoactivation as a function of the amount of kallikrein present at time 0. Prekallikrein was preincubated at 37 °C in 250 μl of a buffer containing 50 mM Hepes (pH 7.2 at 37 °C), 27 mM NaCl, and 0.6 mg/ml ovalbumin. After 5 min, the reaction was started by addition of 50 μl of a buffer containing 50 mM Hepes (pH 7.2 at 37 °C), 60 mM NaCl, and 300 μM sulfatides. The final concentrations were: 50 mM Hepes (pH 7.2 at 37 °C), 32.5 mM NaCl, 0.5 mg/ml ovalbumin, 234 nM prekallikrein, and 50 μM sulfatides. Variation of the initial amount of kallikrein present was achieved by: O—O, preincubation of the stock solution prekallikrein (3.8 μM) with H-D-Phe-Phe-Arg-CH₂Cl (80 μM final concentration) for 20 min at 37 °C to inhibit the major part of the kallikrein present in the prekallikrein preparation (usually approximately 3-4%); ▲—▲, no pretreatment of the prekallikrein stock solution; and ●—●, addition of kallikrein to the reaction mixture to result in an initial kallikrein concentration of 10.5 nM. The amount of kallikrein present in the reaction mixture was determined as described under "Experimental Procedures." The second-order semilogarithmic plots shown in panel B were constructed as described under "Experimental Procedures," using the amount of kallikrein (K) determined in the sample and the final plateau of kallikrein reached (P) to calculate \( \ln([P-K]/K) \).
Prekallikrein Autoactivation

Various amounts of prekallikrein were incubated for 5 min at 37°C in 250 μl of a buffer containing 50 mM Hepes (pH 7.2 at 37°C), 27 mM NaCl, and 0.6 mg/ml ovalbumin. After 5 min, the reaction was started with 50 μl of buffer containing 50 mM Hepes (pH 7.2 at 37°C), 60 mM NaCl, and 300 μM sulfatides. Final concentrations were: 50 mM Hepes (pH 7.2 at 37°C), 32.5 mM NaCl, 0.5 mg/ml ovalbumin, 50 μM sulfatides, and 116 nM prekallikrein (0.87), 167 nM prekallikrein (1.13), 333 nM prekallikrein (3.33), and 531 nM prekallikrein (6.67).

The amount of kallikrein present was determined as described under "Experimental Procedures." The second-order semilogarithmic plots were constructed as described under Kinetic Data Analysis (see "Experimental Procedures") using the amounts of kallikrein (K) and the final kalliplateau level reached (P) to calculate ln([PK]/[K]).

Prekallikrein Activation—The data presented thus far show that the observed spontaneous activation of prekallikrein in the presence of sulfatides or dextran sulfate can be explained by a reaction mechanism in which prekallikrein is activated by its own enzymatically active form, kallikrein. To investigate whether a contaminating protease or the zymogen form of such a protease might significantly contribute to the observed kallikrein formation, we tested the effect of various inhibitors on the rate of prekallikrein autoactivation. Table I shows that autoactivation could be completely blocked by soybean trypsin inhibitor but not by the corn, lima bean, or ovomucoid trypsin inhibitors. The kallikrein-specific inhibitor H-D-Phe-Phe-Arg-CH₂Cl also blocked prekallikrein autoactivation. In the presence of soybean trypsin inhibitor or the chloromethyl ketone inhibitor, no prekallikrein cleavage products were detectable on the gels (data not shown). Thus, the action of these potent inhibitors was due to a total block of the prekallikrein cleavage seen in the activation process and thus not simply due to inhibition of the amidolytic activity of the reaction product, kallikrein.

The reversible inhibitor benzamidine inhibited autoactivation in a concentration-dependent manner. At 500 μM benzamidine a 67% inhibition, of prekallikrein autoactivation was observed. Fig. 5A shows that increasing benzamidine concentrations caused an increased inhibition of prekallikrein autoactivation as judged by the decrease in the slope of the second-order semilogarithmic autoactivation plots. The effect of benzamidine on the apparent kᵦ, calculated from these plots is shown in Fig. 5B. The data points could be fitted well with a simulated curve that was obtained by assuming competitive inhibition by benzamidine with a Kᵦ of 240 μM (see also "Experimental Procedures"). This Kᵦ is identical to the Kᵦ for benzamidine determined by kinetic analysis of the inhibitory effect of benzamidine on kallikrein-catalyzed S 2302 hydrolysis measured under the same experimental conditions.

**Table I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
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<td>None</td>
<td>0.88</td>
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<td>Corn trypsin inhibitor (50 μg/ml)</td>
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<td>Lima bean trypsin inhibitor (50 μg/ml)</td>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>Benzamidine (500 μM)</td>
<td>0.29</td>
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**Fig. 3.** Sulfatide-dependent prekallikrein autoactivation at varying concentrations of prekallikrein. Various amounts of prekallikrein were incubated for 5 min at 37°C in 250 μl of a buffer containing 50 mM Hepes (pH 7.2 at 37°C), 27 mM NaCl, and 0.6 mg/ml ovalbumin. After 5 min, the reaction was started with 50 μl of buffer containing 50 mM Hepes (pH 7.2 at 37°C), 60 mM NaCl, and 300 μM sulfatides. Final concentrations were: 50 mM Hepes (pH 7.2 at 37°C), 32.5 mM NaCl, 0.5 mg/ml ovalbumin, 50 μM sulfatides, and 116 nM prekallikrein (0.87), 167 nM prekallikrein (1.13), 333 nM prekallikrein (3.33), and 531 nM prekallikrein (6.67). The amount of kallikrein present was determined as described under "Experimental Procedures." The second-order semilogarithmic plots were constructed as described under Kinetic Data Analysis (see "Experimental Procedures") using the amounts of kallikrein (K) and the final kalliplat level reached (P) to calculate ln([PK]/[K]).

**Fig. 4.** Influence of NaCl concentration on prekallikrein autoactivation. A, prekallikrein was preincubated at 37°C in 250 μl of buffer containing 50 mM Hepes (pH 7.2 at 37°C), 60 mM NaCl, and 300 μM sulfatides. Final concentrations were: 50 mM Hepes (pH 7.2 at 37°C), 0.5 mg/ml ovalbumin, 234 nM prekallikrein, 50 μM sulfatides, and 17.5 mM NaCl (0.87), 22.5 mM NaCl (3.33), and 27.5 mM NaCl (5.87). At the time intervals indicated (A) the amount of kallikrein present was determined as described under "Experimental Procedures." The second-order semilogarithmic plots were constructed as described under Kinetic Data Analysis (see "Experimental Procedures") using the amounts of kallikrein (K) present and the final plateau of kallikrein reached (P) to calculate ln([PK]/[K]). B, the apparent rate constants were calculated from the slope of the lines and are given in B.

The reversible inhibitor benzamidine inhibited autoactivation in a concentration-dependent manner. At 500 μM benzamidine a 67% inhibition, of prekallikrein autoactivation was observed. Fig. 5A shows that increasing benzamidine concentrations caused an increased inhibition of prekallikrein autoactivation as judged by the decrease in the slope of the second-order semilogarithmic autoactivation plots. The effect of benzamidine on the apparent kᵦ, calculated from these plots is shown in Fig. 5B. The data points could be fitted well with a simulated curve that was obtained by assuming competitive inhibition by benzamidine with a Kᵦ of 240 μM (see also "Experimental Procedures"). This Kᵦ is identical to the Kᵦ for benzamidine determined by kinetic analysis of the inhibitory effect of benzamidine on kallikrein-catalyzed S 2302 hydrolysis measured under the same experimental conditions.

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**Immunoblotting Analysis of Prekallikrein Autoactivation**—During the course of our work it became clear that the final plateau of kallikrein amidolytic activity reached in autoactivation experiments was consistently lower than the amount of prekallikrein added to the reaction mixture. Therefore, apparently not all prekallikrein was available for activation or part of it became inactivated during the time course of the autoactivation.
FIG. 5. Inhibition of prekallikrein autoactivation by benzamidine. A, autoactivation of 290 nM prekallikrein was determined at 37 °C in a total volume of 300 μl of a reaction buffer containing 50 mM Hepes (pH 7.2 at 37 °C), 32.5 mM NaCl, 0.5 mg/ml ovalbumin, 50 μM sulfatides, and no benzamidine (□—□), 0.0625 mM benzamidine (○—○), 0.125 mM benzamidine (■—■), 0.25 mM benzamidine (▲—▲), 0.5 mM benzamidine (●—●), 1.0 mM benzamidine (△—△), and 2.0 mM benzamidine (■—■). The small variation of ionic strength at different benzamidine concentrations was compensated by addition of NaCl. At the time intervals indicated (A) the amount of kallikrein formed was determined as described under “Experimental Procedures.” The second-order semilogarithmic plots were constructed as described under Kinetic Data Analysis (see “Experimental Procedures”) using the amounts of kallikrein (K) determined and the final plateau reached (P) to calculate In([PK]/[K]). The apparent rate constants were calculated from the slope of these lines and plotted as a function of the benzamidine concentration (B). The solid line represents a simulation (calculated as described under “Experimental Procedures”) assuming competitive inhibition of autoactivation by benzamidine with a K of 240 μM.

reaction. To discriminate between these possibilities, experiments were performed in which the activation products formed during autoactivation were subjected to immunoblotting analysis under nonreducing and reducing conditions as described elsewhere (9). The results of an experiment are shown in Fig. 6. Concomitant with the appearance of amidolytic activity between 0 and 15 min (Fig. 6A), the prekallikrein zymogen was cleaved and the heavy and light chains of kallikrein (at M, apparent 45,000, 37,000, and 33,000) became visible on the reduced immunoblots (Fig. 6C). At the time the plateau level of kallikrein amidolytic activity was reached (greater than 15 min, Fig. 6A) allzymogen had been cleaved (Fig. 6C), indicating that the reaction went to completion. However, it is also clear from the appearance of multiple fragments on nonreduced immunoblots for samples taken between 9 and 16 min (Fig. 6B) that substantial proteolytic cleavage of prekallikrein (and/or kallikrein) occurred at peptide bonds outside the disulfide bridge connecting the heavy and light chain region of the molecule. The main fragments showed apparent molecular weights of 43,000 and 38,000 (Fig. 6B) which represent a virtual cutting in half of the molecule. Upon reduction, fragments at M, approximately 55,000 and 27,000 were immunologically detectable in addition to the heavy and light chains (Fig. 6C). Since autolysis of kallikrein into β-kallikrein does not result in a loss of amidolytic activity (17, 18) and since the plateau level of kallikrein amidolytic activity was stable for prolonged incubation up to 1 or 2 h (Fig. 6A and data not shown), we conclude that the degradation products did not originate from kallikrein but that a substantial amount of prekallikrein in the reaction mixture was converted into products that were not subsequently activated by kallikrein to give active enzyme. The inactivated prekallikrein fragments could also not be activated by addition of excess amounts of β-Factor XII, (data not shown) and were, therefore, apparently not available for activation.

DISCUSSION

The data presented in this paper show that, in the presence of a suitable negatively charged surface, purified human plasma prekallikrein can undergo autoactivation by its own enzymatically active form, kallikrein. Several lines of evidence support this conclusion. Kinetic analysis of the time course of activation shows that the observed kallikrein formation can be modelled according to a second-order mechanism of activation of prekallikrein by kallikrein. The linear second-order logarithmic plots obtained throughout our studies and the kinetic effects obtained by varying the initial amount of kallikrein present or by variation of the total amount of prekallikrein added to the reaction mixture are in accord with the proposed model. Further support for the occurrence of autocatalytic prekallikrein activation was obtained from experiments with inhibitors. The characteristics of inhibition of prekallikrein autoactivation are identical to those for kallikrein. Thus, soybean trypsin inhibitor and the kallikrein-specific inhibitor, H-D-Phe-Phe-Arg-CH₂Cl, completely block autoactivation whereas corn trypsin inhibitor (an efficient inhibitor of Factor XIIa), lima bean trypsin inhibitor, and ovomucoid trypsin inhibitor do not affect the autoactivation
Activation is due to the action of kallikrein on prekallikrein. Therefore, these data support the conclusion that the observed spontaneous surface-dependent activation is due to the action of kallikrein on prekallikrein.

The presence of a suitable surface appears to be an absolute requirement since in the absence of surface no activation could be detected. This indicates that the activation of prekallikrein by kallikrein is a surface-bound process. The finding that increasing NaCl concentrations strongly inhibit autoactivation supports this proposal since it has been shown that kallikrein does not bind to sulfatides at high salt concentrations (13). Moreover, addition of the isolated light chain of kallikrein, the fragment containing the active site (7) that lacks affinity for the surface (13) did not influence the time course of autoactivation and was not able to catalyze surface-dependent prekallikrein activation. Therefore, it appears that surface-bound kallikrein cleaves and activates surface-bound prekallikrein.

Immunoblotting analysis of prekallikrein activation mixtures also indicates that kallikrein is capable of proteolytic activation of prekallikrein since on reduced blots the characteristic heavy and light chains of kallikrein can be seen. Thus, it seems likely that kallikrein cleaves the same peptide bond in prekallikrein as Factor XII. However, more peptide bonds in prekallikrein and/or kallikrein appear to be susceptible to proteolysis by kallikrein since observable amounts of prekallikrein appear to become proteolytically degraded during the time course of activation (cf. Fig. 6). It is known that kallikrein is susceptible to autocatalytic proteolysis of at least one peptide bond in the heavy chain region of the molecule resulting in so-called β-kallikrein (18). It is unlikely, however, that the degradation products visible on the immunoblots of prekallikrein autoactivation mixtures are due to the action of kallikrein on prekallikrein. The main degradation product on the reduced gel has a molecular weight of 55,000 and hence cannot be derived from kallikrein. Thus, we conclude that the additional products visible on the gel are the result of proteolytic degradation of prekallikrein. These degradation products are not available for further activation since even with excess amounts of β-Factor XII, no further increase of amidolytic activity was observed. It appears, therefore, that in addition to the activation of prekallikrein by kallikrein, a second reaction (or reactions) must be taken into account in which prekallikrein is degraded by kallikrein into forms which cannot be activated. In this case, the kinetic data analysis as presented under “Experimental Procedures” is not valid. It is shown in the Appendix that second-order logarithmic plots will still yield straight lines, provided the amount of prekallikrein that is converted into kallikrein rather than the total amount of added prekallikrein is used to calculate the natural logarithm of prekallikrein over kallikrein present at a given time.

The physiological significance of the autoactivation of prekallikrein reported here remains open to speculation. Approximation of the rate constant from the slope of the logarithmic plot yields an estimated second-order rate constant of $3 \times 10^4$ M$^{-1}$ s$^{-1}$. This is much smaller than the rate constant reported for prekallikrein activation by α-Factor XII, in the presence of dextran sulfate ($2 \times 10^7$ M$^{-1}$ s$^{-1}$, cf. Ref. 19). Moreover, at higher salt concentrations, prekallikrein autoactivation is greatly diminished. It should be emphasized, however, that we cannot exclude the possibility that procoagulant surfaces other than sulfatides or dextran sulfate or the presence of other proteins may enhance reaction rates of prekallikrein autoactivation to values comparable to the rate of factor XII-catalyzed prekallikrein activation. In such a case, surface-dependent prekallikrein autoactivation may contribute to the initiation of kallikrein-dependent pathways.

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APPENDIX

In the case where a side reaction is occurring in which prekallikrein is inactivated by kallikrein, the mechanism for the reactions between kallikrein and prekallikrein can be written as follows.

$$\text{prekallikrein} + \text{kallikrein} \rightarrow_{k_1} 2 \text{kallikrein}$$

$$\text{kallikrein} + \text{prekallikrein} \rightarrow_{k_2} \text{prekallikrein} + \text{kallikrein}$$

Prekallikrein, (PK), is the degradation product mentioned above. The rate equations are as follows.

$$\frac{d[P]{\text{K}}}{dt} = k_3(\text{PK})_{\text{total}} - \frac{K}{K} - \frac{[\text{PK}][\text{K}]}{[\text{PK}][\text{K}]}$$

(II)

$$\frac{d[\text{PK}]}{dt} = k_4(\text{PK})_{\text{total}} - \frac{K}{K} - \frac{[\text{PK}][\text{K}]}{[\text{PK}][\text{K}]}$$

(III)

$$[\text{K}]$$ can be solved in [PK] to give $d[\text{K}]/d[\text{PK}]=k_3/k_4$, which upon integration and rearrangement yields the following.

$$\text{PK}_0 = \frac{k_4}{k_3}(\text{PK})_{\text{total}} - \frac{[\text{PK}][\text{K}]}{[\text{PK}][\text{K}]}$$

(IV)

$$(\text{PK})_{\text{total}}$$, and $\text{PK}_0$ equal the amounts of kallikrein and degraded prekallikrein present at time 0. Substitution of Equation IV in Equation III yields Equation V.

$$\frac{d[\text{PK}]}{dt} = k_3(\text{PK})_{\text{total}} + \frac{K}{K} - \frac{[\text{PK}][\text{K}]}{[\text{PK}][\text{K}]}$$

(V)

Rearrangement finally yields Equation VI.

$$\frac{d[\text{PK}]}{dt} = (k_3 + k_4)(\text{P} - \frac{[\text{PK}]}{[\text{K}]})$$

(VI)

$P$ is a constant which equals the following.

$$\text{PK}_{\text{total}} + \frac{(k_3 + k_4)}{[\text{PK}]_{\text{total}}}(1 - \frac{[\text{PK}][\text{K}]}{[\text{PK}][\text{K}]})$$

(VII)

Inspection of Equation VI shows that it is of the same form as the equation for a simple autoactivation in which all prekallikrein is converted into kallikrein (see also Equation 3 under “Experimental Procedures”) except that the final plateau of kallikrein reached will not equal $\text{PK}_{\text{total}}$ (i.e. the total amount of prekallikrein added) but it will become equal to $P$. However, as can be seen from the form of Equation VI, the solution will be identical and a plot of $\ln(P - K)/K$ versus time will give a straight line with an intercept at time 0 equal to the ratio of $(P - K)/K$ calculated at time 0 and a slope from which an apparent second-order rate constant can be determined which equals $k_3 + k_4$.

REFERENCES

Prekallikrein Autoactivation