FACTOR Xa (fXa) AND THROMBIN are trypsin-like serine proteinases that play pivotal roles in the clotting cascade. fXa is the enzyme of the prothrombinase complex that activates prothrombin to generate thrombin. Thrombin plays a dual function in the coagulation cascade. As a coagulant enzyme, thrombin activates fibrinogen, platelets, and IV, IVIII, VII, XI, and XII. As an anticoagulant enzyme, thrombin binds to thrombomodulin (TM) and activates the vitamin K-dependent serine proteinase zymogen, protein C (PC) to activated protein C (APC). APC downregulates the coagulation cascade by inactivating the procoagulant cofactors Va and VIIIa by limited proteolysis. PC activation by the thrombin-TM complex occurs optimally on membrane surfaces, but binding of the epidermal-like growth factor domains 4-6 of TM (TM4-6) to the anion binding exosite 1 of thrombin is sufficient to change the macromolecular specificity of thrombin, and accounts for most of the cofactor function of TM for PC activation. fXa is not known to play a significant role in PC activation. Although there is a report demonstrating that bovine fXa can bind rabbit TM and rapidly activate bovine PC, the result has not been confirmed for human fXa in a homologous system. Heparin is a naturally occurring glycosaminoglycan commonly used as an anticoagulant drug. It is believed that the primary antithrombotic effect of heparin is through acceleration of the inhibition of the coagulation serine proteinases, including thrombin, fXa, fXa, fIXa, fIXa, fXIIa, and kallikrein by antithrombin. Results from several laboratories suggest that heparin accelerates antithrombin inhibition of thrombin, fXa, and fXa by a bridging mechanism, whereas in inhibition of fXa, kallikrein, and possibly fXIIa, a heparin induced conformational change in the reactive site loop of antithrombin accounts for the acceleration.

In a recent study, it was demonstrated that heparin and a similar negatively charged substance, dextran sulfate, dramatically enhanced the thrombin activation of fXI by a template mechanism, analogous to acceleration of thrombin inhibition by antithrombin. This result suggested that heparin-like compounds may facilitate the assembly of other enzyme-substrate complexes in plasma for rapid activation if both proteins contained a binding site for heparin. The anion binding exosite 2 of thrombin, located above the active site pocket, contains 11 basic residues that are thought to constitute the heparin binding site of thrombin. Structural data indicate that 7 of the basic residues of this region are conserved in fXa, so that fXa can also bind heparin. The recent crystal structure of activated Gla-domainless PC (GDPC) also showed that PC contains several basic residues that are clustered in the same three-dimensional location analogous to the anion binding exosite 1 of thrombin. It is also known that thrombin accelerates APC inhibition by the serpin, PC inhibitor, by a template mechanism. Based on these observations, it was rationalized that heparin-like compounds could also accelerate PC activation by thrombin and fXa, if the heparin binding site on PC is not masked by the noncatalytic domain.

In the present study, this possibility was tested by measuring the initial rates of PC activation by thrombin and fXa in the absence or presence of heparin and dextran sulfate. Kinetic analysis indicated that these polysaccharides markedly accelerate PC and GDPC activation by both thrombin and fXa in the presence of Ca2+ by reducing the Kmic. Kinetic analysis in the...
The presence of oligosaccharides containing 6 to 64 saccharide units indicated that a chain length with a minimum of 14 to 18 saccharide units was required for the acceleration of PC activation. In EDTA, the polysaccharides accelerated GDPC activation by thrombin, but they inhibited full-length PC activation by thrombin and eliminated the characteristic higher rate of PC activation by the thrombin-TM complex lacking chondroitin sulfate in low Ca$^{2+}$ buffer. These findings are discussed in the context of a model for PC activation under conditions in which the Gla-domain of PC is not stabilized by Ca$^{2+}$ ions.

**MATERIALS AND METHODS**

**Materials.** Plasma-derived human PC was purchased (Haematologic Technologies Inc, Essex Junction, VT) or purified from plasma as described. Human plasma FXa, bovine plasma antithrombin, human recombinant GDPC, rabbit TM, human recombinant TM4-6, and human recombinant soluble TM containing or lacking the chondroitin sulfate moiety were prepared as described previously. Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) or 40% phosphatidylcholine, 20% phosphatidylserine, and 40% phosphatidylethanolamine (PC/PS/PE) were prepared as described previously. Unfractionated heparin from porcine intestinal mucosa, sodium salt (169.2 USP U/mg), dextran sulfate with an average molecular weight of 8,000, and polybrene were purchased from Sigma (St Louis, MO). Spectrozyme PCa (SpPCa) was purchased from American Diagnostica (Greenwich, CT). The oligosaccharides, ranging in size from 6 to 18 saccharide units, were generous gifts from Dr Ingemar Björk (Swedish University of Agricultural Sciences, Uppsala, Sweden) and high-affinity heparin fragments with 22 to 64 saccharide units were generous gifts from Dr Steven Olson (University of Illinois-Chicago, Chicago, IL).

**PC activation.** The initial rate of PC or GDPC (0.75 µmol/L) activation by thrombin (5 µmol/L) was studied as a function of different dextran sulfate or heparin concentrations (0 to 500 µg/mL). The activation reactions were performed in 96-well plates in 0.1 mol/L NaCl, 0.02 mol/L Tris-HCl, pH 7.5 (TBS), buffer containing 1 mg/mL bovine serum albumin (BSA) and 2.5 mmol/L Ca$^{2+}$ or 100 µmol/L EDTA for 10 minutes at room temperature, after which 50 µg/mL antithrombin and 1 U/mL (−5.9 µg/mL) heparin was added to each well to inhibit thrombin activity. At this concentration of antithrombin, the activity of thrombin was rapidly inhibited, whereas the amidolytic activity of APC remained stable for more than 15 minutes. The amidolytic activity of APC in the activation reactions was monitored by hydrolysis of 200 µmol/L SpPCa in TBS buffer containing 1 mg/mL BSA. The rate of hydrolysis was measured at 405 nm at room temperature. For the Km and kcat analysis in the presence of calcium ions, the initial rates of PC activation by thrombin were calculated from the Michaelis-Menten equation using Enzfitter computer program (R.J. Leatherbarrow, Elsevier, Biosoft, London, UK). All data presented are the average of at least two to three independent measurements ± SD.

**RESULTS**

The effect of dextran sulfate and heparin on PC or (GDPC) activation by thrombin was studied both in the presence of Ca$^{2+}$ and EDTA. In the presence of Ca$^{2+}$ and an optimum concentration of heparin or dextran sulfate (~20 to 25 µg/mL), the activation rate of PC by thrombin was enhanced approximately fivefold by heparin and approximately 38-fold by dextran sulfate (Fig 1A). In contrast to acceleration of activation in Ca$^{2+}$, the polysaccharides inhibited PC activation by thrombin in EDTA (Fig 1B). However, with GDPC, the polysaccharides accelerated the activation rates both in Ca$^{2+}$ and EDTA. The acceleration of GDPC activation in Ca$^{2+}$ was more efficient than in EDTA. Under the same experimental conditions as in PC activation, at the optimum concentration of the polysaccharides (~20 to 25 µg/mL), the GDPC activation rate was enhanced approximately 20-fold by heparin and approximately 45-fold by dextran sulfate in the presence of Ca$^{2+}$ (Fig 2A). However, in the presence of EDTA, heparin and dextran sulfate accelerated GDPC activation approximately 10-fold and approximately twofold, respectively (Fig 2B). The optimum concentration of the polysaccharides in the presence of EDTA was approximately 5 µg/mL. These results suggest that Ca$^{2+}$ binding on GDPC is required for optimal acceleration of activation and that the Gla-domain of PC is responsible for the polysaccharides inhibition of PC activation by thrombin in EDTA.

It was previously demonstrated that, at saturating Ca$^{2+}$ (>500 µmol/L), normal PC and GDPC, whether prepared by enzymatic or by the recombinant DNA methods, are activated by thrombin with similar kinetic parameters in solution. It is also known that, in the absence of TM, PC or GDPC activation by thrombin is accelerated by EDTA, but GDPC is activated much slower than wild-type PC in EDTA.
kinetic basis for the lower rate of GDPC activation by thrombin in the absence of Ca\(^{2+}\) has been demonstrated to be primarily due to elevated \(K_m\) of GDPC for thrombin in the reactions.\(^{30}\) The observation that the polysaccharides in EDTA inhibited PC activation but not GDPC activation suggests that the lower \(K_m\) of thrombin for PC in the absence of Ca\(^{2+}\) might be due to binding of the acidic Gla-domain of PC to the heparin binding exosite of thrombin and that the binding of heparin to this site prevents this interaction.

To test the hypothesis that binding of the polysaccharides to the heparin binding exosite 2 of thrombin is responsible for the acceleration of PC activation in the presence of Ca\(^{2+}\) and inhibition of the reaction in the presence of EDTA, the initial rates of PC activation were measured with the anion binding exosite 2 mutant R93,97,101A thrombin, which was previously shown to be unable to bind heparin.\(^{31}\) In the presence of EDTA, heparin neither inhibited full-length PC activation (Fig 3A) nor accelerated GDPC activation by this mutant thrombin (Fig 3B). This result supports the hypothesis that the Gla-domain of PC and heparin are interacting with exosite 2 of thrombin in EDTA. In the presence of Ca\(^{2+}\), heparin accelerated activation of both PC derivatives by the mutant thrombin approximately twofold (data not shown). The optimum heparin concentration for acceleration of reaction in Ca\(^{2+}\) was elevated approximately 10-fold with the exosite 2 mutant of thrombin. This result is consistent with the previous observation that heparin accelerated the inactivation rate of this mutant by antithrombin approximately twofold and that the optimum heparin concentra-

![Graph](image1.png)

**Fig 1.** Heparin and dextran sulfate concentration dependence of PC activation by thrombin. (A) Human plasma-derived PC (0.75 \(\mu\)mol/L) was incubated with thrombin (5 nmol/L) in TBS buffer containing 2.5 mmol/L Ca\(^{2+}\), 1 mg/mL BSA, 0.1% PEG 8000, and indicated concentrations of heparin (■) or dextran sulfate (○) at room temperature for 10 minutes. After inactivation of thrombin activity by antithrombin, the initial rate of PC activation was measured by an amidolytic activity assay using SpPCa as described under the Materials and Methods. Less than 15% substrate was activated in all reactions. (B) The same as (A), except that TBS buffer contained 100 \(\mu\)mol/L EDTA, instead of Ca\(^{2+}\).

![Graph](image2.png)

**Fig 2.** Heparin and dextran sulfate concentration dependence of GDPC activation by thrombin. (A) Recombinant GDPC (0.75 \(\mu\)mol/L) was incubated with thrombin (5 nmol/L) in TBS buffer containing 2.5 mmol/L Ca\(^{2+}\), 1 mg/mL BSA, 0.1% PEG 8000, and indicated concentrations of heparin (■) or dextran sulfate (○) at room temperature for 10 minutes. After inactivation of thrombin activity by antithrombin, the initial rate of PC activation was measured by an amidolytic activity assay using SpPCa as described under the Materials and Methods. Less than 15% substrate was activated in all reactions. (B) The same as (A), except that TBS buffer contained 100 \(\mu\)mol/L EDTA, instead of Ca\(^{2+}\).
be different from that of heparin, PC activation by the thrombin-Arg and Lys residues of exosite 2 with a specificity that might differ from that of heparin. A previous study showed that the chondroitin sulfate moiety of TM binds to exosite 2 with a specificity that is also different from that of heparin. Furthermore, it has been demonstrated that dextran sulfate accelerates thrombin inhibition by antithrombin by a template mechanism similar to heparin, but the acceleration rate by dextran sulfate is approximately 10-fold lower than that of low-affinity heparin. Taken together, these results suggest that these polysaccharides bind to exosite 2 of thrombin with some degree of specificity and that this site is not simply a cationic region that interacts nonspecifically with all anionic polymers.

**Heparin effect on PC activation by the thrombin-TM complex.** The effect of heparin on PC activation by thrombin in complex with the TM forms containing or lacking the chondroitin sulfate moiety was determined. It has been shown previously that the Ca$^{2+}$-dependence of PC activation by thrombin in complex with TM containing or lacking the chondroitin sulfate moiety differs. In the presence of TM lacking chondroitin sulfate, PC activation exhibits a distinct optimum reaching a maximum at approximately 100 µmol/L Ca$^{2+}$, with a considerable decrease occurring at physiological Ca$^{2+}$ concentrations. In contrast, the Ca$^{2+}$-dependence of PC activation by thrombin in complex with TM containing chondroitin sulfate is a simple hyperbolic relationship, reaching saturation at approximately 500 µmol/L Ca$^{2+}$. With TM containing chondroitin sulfate, heparin did not influence PC activation by thrombin (data not shown), but with TM lacking chondroitin sulfate, the characteristic peak rate of PC activation at low Ca$^{2+}$ was largely abolished by heparin (Fig 4).

**PC activation on the phospholipid vesicles.** To examine whether the polysaccharides can also accelerate PC activation on membrane surfaces, the initial rate of PC activation by thrombin was determined in the presence of 100 µg/mL phospholipid vesicles and 5 mmol/L Ca$^{2+}$. Heparin accelerated the PC activation rate by thrombin approximately fivefold in the presence of PC/PS and approximately 10-fold in the presence of PC/PS/PE vesicles (Fig 5A). The acceleration of PC activation by dextran sulfate in the presence of PE containing phospholipids approached approximately 45-fold (Fig 5B). In general, PC activation rate by thrombin on PE containing phospholipids was slightly higher (less than 2-fold) than on PC/PS vesicles. In the presence of heparin or dextran sulfate however, thrombin activated PC twofold to threefold more efficiently on PE containing phospholipids (Fig 5). It is previously shown that the physiological function of activated PC (FVα inactivation) is improved on PE containing phospholipids.
now suggest that PC is also activated more efficiently on this type of phospholipids.

To determine the kinetic step that may be influenced by the polysaccharides in the presence of Ca\(^{2+}\), the \(K_m\) and \(k_{cat}\) constants for PC activation were determined from the initial rates of activation as a function of increasing PC concentrations in the presence of 100 µg/mL PC/PS/PE vesicles and 5 mmol/L Ca\(^{2+}\) (Table 1). Under these experimental conditions, no kinetic analysis for PC activation in the absence of the polysaccharide was possible, because the reaction rate remained linear for up to 12.5 µmol/L PC concentration (the highest concentration available). These results suggest that the effect of the polysaccharides in the acceleration of PC activation is at least partly, if not entirely due to lowering the \(K_m\) of PC for thrombin in the reactions. Similarly, kinetic analysis indicated that heparin and dextran sulfate inhibited PC activation by thrombin in EDTA by increasing the \(K_m\) and \(K_a\) in the reactions. No \(K_m\) determination in the presence of heparin or dextran sulfate was possible (the highest PC concentration was 12.5 µmol/L).

The observation that the polysaccharides lowered the \(K_m\) for PC activation suggested that these compounds may act as templates to which both thrombin and PC bind. To further investigate this possibility, PC activation by thrombin was studied in the presence of oligosaccharides containing 6, 10, 14, or 18 saccharide units or the high-affinity heparin fragments containing 22, 35, 50, and 64 saccharide units. All of these fragments bind thrombin with similar affinity, because Olson et al.\(^3\) previously used these oligosaccharide fragments to characterize the heparin binding site of thrombin and demonstrated that only 5 to 6 charged residues contained within a 3-disaccharide binding site of heparin account for most of the binding energy of heparin in interaction with thrombin. No effect on PC activation by thrombin was observed with either 6- or 10-unit oligosaccharides (up to 300 µmol/L) either in solution using GDPC or on the phospholipid vesicles using full-length PC as the substrate (data not shown). An approximately 2.5-fold enhancement of PC activation rate was observed with the 14-unit oligosaccharide, and the accelerating effect was increased with increasing heparin chain length up to 50 saccharide units. These results are consistent with a template mechanism for heparin in acceleration of PC activation by thrombin. A similar optimal concentration for unfractionated
RAPID PROTEIN C ACTIVATION

Table 1. Kinetic Parameters for PC Activation by Thrombin and fXa on PC/PS/PE Vesicles in the Presence of Heparin or Dextran Sulfate

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µmol/L)</th>
<th>$k_{cat}$ (mol/min/mol)</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (heparin)</td>
<td>2.3 ± 0.4</td>
<td>0.36 ± 0.02</td>
<td>0.16</td>
</tr>
<tr>
<td>Thrombin (dextran sulfate)</td>
<td>0.7 ± 0.1</td>
<td>0.62 ± 0.05</td>
<td>1.17</td>
</tr>
<tr>
<td>fXa (heparin)</td>
<td>0.55 ± 0.04</td>
<td>0.17 ± 0.01</td>
<td>0.31</td>
</tr>
<tr>
<td>fXa (dextran sulfate)</td>
<td>0.45 ± 0.04</td>
<td>0.65 ± 0.19</td>
<td>4.33</td>
</tr>
</tbody>
</table>

The kinetic constants were determined by incubating PC (0.1 to 12.5 µmol/L) with 5 mmol/L thrombin or fXa in the absence or presence of 100 µg/mL PC/PS/PE vesicles in TBS buffer containing 1 mg/mL BSA, 0.1% PEG 8000, and 5 mmol/L Ca$^{2+}$ and 25 µg/mL heparin or dextran sulfate at room temperature. The kinetic values were determined from the rate of APC generated in each reaction as described under the Materials and Methods.

PC activation by fXa. fXa has the capacity to bind heparin$^{35}$; however, fXa is not known to play a significant role in PC activation. To examine if polysaccharides play a role in PC activation by fXa, the initial rate of PC activation by fXa was determined in the presence of these compounds. Interestingly, as shown in Fig 6, human fXa on phospholipid vesicles rapidly activated human PC in the presence of 25 µg/mL heparin or dextran sulfate. Similar to thrombin, PC activation by fXa in the presence of either heparin (Fig 6A) or dextran sulfate (Fig 6B) on the PC/PS/PE vesicles was twofold to threefold better than that on the PC/PS vesicles. Comparison of the initial rates of PC activation by thrombin in Fig 5, and by fXa in Fig 6, indicates that fXa in the presence of the polysaccharides activated PC twofold to fourfold better than thrombin on both types of the phospholipid vesicles. Kinetic analysis indicated that relative to thrombin, a lower $K_m$ of PC for fXa on both types of membranes, accounts for the higher catalytic efficiency of fXa in PC activation in the presence of the polysaccharides (Table 1). Because it was not possible to determine the kinetic constants for PC activation by fXa in the absence of the polysaccharides or phospholipids, it is not known whether the accelerating effect of dextran sulfate or heparin was only to lower the $K_m$ for PC, or they also improved the $k_{cat}$ of the reactions. Similar to the reactions with thrombin, no acceleration of PC activation by fXa was observed with the oligosaccharides containing either 6 or 10 saccharide units. A minimum chain length of 14 saccharide units was also required to observe any significant effect on PC activation by fXa.

To demonstrate how rapidly PC is activated by fXa in the presence of the polysaccharides, the initial rate of PC activation on phospholipid vesicles by thrombin in complex with full-length rabbit TM and by fXa in the presence of heparin or dextran sulfate was compared at near physiological concentration of PC (~80 nmol/L). On the PC/PS/PE vesicles, under conditions of less than 10% substrate utilization, activation of PC in the presence of dextran sulfate was essentially identical by either fXa or the thrombin-rabbit TM complex (Fig 7). However, on PC/PS vesicles, activation by the thrombin-rabbit TM complex was approximately threefold to fourfold better than that by fXa in the presence of dextran sulfate (Fig 7).

A similar (within 75% to 80%) acceleration of PC activation by both thrombin and fXa was observed on the phospholipid vesicles, if the concentration of heparin was reduced to 1.25 µg/mL (~0.2 U/mL), which is the lowest range of heparin concentrations in plasma during antithrombotic therapy.$^{36}$ A threefold to fourfold rate enhancement of GDPC activation by thrombin was also observed with dermatan sulfate and heparan

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Methods.

amidolytic activity assay as described under the Materials and indicated time intervals, the rate of PC activation was measured by an containing 5 mmol/L Ca \(^{2+}\). If 50 µg/mL polybrene was included in the reactions.

was also performed in the presence of hirudin, TM, and other contamination of fXa with thrombin could not result in a significant APC generation. Nevertheless, PC activation by fXa in the presence of 25 µg/mL dextran sulfate or 1 nmol/L thrombin in complex with 100 nmol/L rabbit TM (data not shown). PC activation by thrombin in the presence of phospholipids with 1 nmol/L fXa was not determined with certainty. The mechanism by which heparin or dextran sulfate accelerates PC activation by fXa was identical to the effect observed by the homogeneous high-affinity heparin at the equimolar concentrations. The commercial unfractionated heparin is a mixture of the high- and low-affinity polysaccharide chains, and the high-affinity fraction constitutes only about one third of the molecules. It is known that low-affinity heparin potentiates the anticoagulant action of high-affinity heparin in plasma. The results of this study suggest that the low-affinity heparin that binds antithrombin poorly may contribute to the anticoagulant effect of therapeutic heparin by catalyzing PC activation, particularly on the surfaces of the activated platelets.

The observation that the polysaccharides have an optimal concentration for acceleration of the reactions, with the activities decreasing at concentrations above and below this value, suggests that these negatively charged substances might serve as templates to which both thrombin and PC bind. Further support for a template mechanism for the polysaccharides in acceleration of PC activation by thrombin is provided by the observation that these compounds lower the K_m for PC in the reactions. This resembles the mechanism of heparin acceleration of thrombin inhibition by antithrombin, in which heparin lowers the K_d for the analogous enzyme-inhibitor Michaelis complex formation by a template mechanism. Finally, this proposal is consistent with the observation that a minimum chain length for the oligosaccharides was required for acceleration of the reactions. However, in contrast to thrombin, the mechanism by which heparin or dextran sulfate accelerates PC activation by fXa was not determined with certainty. The observations that the K_m for PC activation by fXa in the presence of the polysaccharides was very low (Table 1) and that the oligosaccharides containing 6 or 10 saccharide units did not accelerate the reaction suggest that the primary effect of these compounds may also be through an approximation effect by a template mechanism. However, a bell-shaped concentration dependence curve for heparin acceleration was not observed for PC activation by fXa. Although this could be due to the weak nature of the fXa-heparin interaction, other mechanisms for the polysaccharides acceleration of PC activation by fXa cannot be ruled out in this study. Particularly, in a different study, it was previously reported that another polysaccharide, the pentosan polysulfate, accelerated PC activation by fXa on phospholipids by increasing the V_max value fourfold with no influence on the K_m value. Because, in this study, it was not possible to accurately determine the kinetic constants for PC activation by fXa in the absence of the polysaccharide, such a modest effect on the V_max of activation by the polysaccharides used in the current study cannot be ruled out.

At the biochemical level, the results of this study provide insight into several aspects of PC activation by thrombin in the absence or the presence of TM. First, it is known that, in the absence of TM, thrombin exhibits a low K_m for PC in the presence of EDTA (1.2 µmol/L). The observation that heparin and dextran sulfated both accelerated GDPC activation, but
inhibited full-length PC activation in EDTA suggests that the Gla-domain of PC interacts with the anion binding exosite 2 of thrombin lowering the $K_m$ for PC in EDTA and that heparin binding to this site prevents this interaction. Further support for this hypothesis was provided by the observation that heparin in the presence of EDTA neither accelerated nor inhibited PC activation with the exosite 2 mutant R93,97,101A thrombin, which is known to not bind heparin. Second, it is known that the Ca$^{2+}$-dependence of PC activation by thrombin in complex with TM containing or lacking the chondroitin sulfate differs. The Ca$^{2+}$-dependence of PC activation with TM containing chondroitin sulfate is a simple saturation curve with a $K_{d(app)}$ of approximately 300 μmol/L Ca$^{2+}$, whereas with TM lacking chondroitin sulfate it has a characteristic high peak of activation at approximately 50 to 100 μmol/L Ca$^{2+}$, which decreases thereafter to a more normal rate at physiological Ca$^{2+}$ levels. A low $K_m$ for PC at approximately 50 to 100 μmol/L Ca$^{2+}$ accounts for the high rate of activation by thrombin-TM lacking chondroitin sulfate. The result of this study showed that heparin eliminated this peculiar Ca$^{2+}$-dependence of PC activation, suggesting that the Gla-domain of PC, when present as in not fully Ca$^{2+}$-stabilized conformer, binds to the anion binding exosite 2 of thrombin similar to that in EDTA and that heparin prevents this interaction. It should be noted that the Gla-domain has a $K_{d(app)}$ of approximately 250 to 300 μmol/L for Ca$^{2+}$ binding; therefore, the Gla-domain of PC is not expected to be in the fully stabilized conformer at 50 to 100 μmol/L Ca$^{2+}$. Furthermore, because the occupancy of the high-affinity Ca$^{2+}$ binding site in the protease domain [$K_{d(app)}$ ~50 to 100 μmol/L] is a requirement for the cofactor function of TM, it follows that the consequence of the Gla-domain interaction with the anion binding exosite 2 of thrombin (lower $K_m$) is only manifested at approximately 50 to 100 μmol/L Ca$^{2+}$. This hypothesis is also consistent with the previous results that the Ca$^{2+}$-dependence of PC activation by the R93,97,101A thrombin-TM complex follows a simple saturation curve whether or not TM contains the chondroitin sulfate moiety. It has been reported that platelet factor 4 stimulates the cofactor activity of TM containing chondroitin sulfate at low Ca$^{2+}$ and changes the Ca$^{2+}$-dependence of PC activation from simple saturation to a distinct high peak at the lower Ca$^{2+}$. This stimulatory effect of platelet factor 4 was not observed with GDPC and was minimal when TM lacking chondroitin sulfate was used in the activation assay. The authors of this report hypothesized several possibilities for their observation but did not consider the PC activation model in low Ca$^{2+}$, which may play in PC activation and rapid activation of PC by the thrombin-TM complex. The results of this study suggest that the Ca$^{2+}$-induced conformational changes in the protease domain also involves the basic residues of PC that interact with dextran sulfate and heparin. Such a conformational change could provide binding sites critical for interaction of PC with the thrombin-TM complex for rapid zymogen activation. In support of this hypothesis, it was recently shown that a PC mutant in which Lys$^{77}$, Lys$^{86}$, and Lys$^{89}$ (chymotrypsin numbering) are substituted was activated normally by thrombin, but the activation rate was no longer accelerated by TM. In a previous study with an antithrombin sensitive mutant of APC (T99Y), it was also demonstrated that heparin acceleration of the APC T99Y inhibition by antithrombin was Ca$^{2+}$-dependent. Taken together, these results suggest that, in contrast to thrombin, in which basic residues of the heparin binding site are masked by the noncatalytic prothrombin fragment 2 domain in its zymogen form, these residues are available in PC and may be critical for interaction with cofactors and other macromolecules in both the active and zymogen forms of the molecule.

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Rapid Activation of Protein C by Factor Xa and Thrombin in the Presence of Polyanionic Compounds

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