




ORIGINAL ARTICLE

Polyphosphate, Zn²⁺ and high molecular weight kininogen modulate individual reactions of the contact pathway of blood clotting

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Abstract

Background: Inorganic polyphosphate modulates the contact pathway of blood clotting, which is implicated in thrombosis and inflammation. Polyphosphate polymer lengths are highly variable, with shorter polymers (approximately 60-100 phosphates) secreted from human platelets, and longer polymers (up to thousands of phosphates) in microbes. We previously reported that optimal triggering of clotting via the contact pathway requires very long polyphosphates, although the impact of shorter polyphosphate polymers on individual proteolytic reactions of the contact pathway was not interrogated.

Objectives and methods: We conducted in vitro measurements of enzyme kinetics to investigate the ability of varying polyphosphate sizes, together with high molecular weight kininogen and Zn²⁺, to mediate four individual proteolytic reactions of the contact pathway: factor XII autoactivation, factor XII activation by kallikrein, prekallikrein activation by factor XIIa, and prekallikrein autoactivation.

Results: The individual contact pathway reactions were differentially dependent on polyphosphate length. Very long-chain polyphosphate was required to support factor XII autoactivation, whereas platelet-size polyphosphate significantly accelerated the activation of factor XII by kallikrein, and the activation of prekallikrein by factor XIIa. Intriguingly, polyphosphate did not support prekallikrein autoactivation. We also report that high molecular weight kininogen was required only when kallikrein was the enzyme (ie, FXII activation by kallikrein), whereas Zn²⁺ was required only when FXII was the substrate (ie, FXII activation by either kallikrein or FXIIa). Activation of prekallikrein by FXIIa required neither Zn²⁺ nor high molecular weight kininogen.

Conclusions: Platelet polyphosphate and Zn²⁺ can promote subsets of the reactions of the contact pathway, with implications for a variety of disease states.

KEYWORDS

blood coagulation factors, polyphosphates, prekallikrein, thrombosis, zinc

1 | INTRODUCTION

The contact pathway of blood clotting—also known as the plasma kallikrein-kinin system—includes the circulating serine protease zymogens, factor XII (FXII) and plasma prekallikrein (PK), as well as high-molecular-weight kininogen (HK), the non-enzymatic cofactor for PK and a substrate for plasma kallikrein (PKa).^{1,2} This pathway, a schematic of which appears in Figure 1, is initiated when blood or plasma is exposed to certain charged polymers or suitable surfaces.³ This interaction is thought to induce a conformational change in FXII that produces small amounts of enzymatically active FXIIa via autoactivation (more precisely, activation of FXII by FXIIa).⁴ FXIIa then cleaves PK to PKa, which can reciprocally activate more FXII to FXIIa.⁵ PK was also reported to undergo autoactivation to form PKa in the presence of a suitable polymer or surface.⁶ Once significant concentrations of FXIIa accumulate, FXIIa activates factor XI (FXI) to FXIa, triggering the intrinsic cascade of coagulation that leads to thrombin generation and fibrin formation.⁷ The contact pathway is also a critical connection for crosstalk between coagulation and inflammation.^{2,8,9} However, this pathway is completely dispensable for hemostasis, as individuals deficient in either FXII^{10,11} or PK¹² have no bleeding tendencies. On the other hand, animal studies have shown that targeting FXII or PK is protective in thrombosis models,¹³⁻¹⁷ suggesting that the contact pathway could contribute to the development of thrombosis in humans.

While several artificial surfaces and polymers may activate the contact pathway *in vitro*, the relevant (patho)physiologic activators for this system *in vivo* have only recently been suggested.¹ Long-chain polyphosphate (polyP), in particular, has emerged as an

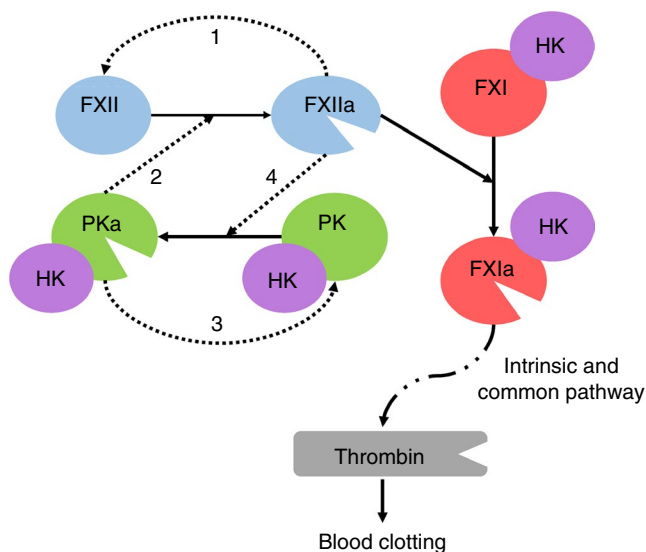


FIGURE 1 The following numbered reactions that contribute to initiation of the contact pathway of blood clotting were examined in this study: 1, activation of FXII by FXIIa (FXII autoactivation); 2, activation of FXII by PKa; 3, activation of PK by PKa (PK autoactivation); and 4, activation of PK by FXIIa. Propagation of the clotting cascade happens when FXIIa activates FXI, leading ultimately to thrombin generation

Essentials

- How individual contact pathway reactions depend on polyphosphate length was not known.
- Effect of polyphosphate, high-MW kininogen and Zn^{2+} on each reaction was investigated.
- The only reaction requiring very long-chain polyphosphate was factor XII autoactivation.
- Platelet-size polyphosphate strongly promoted subsets of reactions of the contact pathway.

especially potent trigger of the contact pathway.¹⁸ In this study, we aimed to investigate the ability of various sizes of polyP, together with two important cofactors, HK and Zn^{2+} , to modulate four proteolytic reactions of the contact pathway: FXII autoactivation, FXII activation by PKa, PK activation by FXIIa, and PK autoactivation.

PolyP is a highly negatively charged polymer of inorganic phosphates that varies in length, depending on biological source. PolyP released from platelets has a fairly narrow size range of approximately 60-100 phosphates long,¹⁹ whereas unicellular organisms contain heterogeneous polyP that can range up to thousands of phosphates in length.²⁰ Roles for polyP in prokaryotes and unicellular eukaryotes have been extensively studied²¹ but only recently have polyP's functions been explored in multicellular eukaryotes (particularly in mammals). Our previous work demonstrated that polyP acts at multiple stages of the clotting cascade, with pro-hemostatic, pro-thrombotic, and pro-inflammatory effects that are highly dependent on polyP chain length.^{18,22} In particular, we reported that optimal triggering of plasma clotting via the contact pathway requires very long polyP polymers,¹⁸ although the polymer length dependence of each individual reaction of the contact pathway was not investigated. In the present study we investigated the details of the requirement for very long-chain polyP to trigger clotting via the contact pathway. Individual enzyme reactions within the contact pathway can contribute to the complement cascade, fibrinolysis, bradykinin generation, and inflammation.^{1,2} Accordingly, we hypothesized that the polyP length requirement might not be the same for all four of the individual reactions in Figure 1, with platelet-sized polyP possibly supporting subsets of these reactions. We now report that only FXII autoactivation required very long-chain polyP, whereas the reciprocal enzyme activation reactions (FXII activation by kallikrein; prekallikrein activation by FXIIa) were accelerated to significant extents by platelet-sized polyP. Furthermore, HK and Zn^{2+} are required only for specific subsets of these reactions.

2 | MATERIALS AND METHODS

2.1 | Materials

Polybrene (hexadimethrine bromide), polyethylene glycol and soybean trypsin inhibitor were from Sigma. Human FXII, α -FXIIa, PK,

PKa, and single-chain HK were from Enzyme Research Laboratories. CTI was from Haematologic Technologies (Essex Junction). Polyclonal goat anti-human PK antibody (IgG) was from Affinity Biologicals. The chromogenic substrate for FXIIa or PKa, H₇-D-Pro-Phe-Arg-pNA (L-2120), was from Bachem. Chelex® 100 Resin was from Bio-Rad Laboratories. Ultra-Low Attachment microplates were from Corning Inc.

Two preparations of heterogeneous, long-chain polyP employed in this study were prepared starting from chemically synthesized, high molecular weight polyP (Sigma-Aldrich). One preparation, termed polyP₇₀₀, had a modal length of 700 phosphates (range, 200-1300 phosphates) and was solubilized from high molecular weight polyP as described.²³ The other, termed polyP₁₂₀₀, had a modal length of 1200 phosphates (range, 595-1935 phosphates). It was produced from solubilized high molecular weight polyP²³ after precipitation with 10 mmol/L NaCl and 25% isopropanol. Narrowly size-fractionated polyP preparations were produced from chemically synthesized polyP by preparative PAGE as previously described,^{18,24} and are referred to by their polymer lengths, which were: 28, 42, 65, 68, 79, 110, 143, 211, 315, and 415 phosphates. PolyP concentrations were quantified by measuring inorganic phosphate following hydrolysis in 1 mol/L HCl at 100°C for 10 minutes.²⁴ Throughout this study, polyP concentrations are given in terms of molar concentrations of phosphate monomer.

2.2 | FXII autoactivation

Reactions were performed at 37°C in ultra-low attachment microplate wells with 100 nmol/L FXII in HBSP solution (20 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.4), 100 mmol/L NaCl, and 1% w/v polyethylene glycol, 8000 MW). Pilot experiments evaluating the polyP dose dependence included 5 μmol/L ZnCl₂ in the presence of 0 to 100 μmol/L polyP₁₂₀₀ or polyP₇₉. Experiments evaluating the polyP size dependence included 5 μmol/L ZnCl₂ in the absence or presence of 10 μmol/L narrowly sized-fractionated polyP. Experiments evaluating the impact of ZnCl₂ included 10 μmol/L polyP₁₂₀₀ and either 2 mmol/L EDTA or 0 to 20 μmol/L ZnCl₂. Experiments evaluating the impact of HK included 10 μmol/L polyP₁₂₀₀, 5 μmol/L ZnCl₂, and 0 to 900 nmol/L HK. Timed aliquots (10 μL) were removed and quenched in 70 μL ice-cold Quench Buffer I (20 mmol/L HEPES-NaOH [pH 7.4], 5 mmol/L EDTA, and 1 mol/L NaCl). Following quenching, FXIIa amidolytic activities were quantified by measuring A₄₀₅ at 37°C and converting to FXIIa concentrations by reference to a standard curve. Second-order rate constants for FXII autoactivation were calculated according to the method of Tans et al.,⁶ based on the equation, $d[\text{FXIIa}]/dt = k_2[\text{FXII}][\text{FXIIa}]$.

2.3 | FXII activation by PKa

These reactions were also conducted in HBSP at 37°C in ultra-low attachment microplate wells. Pilot experiments evaluating the polyP dose dependence contained 100 nmol/L FXII, 100 pmol/L

PKa, 100 nmol/L HK, and 10 μmol/L ZnCl₂, in the presence of 0 to 100 μmol/L polyP₁₂₀₀ or polyP₇₉. Experiments evaluating the polyP size-dependence contained 100 nmol/L HK and 10 μmol/L ZnCl₂, in the absence or presence of 10 μmol/L narrowly size-fractionated polyP. Experiments evaluating the impact of ZnCl₂ included 10 μmol/L polyP₁₂₀₀, 100 nmol/L HK, and either 2 mmol/L EDTA or 0 to 20 μmol/L ZnCl₂. Experiments evaluating the impact of HK included 10 μmol/L polyP₁₂₀₀, 10 μmol/L ZnCl₂, and 0 to 900 nmol/L HK. Timed aliquots (10 μL) were removed and quenched in 70 μL ice-cold Quench Buffer II (20 mmol/L HEPES-NaOH pH 7.4, 5 mmol/L EDTA, 50 μg/mL soybean trypsin inhibitor, and 15 μg/mL polybrene). Following quenching, FXIIa amidolytic activities were quantified by measuring A₄₀₅ at 37°C and converting to FXIIa concentrations by reference to a standard curve.

2.4 | PK activation by FXIIa

These reactions were also conducted in HBSP at 37°C in ultra-low attachment microplate wells. Pilot experiments evaluating the polyP dose dependence contained 100 nmol/L PK, 100 pmol/L FXIIa and 10 μmol/L ZnCl₂, in the presence of 0 to 100 μmol/L polyP₁₂₀₀ or polyP₇₉. Experiments evaluating polyP size-dependence included 10 μmol/L ZnCl₂, in the absence or presence of 10 μmol/L narrowly size-fractionated polyP. For experiments evaluating the impact of ZnCl₂, HBSP was pretreated with Chelex® 100 resin (buffered to pH 7.4) to deplete trace divalent metal ions. These assays included 10 μmol/L polyP₁₂₀₀ and either 0 to 10 mmol/L EDTA or 0 to 10 μmol/L ZnCl₂. Experiments evaluating the impact of HK included 10 μmol/L P₁₂₀₀, 10 μmol/L ZnCl₂, and 0-900 nmol/L HK. Timed aliquots (10 μL) were removed and quenched in 70 μL ice-cold Quench Buffer I. Following quenching, amidolytic activities were quantified by measuring A₄₀₅ at 37°C (to prevent temperature-dependent auto-inhibition of PKa²⁵). Amidolytic activities were converted to PKa concentrations by reference to a standard curve.

2.5 | PK autoactivation

Apparent PK autoactivation was evaluated by quantifying PKa after incubating 100 nmol/L PK with 100 nmol/L HK, 10 μmol/L ZnCl₂, 10 μmol/L polyP₁₂₀₀, and 0 to 2.5 μmol/L CTI in HBSP at 37°C. Timed, 10 μL-aliquots were quenched in 70 μL ice-cold Quench Buffer I. Following quenching, PKa amidolytic activities were quantified by measuring A₄₀₅ at 37°C and converting to PKa concentrations by reference to a standard curve.

Additional experiments employed an inactive mutant form of PK with the active-site serine replaced with alanine (PK-S559A).²⁶ PK-S559A cDNA²⁷ was expressed in HEK293 cells using vector pJVCMV under serum-free conditions, and PK-S559A protein was purified by anion-exchange chromatography as described.²⁶ Enzymatic reactions were then conducted in polypropylene tubes coated with polyethylene glycol (20 000 MW). 200 nmol/L PK-S559A, with or without 70 μmol/L polyP₇₀₀, 200 nmol/L HK, or 25 nmol/L PKa, was incubated at 37°C in HBSP containing 10 μmol/L ZnCl₂ and

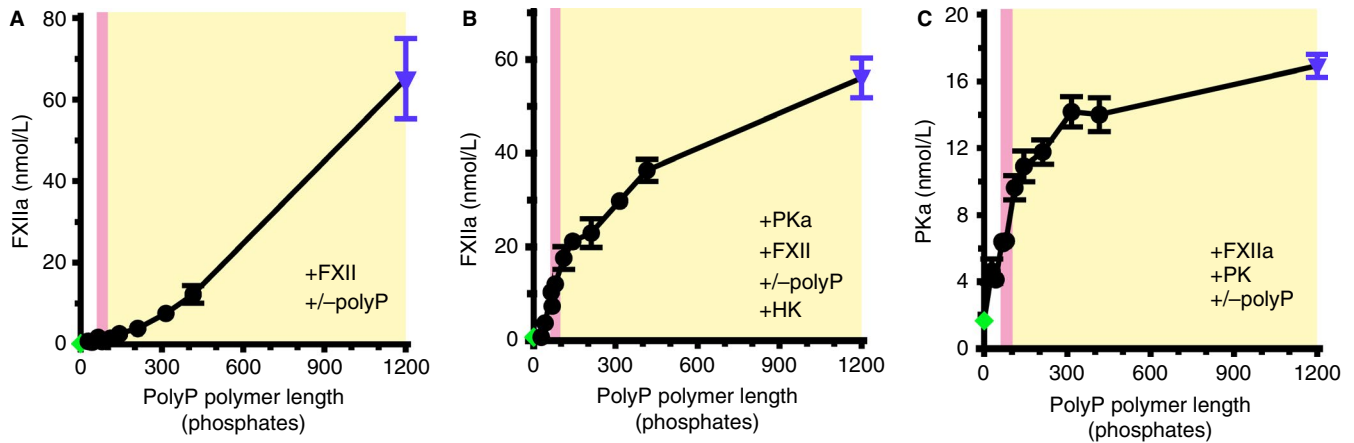


FIGURE 2 The ability of polyP to accelerate the individual reactions of the contact pathway depends on its polymer length. In each panel, reactions were conducted without (◆) or with 10 $\mu\text{mol/L}$ polyP (●, ▼). Data points for size-fractionated polyP preparations (●) are plotted on the x-axes according to their polymer lengths, whereas data points for heterogeneous polyP₁₂₀₀ (▼) are plotted on the x-axes at the modal length of this preparation (1200 phosphate units). In each panel, a pink rectangle represents the approximate length range of platelet polyP (60–100 phosphates),¹⁹ and a yellow rectangle indicates the length range of microbial polyP.⁵⁴ A, PolyP length requirement for FXII autoactivation. In an endpoint assay (stopped at 20 min), 100 nmol/L FXII was incubated with 5 $\mu\text{mol/L}$ ZnCl₂ without polyP (◆) or with 10 $\mu\text{mol/L}$ polyP (●, ▼). HK was not included in this experiment. B, PolyP length requirement for FXII activation by PKa. In an endpoint assay (stopped at 4 min), 100 nmol/L FXII was incubated with 100 pmol/L PKa, 100 nmol/L HK and 10 $\mu\text{mol/L}$ ZnCl₂, without polyP (◆) or with 10 $\mu\text{mol/L}$ polyP (●, ▼). C, PolyP length requirement for PK activation by FXIIa. In an endpoint assay (stopped at 3 min), 100 nmol/L PK was incubated with 100 pmol/L FXIIa and 10 $\mu\text{mol/L}$ ZnCl₂, without polyP (◆) or with 10 $\mu\text{mol/L}$ polyP (●, ▼). HK was not included in this experiment. Data in all panels are mean \pm SE ($n \geq 3$)

50 nmol/L of the blocking anti-FXII(a) monoclonal antibody, 1B2.²⁸ At various time points, aliquots were removed and mixed with reducing SDS sample buffer. Samples were resolved on SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. Blots were probed with polyclonal IgG to PK. Detection was with a horseradish peroxidase-conjugated secondary antibody and chemiluminescence.

3 | RESULTS

The four individual enzyme activation reactions that can contribute to activation of plasma clotting via the contact pathway are numbered in Figure 1. The goal of the present study was to understand how the polymer length of polyP influences its ability to accelerate these four enzyme reactions individually. Because HK and Zn²⁺ are known modulators of the contact pathway, we also examined their contributions to each reaction, together with polyP. Pilot experiments were performed to determine the optimal concentrations of long-chain and platelet-size polyP to drive individual reactions of the contact pathway (Figures S1, S3, and S4, available in the online supplemental information). For all three reactions, we used 10 $\mu\text{mol/L}$ polyP (in terms of phosphate monomer), which was within the concentration range that gave maximal reaction rates.

3.1 | PolyP length-requirement for contact pathway reactions

PolyP accelerated FXII autoactivation in a manner that was strongly dependent on polyP polymer length and concentration (Figures 2A

and S1). Under the experimental conditions tested (100 nmol/L FXII in the presence of 5 $\mu\text{mol/L}$ ZnCl₂, examined in a 20-minute endpoint assay), only 0.15 ± 0.03 nmol/L FXIIa was generated in the absence of polyP, whereas 420-fold more FXIIa was generated in the presence of polyP₁₂₀₀. Platelet-size polyP (79 phosphates) supported only about fourfold more FXIIa generation than in the absence of polyP. Compared with polyP₁₂₀₀, platelet-size polyP did not detectably support this reaction in a 45-minute kinetic assay (Figure S2). Our finding that the amount of FXII autoactivation increased continually with increasing polyP polymer length is highly reminiscent of the polymer length-dependence by which polyP triggers plasma clotting via the contact pathway.¹⁸ It is also consistent with our previous finding that platelet-size polyP is very weak at triggering clotting via the contact pathway.¹⁸

PolyP was required for efficient FXII activation by PKa, in a manner that depended on polyP polymer length and concentration (Figures 2B and S3). In a 4-minute endpoint assay (using 100 nmol/L FXII with 100 pmol/L PKa, 100 nmol/L HK, and 10 $\mu\text{mol/L}$ ZnCl₂), only 0.72 ± 1.11 nmol/L FXIIa was generated in the absence of polyP. Inclusion of polyP₁₂₀₀ increased FXIIa generation approximately 77-fold. In contrast to FXII autoactivation, however, the effect of polyP polymer length on activation of FXII by PKa showed signs of plateauing at longer polymer lengths. Furthermore, platelet-size polyP (79 phosphates) supported 16-fold more FXIIa generation than in the absence of polyP. These data demonstrated that platelet-size polyP can accelerate FXII activation by PKa, although the reaction was fastest with long-chain polyP.

PolyP was not absolutely required for PK activation by FXIIa, although the reaction was accelerated by polyP (Figures 2C and S4). Under the experimental conditions tested (100 nmol/L PK with 100 pmol/L FXIIa in the presence of 10 $\mu\text{mol/L}$ ZnCl₂, examined in

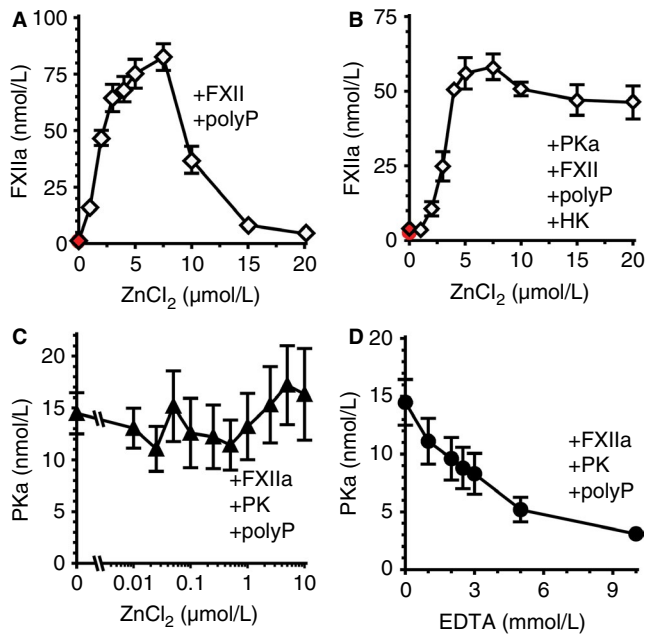


FIGURE 3 Zn^{2+} differentially influences the ability of polyP to accelerate individual reactions of the contact pathway. A, Zn^{2+} requirement for polyP-mediated FXII autoactivation. In an endpoint assay (stopped at 20 min), 100 nmol/L FXII without HK was incubated with 10 μ mol/L polyP₁₂₀₀ in the presence of either 2 mmol/L EDTA (●) or varying $ZnCl_2$ concentrations (◇). B, Zn^{2+} requirement for polyP-mediated FXII activation by PKa. In an endpoint assay (stopped at 4 min), 100 nmol/L FXII and 100 pmol/L PKa were incubated with 100 nmol/L HK and 10 μ mol/L polyP₁₂₀₀ in the presence of either 2 mmol/L EDTA (●) or varying $ZnCl_2$ concentrations (◇). C, Zn^{2+} requirement for polyP-mediated PK activation by FXIIa. In an endpoint assay (stopped at 3 min), 100 nmol/L PK and 100 pmol/L FXIIa without HK were incubated with 10 μ mol/L polyP₁₂₀₀ in divalent metal ion-depleted buffer to which varying $ZnCl_2$ concentrations were added. D, EDTA inhibits polyP-mediated PK activation by FXIIa in a concentration-dependent manner. In an endpoint assay (stopped at 3 min), 100 nmol/L PK and 100 pmol/L FXIIa without HK were incubated with 10 μ mol/L polyP₁₂₀₀ in the presence of varying EDTA concentrations. Data in all panels are mean \pm SE ($n \geq 3$)

a 3-minute endpoint assay), 1.67 ± 0.03 nmol/L PKa was generated in the absence of polyP, whereas 10-fold more PKa was generated in the presence of polyP₁₂₀₀. As a function of polyP polymer length, this reaction tended to plateau at even shorter polyP polymer lengths (approximately 315 phosphates) compared with FXII activation by PKa. Platelet-size polyP (79 phosphates) supported approximately 3.8-fold more PKa generation than in the absence of polyP. Thus, as with FXII activation by PKa, these data demonstrated that platelet-size polyP can accelerate FXII activation by PKa, although the reaction was fastest with longer-chain polyP.

3.2 | Influence of Zn^{2+} on contact pathway reactions

Varying the $ZnCl_2$ concentration (in the absence of HK) demonstrated that Zn^{2+} was required for polyP-mediated FXII autoactivation (Figure 3A). FXII autoactivation was essentially undetectable in

the presence of 2 mmol/L EDTA in the 20-minute endpoint assay, whereas the optimal $ZnCl_2$ concentration was approximately 3–7.5 μ mol/L. Because Zn^{2+} was required for this reaction, 5 μ mol/L $ZnCl_2$ was included in FXII autoactivation reactions unless otherwise noted.

FXII activation by PKa in the presence of HK was also Zn^{2+} -dependent, and only minimal amounts of FXIIa (2.74 ± 0.36 nmol/L) were generated in the presence of EDTA in the 4-minute endpoint assay (Figure 3B). Optimal $ZnCl_2$ concentrations were 4 μ mol/L or higher, resulting in FXIIa generation that was approximately 20-fold higher than in the absence of $ZnCl_2$. Accordingly, 10 μ mol/L $ZnCl_2$ was included in reactions of FXII activation by PKa, unless otherwise noted.

Activation of PK by FXIIa in the absence of HK was not modulated significantly by added $ZnCl_2$, even when we used solutions that had been pretreated with Chelex beads to deplete trace amounts of divalent metal ions (Figure 3C). On the other hand, inclusion of 2 mmol/L EDTA decreased the amount of FXIIa generated by 44% compared to the absence of added $ZnCl_2$ (data not shown). To further investigate this, PK activation by FXIIa was studied in an endpoint assay in the presence of varying EDTA concentrations (Figure 3D). We observed that EDTA inhibited this reaction in a concentration-dependent manner over the range of 0 to 10 mmol/L EDTA. We propose that anionic EDTA molecules may compete with polyP for interaction with PK and/or FXIIa, independent of the ability of EDTA to chelate divalent metal ions.

3.3 | Influence of HK on contact pathway reactions

Although FXII is not thought to circulate bound to HK in vivo, we tested if HK could influence rates of polyP-mediated FXII autoactivation (Figure 4A). Without HK, polyP₁₂₀₀ readily supported FXII autoactivation in the presence of 5 μ mol/L $ZnCl_2$. Progress curves for FXII autoactivation were sigmoidal (Figure S2), consistent with models of this process.^{29,30} Second-order rate constants for FXII autoactivation were derived from analyses of the reaction data replotted in Figure 4B, then given in Figure 4C vs HK concentration. Although HK was not required for polyP-mediated FXII autoactivation, up to 100 nmol/L HK accelerated the reaction somewhat, whereas higher concentrations were inhibitory.

HK substantially increased rates of polyP-mediated FXII activation by PKa (Figure 4D). Thus, in the absence of HK, the FXII activation rate was only 10.44 ± 4.36 (nmol/L)/min/(nmol/L) while in the presence of 50 to 200 nmol/L HK, this rate was accelerated approximately 13.4-fold relative to no HK. As little as 0.5 nmol/L HK supported the reaction to a detectable level (~3.2-fold increase over no HK), while HK concentrations of 300 nmol/L and higher reduced the reaction rate. Because the activation of FXII by PKa was readily accelerated by HK, 100 nmol/L HK was generally included in other studies of this reaction.

While HK was not required for polyP-mediated PK activation by FXIIa, the reaction was stimulated by about 34% in the presence of 200–400 nmol/L HK (Figure 4E). Higher HK concentrations were inhibitory.

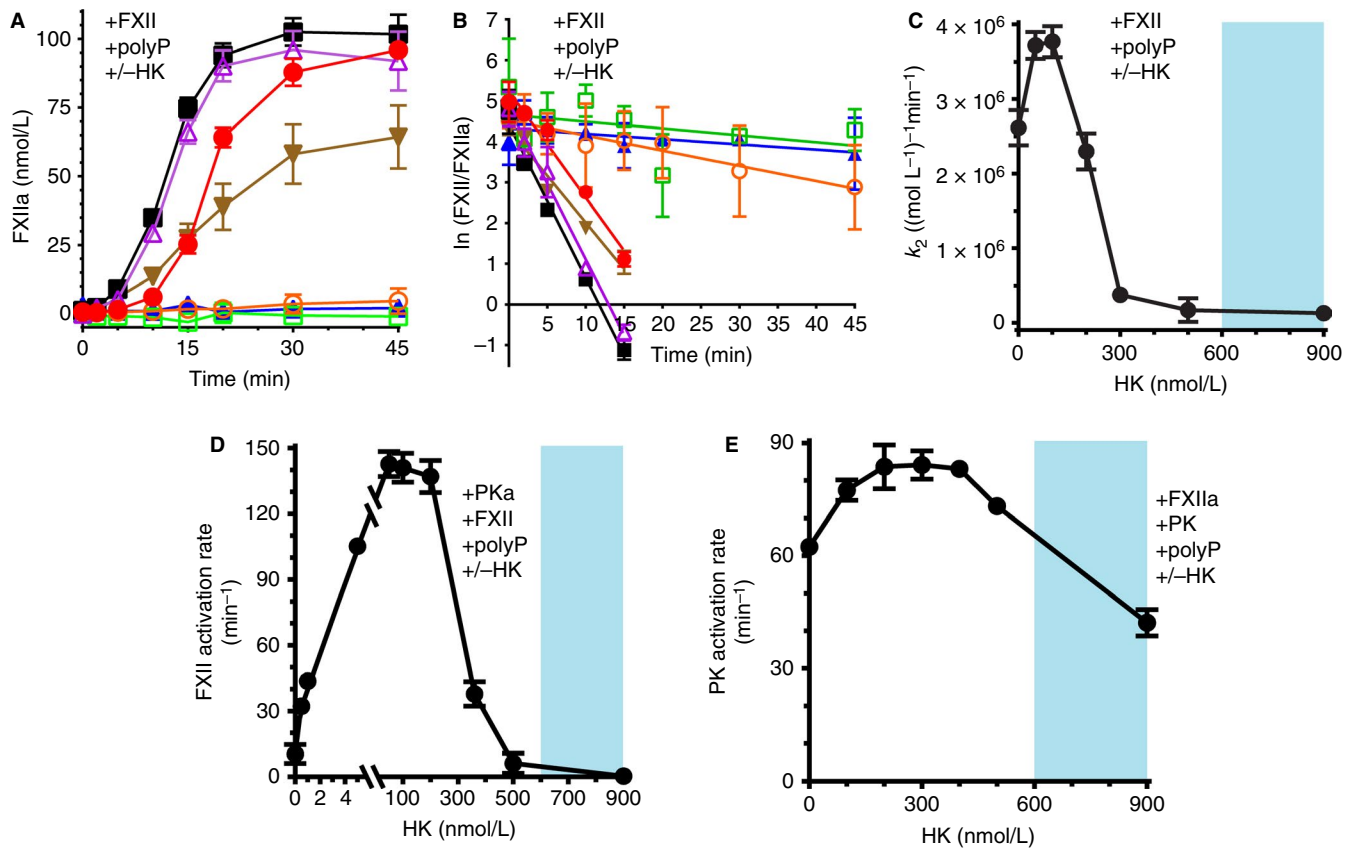


FIGURE 4 HK differentially influences the ability of polyP to accelerate individual reactions of the contact pathway. A, Influence of HK on polyP-mediated FXII autoactivation. FXIIa levels were measured as a function of time after incubating 100 nmol/L FXII with 10 $\mu\text{mol/L}$ polyP₁₂₀₀ in the presence of 5 $\mu\text{mol/L}$ ZnCl₂ and 0 (●), 50 (△), 100 (■), 200 (▼), 300 (○), 500 (□), or 900 (▲) nmol/L HK. B-C, Calculation of second-order rate constants of polyP-mediated FXII autoactivation at various HK concentrations. Data from panel A (time course of FXII autoactivation) were replotted in panel B as $\ln(\text{FXII}/\text{FXIIa})$ values vs time, to which lines were fitted by linear regression. Second-order rate constants, k_2 , were derived from the slopes of these lines as described in *Experimental Procedures*, and plotted in panel C as a function of HK concentration. D, Influence of HK on polyP-mediated FXII activation by PKa. FXIIa levels were measured as a function of time after incubating 100 nmol/L FXII and 100 pmol/L PKa with 10 $\mu\text{mol/L}$ polyP₁₂₀₀ in the presence of 10 $\mu\text{mol/L}$ ZnCl₂ and varying HK concentrations. Initial FXII activation rates are plotted as nmol/L FXII activated per min divided by nmol/L PKa used. E, Influence of HK on polyP-mediated PK activation by FXIIa. PKa levels were measured as a function of time after incubating 100 nmol/L PK and 100 pmol/L FXIIa with 10 $\mu\text{mol/L}$ polyP₁₂₀₀ in the presence of 10 $\mu\text{mol/L}$ ZnCl₂ and varying HK concentrations. Initial PK activation rates were plotted as nmol/L PK activated per min divided by nmol/L FXIIa used. In panel C-E, blue rectangles represent the approximate concentration range of total HK in plasma. Data in all panels are mean \pm SE ($n \geq 3$)

3.4 | PK autoactivation

Autoactivation of PK has been reported to occur in the presence of anionic substances such as dextran sulfate or sulfatides,⁶ so it was of interest to examine whether polyP could mediate PK autoactivation. Initial experiments showed that incubating 100 nmol/L PK with 100 nmol/L HK plus 10 $\mu\text{mol/L}$ polyP₁₂₀₀ resulted in time-dependent generation of readily detectable PKa (Figure 5A). We observed no detectable PKa in the absence of HK (data not shown), indicating that this reaction was highly HK-dependent. We noted, however, that the progress curve for PKa generation was not sigmoidal (Figure 5A, solid black circles), as would have been expected for autoactivation kinetics. We therefore considered the possibility that plasma-derived HK might be contaminated with traces of FXII or FXIIa. If so, we may be observing PK activation by traces of FXIIa, and/or reciprocal activation of PK and contaminating

FXII. To test this hypothesis, we examined the effect of including corn trypsin inhibitor (CTI) in this same experiment (Figure 5A). CTI inhibits FXIIa activity with minimal effect on the enzymatic activity of PKa.^{31,32} Including 0.5 or 2.5 $\mu\text{mol/L}$ CTI in this reaction reduced the amount of PKa generated at 90 minutes by 38% or 83%, respectively.

In further experiments, we used recombinant PK with the active-site serine mutated to alanine (PK-S559A). In the presence of an antibody that specifically inhibits FXIIa enzymatic activity (preventing reciprocal PK activation in the presence of FXII), this mutant PK zymogen remained single-chain over a 3-hr period, as visualized on western blot, whether incubated in the presence or absence of polyP or HK (Figure 5B). Even when 25 nmol/L PKa was coincubated with 200 nmol/L PK-S559A (in the presence or absence of polyP or HK), no PK-S559A cleavage was observed over a 3-hour time course (Figure 5C). These findings argue against polyP supporting detectable PK activation by

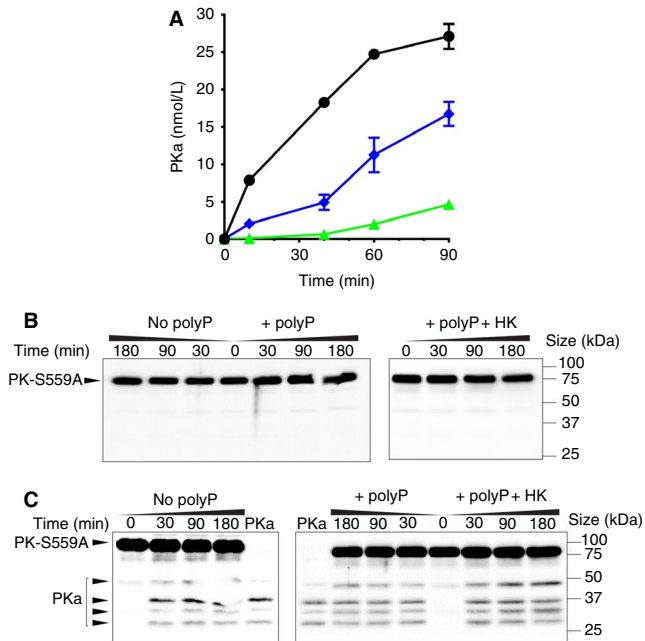


FIGURE 5 Activation of PK in the presence of polyP and HK appears to be due to contamination of HK with traces of FXII(a), rather than PK autoactivation. **A**, Apparent PK autoactivation was inhibited by CTI. PKa levels were measured as a function of time after incubating 100 nmol/L PK with 100 nmol/L HK, 10 $\mu\text{mol/L}$ polyP₁₂₀₀ and 10 $\mu\text{mol/L}$ ZnCl₂ in presence of 0 (●), 0.5 (◆) or 2.5 (▲) $\mu\text{mol/L}$ CTI. Data are mean \pm SE ($n \geq 2$). **B**, In the presence of an inhibitory anti-FXII antibody, activation of PK-S559A was undetectable by western blot. *Left panel*: 200 nmol/L PK-S559A without HK was incubated with or without 70 $\mu\text{mol/L}$ polyP₇₀₀ in the presence of 10 $\mu\text{mol/L}$ ZnCl₂ and 50 nmol/L anti-FXII IgG (1B2). At indicated times, samples were removed into reducing SDS sample buffer. Proteins were resolved by SDS-PAGE, followed by western blot analysis with a polyclonal antibody to human PK. *Right panel*: Parallel time courses of the same reaction conditions as in the *left panel*, but performed in the presence of 200 nmol/L HK. In both panels, $t = 0$ represents the reactants without polyP or HK. **C**, In the presence of an inhibitory anti-FXII antibody, cleavage of PK-S559A by added PKa was undetectable by western blot, in the presence or absence of polyP. *Left panel*: 200 nmol/L PK-S559A and 25 nmol/L purified PKa were incubated without polyP or HK, in the presence of 10 $\mu\text{mol/L}$ ZnCl₂ and 50 nmol/L anti-FXII IgG (1B2). Samples were analyzed by western blot with the same polyclonal antibody to human PK as in *panel B*. *Right panel*: Parallel time courses of the reaction performed in the presence of 70 $\mu\text{mol/L}$ long-chain polyP₇₀₀, with or without 200 nmol/L HK. In both panels, $t = 0$ represents the reaction without HK, polyP or PKa. PKa alone is resolved on the gels in the both panels for comparison purposes. Blots are representative of two independent experiments

PKa, and that contamination with traces of FXII(a) was responsible for the apparent PK autoactivation in the presence of polyP plus HK.

4 | DISCUSSION

We reported previously that polyP is a novel trigger of plasma clotting via the contact pathway,³³ that polyP is orders of magnitude

more potent at triggering clotting than other proposed pathophysiologic contact activators such as nucleic acids,³⁴ and that the longer the polyP, the greater its ability to activate clotting via the contact pathway.¹⁸ As summarized in Table 1, we now report how the individual enzyme reactions responsible for initiating the contact pathway are influenced by polyP, Zn²⁺, and HK. We found that FXII autoactivation, FXII activation by PKa, and PK activation by FXIIa were all profoundly accelerated by polyP, in a manner that was dependent on polyP polymer length. In contrast, we found no evidence of detectable PK autoactivation in the presence of polyP, with the apparent autoactivation reaction most likely resulting from trace contamination of FXII(a) in the HK preparation.

Efficient FXII autoactivation required very long polyP polymers, with a dependence on polyP length that was highly reminiscent of the polyP length-dependence for triggering the clotting of plasma via the contact pathway.¹⁸ In contrast, we found that the two reciprocal enzyme activation reactions (FXII activation by PKa, and PK activation by FXIIa) exhibited patterns of polyP length dependence that differed from that of FXII autoactivation. In particular, platelet-size polyP accelerated both FXII activation by PKa, and PK activation by FXIIa, but had little effect on FXII autoactivation. Our findings suggest that FXII autoactivation is the step in the initiation of the contact pathway that is critically dependent on polyP polymer length, and that it is the inability to readily promote FXII autoactivation that underlies the low plasma clotting activity of platelet-size polyP.¹⁸

Although a recent study reported that platelet polyP can potentially activate the contact pathway,³⁵ that study used silica-based methods for isolating polyP, which we have now shown can result in contamination with highly procoagulant silica particles.^{23,34} Furthermore, decades of studies have shown that activated platelets only very weakly activate the contact pathway of clotting.³⁶

It has long been assumed that any enzymatic activity of preparations of zymogen FXII derived from plasma were due to contamination of these preparations with traces of FXIIa. However, when Ivanov et al²⁶ used recombinant FXII proteins that were locked in an uncleavable conformation, they reported weak proteolytic activity of single-chain FXII, which was enhanced by the presence of polyP. In the present study, we observed polyP-mediated conversion of FXII to FXIIa, as quantified by quenching the reactions to displace bound polyP. The polyP-mediated autoactivation we observed could be initiated by the weak proteolytic activity of single-chain FXII bound to polyP, traces of FXIIa in the FXII preparation, or both.

One may wonder why maximal rates of FXII autoactivation required such long polyP polymers. The molecular mass of FXII(a) is about 80 kDa, with an estimated diameter of 5–6 nm, assuming a spherical shape.³⁷ A polyP polymer of 1200 phosphates would have a fully extended length of about 240 nm,³⁸ which is some fortyfold longer than the diameter of FXII. A possible explanation is that efficient FXII autoactivation may require assembly of many FXII and FXIIa molecules together on the same polyP scaffold. Another possibility is that Zn²⁺ binding to polyP alters its structure such that it does not exist

TABLE 1 Summary of the influence of polyP polymer length, HK, and ZnCl₂ on four reactions of the contact pathway

Reaction number ^a	Enzyme	Substrate	Optimal condition		
			PolyP length (phosphates)	HK (nmol/L)	ZnCl ₂ (μmol/L)
1	FXIIa	FXII	1200	Not required	3-7.5
2	PKa	FXII	400-1200	50-200	4-20
3	PKa	PK	No reaction	No reaction	No reaction
4	FXIIa	PK	300-1200	Not required	Not required

^aReaction numbers correspond to those in Figure 1.

in a fully extended conformation. A parallel finding from a previous study is that hairpin-forming RNA oligomers are more procoagulant than linear RNA, and in particular, structure-forming RNA aptamers were much better at mediating PK autoactivation.³⁹ There are at least four putative Zn²⁺ binding sites on the heavy chain of FXII,⁴⁰ so interactions between Zn²⁺, polyP and FXII(a) could be quite complex.

Zn²⁺ enhances the binding affinities and proteolytic activities of many of the proteins in the contact pathway.^{29,41,42} Zn²⁺ induces conformational changes in HK,⁴³ and a binding site for Zn²⁺ has been described in the D5 domain of HK, which also contains the binding site for anionic polymers.^{44,45} As summarized in Table 1, we found that micromolar concentrations of ZnCl₂ greatly enhanced both of the enzymatic reactions in which FXII was the substrate (ie, polyP-mediated FXII autoactivation and activation of FXII by PKa), but ZnCl₂ had little effect on the enzymatic reaction in which PK was the substrate (ie, polyP-mediated activation of PK by FXIIa). Interestingly, although we found no requirement for ZnCl₂ in supporting polyP-mediated PK activation by FXIIa, this reaction was inhibited by EDTA in a concentration-dependent manner (Figure 3D). EDTA chelates divalent metal ions, but it also contains 4 anionic carboxylate groups. We suspect that millimolar concentrations of anionic EDTA might compete with anionic polyP for binding to PK, FXIIa or both. Indeed, higher polyP₁₂₀₀ concentrations progressively reduced the inhibitory effect of EDTA (Figure S5).

In the present study, HK played different roles in each reaction in the contact pathway (Table 1). In fact, the only reaction strongly requiring HK was the one in which PKa served as the enzyme (ie, polyP-mediated activation of FXII by PKa). This is consistent with our previous finding that at physiologic salt concentrations, PKa did not bind appreciably to polyP unless HK was present.⁴⁶ HK was not required in reactions in which FXIIa was the active enzyme (ie, polyP-mediated FXII autoactivation and FXIIa activation of PK), although HK did modulate these reactions to some degree. In all three reactions investigated in this study, higher HK concentrations (≥500 nmol/L) were inhibitory. HK circulates at a plasma concentration of about 650 to 900 nmol/L,⁴⁷ which falls in the range that inhibited polyP-driven contact pathway reactions. However, in plasma, much of the HK circulates bound to FXI and PK (and possibly other partners), so the free HK concentration is not known with certainty. In this study, the concentrations of HK and ZnCl₂ were chosen to optimize the biochemical assays and not necessarily mimic

physiologic concentrations. The effect of HK on FXII autoactivation might be particularly surprising, since binding interactions between HK and FXII(a) have not been shown. However, one could rationalize the effect of HK on FXII autoactivation by postulating that HK competes with FXII(a) for binding to polyP, so that high HK concentrations would alter the effective concentration of polyP available to interact with FXII. This might also explain the mildly stimulatory and inhibitory effects of HK on polyP-mediated PK activation by FXIIa, depending on HK concentration. Exploring this further would require systematically varying both the polyP and HK concentrations. Reaction rates reported here are maximal rates under fixed conditions and rates in vivo may vary. In particular, the apparent ability of high HK concentrations to inhibit reactions such as FXII autoactivation is likely to be reversed at higher polyP concentrations, although it was not practical to explore all possible combinations of reactant concentrations in the present study.

In summary, this study shows that three of the four possible enzyme activation reactions involved in triggering the contact pathway of clotting were greatly accelerated by polyP, in a manner that depended strongly on polyP polymer length. Of these three polyP-mediated reactions, Zn²⁺ was only required when FXII was the substrate, whereas HK was only required when PKa was the enzyme. PolyP polymer lengths are known to vary substantially depending on the biological source, with shorter polyP chains (60 to 100 phosphates long) secreted from activated platelets, mast cells and basophils.^{19,48} On the other hand, microbial polyP polymers can range in length up to thousands of phosphates,²⁰ and mammalian brain is also reported to contain long-chain polyP (approximately 800 phosphates long).⁴⁹ Furthermore, platelets secrete Zn²⁺, such that the local concentration of this metal ion in the vicinity of platelet aggregates could greatly exceed that of normal plasma.^{42,50}

Our findings therefore identify conditions under which these three polyP-mediated reactions (FXII autoactivation, FXII activation by PKa, and PK activation by FXIIa) may be differentially regulated via secretion of shorter-chain polyP and Zn²⁺. Thus, platelets, mast cells and basophils may be able to orchestrate subsets of the contact pathway reactions that could lead, for example, to bradykinin generation without activation of the final common pathway of the clotting cascade. Mechanisms for activating subsets of these enzyme reactions without necessarily triggering plasma clotting could have implications for inflammatory reactions and diseases

such as hereditary angioedema, which are driven by highly dysregulated bradykinin formation via the contact pathway, but without thrombosis.^{1,2,51} Other precedents for this exist. For example, previous studies that have reported that endothelial cell prolyl carboxypeptidase can activate PK independent of FXIIa.^{52,53} On the other hand, elaboration of long-chain polyP from microbes may be capable of activating the contact pathway to participate in both inflammatory reactions and the full plasma clotting system as part of the innate immune response.

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CONFLICT OF INTEREST

S.A.S. and J.H.M. are co-inventors on patents and pending patent applications on medical uses of polyP and polyP inhibitors. The research laboratory of J.H.M. receives revenues from sales of polyP and polyP derivatives through Kerafast, Inc. D.G. serves as a consultant and receives consultant's fees from Bayer AG, Bristol-Myers Squibb, Ionis Pharmaceuticals, Janssen Pharmaceuticals, and Novartis. Y.W. and I.I. declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Y.W. performed most of the experiments, analyzed results, and wrote most of the report. I.I. performed western blot experiments for PK autoactivation, analyzed results, and helped write the report. S.A.S. participated in designing experiments, preparing figures, and writing the report. D.G. and J.H.M. conceived the idea of the project, helped design experiments, and analyzed the results, and wrote the report. All authors reviewed the results and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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