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Polyphosphate, Zn^{2+} and high-molecular-weight kininogen modulate individual reactions of the contact pathway of blood clotting

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Running head: *Modulators of contact pathway reactions*

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Essentials

- How individual contact pathway reactions depend on polyphosphate length was not known
- Effect of polyphosphate, high-MW kininogen and Zn^{2+} on individual reactions 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

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 Zn^{2+}, 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 (i.e., FXII activation by kallikrein), whereas Zn^{2+} was required only when FXII was the substrate (i.e., FXII activation by either kallikrein or FXIIa). Activation of prekallikrein by FXIIa required neither Zn^{2+} nor high-molecular-weight kininogen.

Conclusions: Platelet polyphosphate and Zn^{2+} 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

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 Fig. 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 PK [12] 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 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], while 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 studied [21] 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 Fig. 1, with platelet-sized polyP possibly supporting subsets of these reactions. We now report that only FXII autoactivation required very long-chain polyP, while the reciprocal enzyme activation reactions (FXII activation by kallikrein, and 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.

Materials and Methods

Materials

Polybrene (hexadimethrine bromide), polyethylene glycol and soybean trypsin inhibitor were from Sigma (St. Louis, MO). Human FXII, α-FXIIa, PK, PKa and single-chain HK were from Enzyme Research Laboratories (South Bend, IN). CTI was from Haematologic Technologies (Essex Junction). Polyclonal goat anti-human PK antibody (IgG) was from Affinity Biologicals (Ancaster, Canada). The chromogenic substrate for FXIIa or PKa, H-$_D$-Pro-Phe-Arg-pNA (L-2120), was from Bachem (Bubendorf, Switzerland). Chelex® 100 Resin was from Bio-Rad Laboratories (Hercules, CA). Ultra-Low Attachment microplates were from Corning Inc (Tewksbury, MA).

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 $polyP_{700}$, had a modal length of 700 phosphates (range, 200 to 1300 phosphates) and was solubilized from high-molecular-weight polyP as described [23]. The other, termed $polyP_{1200}$, had a modal length of 1200 phosphates (range, 595 to 1935 phosphates). It was produced from solubilized high-molecular-weight polyP [23] after precipitation with 10 mM 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 M HCl at 100°C for 10 min [24]. Throughout this study, polyP concentrations are given in terms of molar concentrations of phosphate monomer.

FXII autoactivation

Reactions were performed at 37°C in ultra-low attachment microplate wells with 100 nM FXII in HBSP solution (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.4), 100 mM NaCl, and 1% w/v polyethylene glycol, 8000 MW). Pilot experiments evaluating the polyP dose dependence included 5 μM $ZnCl_2$ in the presence of 0 to100 μM $polyP_{1200}$ or $polyP_{79}$. Experiments evaluating the polyP size dependence included 5 μM $ZnCl_2$ in the absence or presence of 10 μM narrowly sized-fractionated polyP. Experiments evaluating the impact of $ZnCl_2$ included 10 μM $polyP_{1200}$ and either 2 mM EDTA or 0 to 20 μM $ZnCl_2$. Experiments evaluating the impact of HK included 10 μM $polyP_{1200}$, 5 μM $ZnCl_2$, and 0 to 900 nM HK. Timed aliquots (10 μL) were removed and quenched in 70

µl ice-cold Quench Buffer I (20 mM HEPES-NaOH (pH 7.4), 5 mM EDTA, and 1 M NaCl). Following quenching, FXIIa amidolytic activities were quantified by measuring A_{405} 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, $d[\text{FXIIa}]/dt = k_2[\text{FXII}][\text{FXIIa}]$.

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 nM FXII, 100 pM PKa, 100 nM HK, and 10 µM $ZnCl_2$, in the presence of 0 to 100 µM $polyP_{1200}$ or $polyP_{79}$. Experiments evaluating the polyP size-dependence contained 100 nM HK and 10 µM $ZnCl_2$, in the absence or presence of 10 µM narrowly size-fractionated polyP. Experiments evaluating the impact of $ZnCl_2$ included 10 µM $polyP_{1200}$, 100 nM HK, and either 2 mM EDTA or 0 to 20 µM $ZnCl_2$. Experiments evaluating the impact of HK included 10 µM $polyP_{1200}$, 10 µM $ZnCl_2$, and 0 to 900 nM HK. Timed aliquots (10 µL) were removed and quenched in 70 µl ice-cold Quench Buffer II (20 mM HEPES-NaOH pH 7.4, 5 mM EDTA, 50 µg/ml soybean trypsin inhibitor, and 15 µg/ml polybrene). Following quenching, FXIIa amidolytic activities were quantified by measuring A_{405} at 37°C and converting to FXIIa concentrations by reference to a standard curve.

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 nM PK, 100 pM FXIIa and 10 µM $ZnCl_2$, in the presence of 0 to 100 µM $polyP_{1200}$ or $polyP_{79}$. Experiments evaluating polyP size-dependence included 10 µM $ZnCl_2$, in the absence or presence of 10 µM narrowly size-fractionated polyP. For experiments evaluating the impact of $ZnCl_2$, HBSP was pretreated with Chelex® 100 resin (buffered to pH 7.4) to deplete trace divalent metal ions. These assays included 10 µM $polyP_{1200}$ and either 0 to 10 mM EDTA or 0 to 10 µM $ZnCl_2$. Experiments evaluating the impact of HK included 10 µM P_{1200}, 10 µM $ZnCl_2$, and 0-900 nM 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 A_{405} at 37°C (to prevent temperature-dependent auto-inhibition of PKa [25]). Amidolytic activities were converted to PKa concentrations by reference to a standard curve.

PK autoactivation

Apparent PK autoactivation was evaluated by quantifying PKa after incubating 100 nM PK with 100 nM HK, 10 μM $ZnCl_2$, 10 μM $polyP_{1200}$, and 0 to 2.5 μM 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 A_{405} 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 cDNA [27] 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 nM PK-S559A, with or without 70 μM $polyP_{700}$, 200 nM HK, or 25 nM PKa, was incubated at 37°C in HBSP containing 10 μM $ZnCl_2$ and 50 nM 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.

Results

The four individual enzyme activation reactions that can contribute to activation of plasma clotting via the contact pathway are numbered in Fig. 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. Since HK and Zn^{2+} 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 (Fig. S1, S3 & S4, available in the online supplemental information). For all three reactions, we used 10 μM polyP (in terms of phosphate monomer), which was within the concentration range that gave maximal reaction rates.

PolyP length-requirement for contact pathway reactions

PolyP accelerated FXII autoactivation in a manner that was strongly dependent on polyP polymer length and concentration (Fig. 2A & S1). Under the experimental conditions tested (100 nM FXII in the presence of 5 μM $ZnCl_2$, examined in a 20-minute endpoint assay), only 0.15 ± 0.03 nM FXIIa was generated in the absence of polyP, whereas 420-fold more FXIIa was generated in the presence of $polyP_{1200}$. Platelet-size polyP (79 phosphates) supported only about fourfold more FXIIa generation than in the absence of polyP. As compared to $polyP_{1200}$, platelet-size polyP did not detectably support this reaction in a 45-minute kinetic assay (Fig. 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 (Fig. 2B & S3). In a 4-minute endpoint assay (employing 100 nM FXII with 100 pM PKa, 100 nM HK, and 10 μM $ZnCl_2$), only 0.72 ± 1.11 nM FXIIa was generated in the absence of polyP. Inclusion of $polyP_{1200}$ 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 (Figs. 2C & S4). Under the experimental conditions tested (100 nM PK with 100 pM FXIIa in the presence of 10 μM $ZnCl_2$, examined in a 3-minute endpoint assay), 1.67 ± 0.03 nM PKa was generated in the absence of polyP, whereas 10-fold more PKa was generated in the presence of $polyP_{1200}$. 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.

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 (Fig. 3A). FXII autoactivation was essentially undetectable in the presence of 2 mM EDTA in the 20-minute endpoint assay, while the optimal $ZnCl_2$ concentration was approximately 3 to 7.5 μM. Since Zn^{2+} was required for this reaction, 5 μM $ZnCl_2$ 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 nM) were generated in the presence of EDTA in the 4-minute endpoint assay (Fig. 3B). Optimal $ZnCl_2$ concentrations were 4 μM or higher, resulting in FXIIa generation that was approximately 20-fold higher than in the absence of $ZnCl_2$. Accordingly, 10 μM $ZnCl_2$ 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 (Fig. 3C). On the other hand, inclusion of 2 mM 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 (Fig. 3D). We observed that EDTA inhibited this reaction in a concentration-dependent manner over the range of 0 to 10 mM 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.

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 (Fig. 4A). Without HK, $polyP_{1200}$ readily supported FXII autoactivation in the presence of 5 μM $ZnCl_2$. Progress curves for FXII autoactivation were sigmoidal (Fig. 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 Fig. 4B, then given in Fig. 4C *versus* HK concentration. Although HK was not required for polyP-mediated FXII autoactivation, up to 100 nM HK accelerated the reaction somewhat, while higher concentrations were inhibitory.

HK substantially increased rates of polyP-mediated FXII activation by PKa (Fig. 4D). Thus, in the absence of HK, the FXII activation rate was only 10.44 ± 4.36 nM/min/nM, while in the presence of 50-200 nM HK, this rate was accelerated approximately 13.4-fold relative to no HK. As little as 0.5 nM HK supported the reaction to a detectable level (~ 3.2-fold increase over no HK), while HK concentrations of 300 nM and higher reduced the reaction rate. Since the activation of FXII by PKa was readily accelerated by HK, 100 nM 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 to 400 nM HK (Fig. 4E). Higher HK concentrations were inhibitory.

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 nM PK with 100 nM HK plus 10 μM $polyP_{1200}$ resulted in time-dependent generation of readily detectable PKa (Fig. 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 (Fig. 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 (Fig. 5A). CTI inhibits FXIIa activity with minimal effect on the enzymatic activity of PKa [31, 32]. Including 0.5 or 2.5 μM CTI in this reaction reduced the amount of PKa generated at 90 min by 38% or 83%, respectively.

In further experiments, we employed 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 (Fig. 5B). Even when 25 nM PKa was coincubated with 200 nM PK-S559A (in the presence or absence of polyP or HK), no PK-S559A cleavage was observed over a 3-hr time course (Fig. 5C). These findings argue against polyP supporting detectable PK activation by PKa, and that contamination with traces of FXII(a) was responsible for the apparent PK autoactivation in the presence of polyP plus HK.

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, Zn^{2+} 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 due to 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 potently activate the contact pathway [35], that study employed 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 al*. 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 [26]. 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 Zn^{2+} binding to polyP alters its structure such that it does not exist 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 4 putative Zn^{2+} binding sites on the heavy chain of FXII [40], so interactions between Zn^{2+}, polyP and FXII(a) could be quite complex.

Zn^{2+} enhances the binding affinities and proteolytic activities of many of the proteins in the contact pathway [29, 41, 42]. Zn^{2+} induces conformational changes in HK [43], and a binding site for Zn^{2+} 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 $ZnCl_2$ greatly enhanced both of the enzymatic reactions in which FXII was the substrate (i.e., polyP-mediated FXII autoactivation and activation of FXII by PKa), but $ZnCl_2$ had little effect on the enzymatic reaction in which PK was the substrate (i.e., polyP-mediated activation of PK by FXIIa). Interestingly, although we found no requirement for $ZnCl_2$ in supporting polyP-mediated PK activation by FXIIa, this reaction was inhibited by EDTA in a concentration-dependent manner (Fig. 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 $polyP_{1200}$ concentrations progressively reduced the inhibitory effect of EDTA (Fig. 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 (i.e., 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 (i.e., 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 nM) were inhibitory. HK circulates at a plasma concentration of about 650-900 nM [47], which falls in the range that inhibited polyP-driven contact pathway reactions. However, one should note that 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 $ZnCl_2$ 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, Zn^{2+} was only required when FXII was the substrate, while 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 Zn^{2+}, 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 Zn^{2+}. 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.

Supporting Information

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

Figure S1. Ability of polyP to accelerate FXII autoactivation depends on polyP concentration.

Figure S2. Progress curves of polyP-mediated FXII autoactivation.

Figure S3. Ability of long-chain and platelet-size polyP to accelerate FXII activation by PKa depends on polyP concentration.

Figure S4. Ability of long-chain and platelet-size polyP to accelerate PK activation by FXIIa depends on polyP concentration.

Figure S5. Influence of EDTA on polyP-mediated PK activation by FXIIa.

References

1 Wu Y. Contact pathway of coagulation and inflammation. *Thromb J* 2015; **13**: 17.

2 Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost* 2016; **14**: 28-39.

3 Griffin JH. Role of surface in surface-dependent activation of Hageman factor (blood coagulation factor XII). *Proc Natl Acad Sci U S A* 1978; **75**: 1998-2002.

4 Samuel M, Pixley RA, Villanueva MA, Colman RW, Villanueva GB. Human factor XII (Hageman factor) autoactivation by dextran sulfate. Circular dichroism, fluorescence, and ultraviolet difference spectroscopic studies. *J Biol Chem* 1992; **267**: 19691-7.

5 Long AT, Kenne E, Jung R, Fuchs TA, Renné T. Contact system revisited: an interface between inflammation, coagulation, and innate immunity. *J Thromb Haemost* 2016; **14**: 427-37.

6 Tans G, Rosing J, Berrettini M, Lämmle B, Griffin JH. Autoactivation of human plasma prekallikrein. *J Biol Chem* 1987; **262**: 11308-14.

7 Bouma BN, Griffin JH. Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. *J Biol Chem* 1977; **252**: 6432-7.

8 Schulze-Topphoff U, Prat A, Bader M, Zipp F, Aktas O. Roles of the kallikrein/kinin system in the adaptive immune system. *Int Immunopharmacol* 2008; **8**: 155-60.

9 Foley JH, Conway EM. Cross talk pathways between coagulation and inflammation. *Circul Res* 2016; **118**: 1392-408.

10 Ratnoff OD, Colopy JE. A familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma. *J Clin Invest* 1955; **34**: 602-13.

11 Lämmle B, Wuillemin WA, Huber I, Krauskopf M, Zurcher C, Pflugshaupt R, Furlan M. Thromboembolism and bleeding tendency in congenital factor XII deficiency--a study on 74 subjects from 14 Swiss families. *Thromb Haemost* 1991; **65**: 117-21.

12 Kokoye Y, Ivanov I, Cheng Q, Matafonov A, Dickeson SK, Mason S, Sexton DJ, Renné T, McCrae K, Feener EP, Gailani D. A comparison of the effects of factor XII deficiency and prekallikrein deficiency on thrombus formation. *Thromb Res* 2016; **140**: 118-24.

13 Renné T, Pozgajová M, Grüner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 2005; **202**: 271-81.

14 Revenko AS, Gao D, Crosby JR, Bhattacharjee G, Zhao C, May C, Gailani D, Monia BP, MacLeod AR. Selective depletion of plasma prekallikrein or coagulation factor XII inhibits thrombosis in mice without increased risk of bleeding. *Blood* 2011; **118**: 5302-11.

15 Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, Renné C, Gailani D, Nieswandt B, Renné T. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J Exp Med* 2006; **203**: 513-8.

16 Matafonov A, Leung PY, Gailani AE, Grach SL, Puy C, Cheng Q, Sun M-f, McCarty OJT, Tucker EI, Kataoka H, Renné T, Morrissey JH, Gruber A, Gailani D. Factor XII inhibition reduces thrombus formation in a primate thrombosis model. *Blood* 2014; **123**: 1739-46.

17 Cheng Q, Tucker EI, Pine MS, Sisler I, Matafonov A, Sun MF, White-Adams TC, Smith SA, Hanson SR, McCarty OJ, Renné T, Gruber A, Gailani D. A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo. *Blood* 2010; **116**: 3981-9.

18 Smith SA, Choi SH, Davis-Harrison R, Huyck J, Boettcher J, Rienstra CM, Morrissey JH. Polyphosphate exerts differential effects on blood clotting, depending on polymer size. *Blood* 2010; **116**: 4353-9.

19 Ruiz FA, Lea CR, Oldfield E, Docampo R. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *J Biol Chem* 2004; **279**: 44250-7.

20 Brown MR, Kornberg A. Inorganic polyphosphate in the origin and survival of species. *Proc Natl Acad Sci U S A* 2004; **101**: 16085-7.

21 Kornberg A, Rao NN, Ault-Riché D. Inorganic polyphosphate: a molecule of many functions. *Annu Rev Biochem* 1999; **68**: 89-125.

22 Morrissey JH. Polyphosphate: a link between platelets, coagulation and inflammation. *Int J Hematol* 2012; **95**: 346-52.

23 Smith SA, Baker CJ, Gajsiewicz JM, Morrissey JH. Silica particles contribute to the procoagulant activity of DNA and polyphosphate isolated using commercial kits. *Blood* 2017; **130**: 88-91.

24 Smith SA, Morrissey JH. Sensitive fluorescence detection of polyphosphate in polyacrylamide gels using 4',6-diamidino-2-phenylindol. *Electrophoresis* 2007; **28**: 3461-5.

25 Levison PR, Tomalin G. Studies on the temperature-dependent autoinhibition of human plasma kallikrein I. *Biochem J* 1982; **205**: 529-34.

26 Ivanov I, Matafonov A, Sun MF, Cheng Q, Dickeson SK, Verhamme IM, Emsley J, Gailani D. Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. *Blood* 2017; **129**: 1527-37.

27 Chung DW, Fujikawa K, McMullen BA, Davie EW. Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry* 1986; **25**: 2410-7.

28 Wallisch M, Tucker EI, Lorentz CU, Johnson J, Carris MR, McCarty OJT, Hinds MT, Gruber A. The anti-factor XII antibody AB052 is antithrombotic without hemostatic impairment in a primate model of extracorporeal membrane oxygenation. *Blood* 2017; **130**: 236.

29 Røjkjaer R, Schousboe I. The surface-dependent autoactivation mechanism of factor XII. *Eur J Biochem* 1997; **243**: 160-6.

30 Wu JW, Wu Y, Wang ZX. Kinetic analysis of a simplified scheme of autocatalytic zymogen activation. *Eur J Biochem* 2001; **268**: 1547-53.

31 Hojima Y, Pierce JV, Pisano JJ. Plant inhibitors of serine proteinases: Hageman factor fragment, kallikreins, plasmin, thrombin, factor Xa, trypsin, and chymotrypsin. *Thromb Res* 1980; **20**: 163-71.

32 Chong GL, Reeck GR. Interaction of trypsin, beta-factor XIIa, and plasma kallikrein with a trypsin inhibitor isolated from barley seeds: a comparison with the corn inhibitor of activated Hageman factor. *Thromb Res* 1987; **48**: 211-21.

33 Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. *Proc Natl Acad Sci U S A* 2006; **103**: 903-8.

34 Smith SA, Gajsiewicz JM, Morrissey JH. Ability of polyphosphate and nucleic acids to trigger blood clotting: Some observations and caveats. *Front Med* 2018; **5**: 107.

35 Verhoef JJ, Barendrecht AD, Nickel KF, Dijkxhoorn K, Kenne E, Labberton L, McCarty OJ, Schiffelers R, Heijnen HF, Hendrickx AP, Schellekens H, Fens MH, de Maat S, Renné T, Maas C. Polyphosphate nanoparticles on the platelet surface trigger contact system activation. *Blood* 2017; **129**: 1707-17.

36 Caen J, Wu Q. Hageman factor, platelets and polyphosphates: early history and recent connection. *J Thromb Haemost* 2010; **8**: 1670-4.

37 Erickson HP. Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. *Biol Proced Online* 2009; **11**: 32-51.

38 Bolesch DG, Keasling JD. Polyphosphate binding and chain length recognition of *Escherichia coli* exopolyphosphatase. *J Biol Chem* 2000; **275**: 33814-9.

39 Gansler J, Jaax M, Leiting S, Appel B, Greinacher A, Fischer S, Preissner KT. Structural requirements for the procoagulant activity of nucleic acids. *PLoS ONE* 2012; **7**: e50399.

40 Stavrou E, Schmaier AH. Factor XII: what does it contribute to our understanding of the physiology and pathophysiology of hemostasis & thrombosis. *Thromb Res* 2010; **125**: 210-5.

41 Bernardo MM, Day DE, Halvorson HR, Olson ST, Shore JD. Surface-independent acceleration of factor XII activation by zinc ions. II. Direct binding and fluorescence studies. *J Biol Chem* 1993; **268**: 12477-83.

42 Vu TT, Fredenburgh JC, Weitz JI. Zinc: An important cofactor in haemostasis and thrombosis. *Thromb Haemost* 2013; **109**: 421-30.

43 Herwald H, Morgelin M, Svensson HG, Sjöbring U. Zinc-dependent conformational changes in domain D5 of high molecular mass kininogen modulate contact activation. *Eur J Biochem* 2001; **268**: 396-404.

44 Hasan AA, Cines DB, Herwald H, Schmaier AH, Muller-Esterl W. Mapping the cell binding site on high molecular weight kininogen domain 5. *J Biol Chem* 1995; **270**: 19256-61.

45 DeLa Cadena RA, Colman RW. The sequence HGLGHGHEQQHGLGHGH in the light chain of high molecular weight kininogen serves as a primary structural feature for zinc-dependent binding to an anionic surface. *Protein Sci* 1992; **1**: 151-60.

46 Gajsiewicz JM, Smith SA, Morrissey JH. Polyphosphate and RNA differentially modulate the contact pathway of blood clotting. *J Biol Chem* 2017; **292**: 1808-14.

47 Kleniewski J. Plasma high molecular weight kininogen concentration in health and in chosen impairments of haemostasis. Evidence that plasmin uncovers a new antigenic site in high molecular weight kininogen. *Thromb Haemost* 1979; **42**: 1046-55.

48 Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA, Docampo R. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. *J Biol Chem* 2012; **287**: 28435-44.

49 Kumble KD, Kornberg A. Inorganic polyphosphate in mammalian cells and tissues. *J Biol Chem* 1995; **270**: 5818-22.

50 Marx G, Korner G, Mou X, Gorodetsky R. Packaging zinc, fibrinogen, and factor XIII in platelet a-granules. *J Cell Physiol* 1993; **156**: 437-42.

51 Schmaier AH. Plasma prekallikrein: Its role in hereditary angioedema and health and disease. *Front Med* 2018; **5**: 3.

52 Shariat-Madar Z, Mahdi F, Schmaier AH. Recombinant prolylcarboxypeptidase activates plasma prekallikrein. *Blood* 2004; **103**: 4554-61.

53 Moreira CR, Schmaier AH, Mahdi F, da MG, Nader HB, Shariat-Madar Z. Identification of prolylcarboxypeptidase as the cell matrix-associated prekallikrein activator. *FEBS Lett* 2002; **523**: 167-70.

54 Brown MR, Kornberg A. The long and short of it – polyphosphate, PPK and bacterial survival. *Trends Biochem Sci* 2008; **33**: 284-90.

Reaction Number*	Enzyme	Substrate	Optimal Condition		
			PolyP length (phosphates)	**HK (nM)**	**$ZnCl_2$ (µM)**
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

Table 1. Summary of the influence of polyP polymer length, HK, and $ZnCl_2$ on four reactions of the contact pathway.

*Reaction numbers correspond to those in Fig. 1.

FIGURE LEGENDS

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.

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 μM polyP (●,▼). *Data points* for size-fractionated polyP preparations (●) are plotted on the *x*-axes according to their polymer lengths, while *data points* for heterogeneous $polyP_{1200}$ (▼) 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 nM FXII was incubated with 5 μM $ZnCl_2$ without polyP (◆) or with 10 μM 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 nM FXII was incubated with 100 pM PKa, 100 nM HK and 10 μM $ZnCl_2$, without polyP (◆) or with 10 μM polyP (●,▼). *C*, PolyP length requirement for PK activation by FXIIa. In an endpoint assay (stopped at 3 min), 100 nM PK was incubated with 100 pM FXIIa and 10 μM $ZnCl_2$, without polyP (◆) or with 10 μM polyP (●,▼). HK was not included in this experiment. Data in all *panels* are mean ± S.E. ($n \geq 3$).

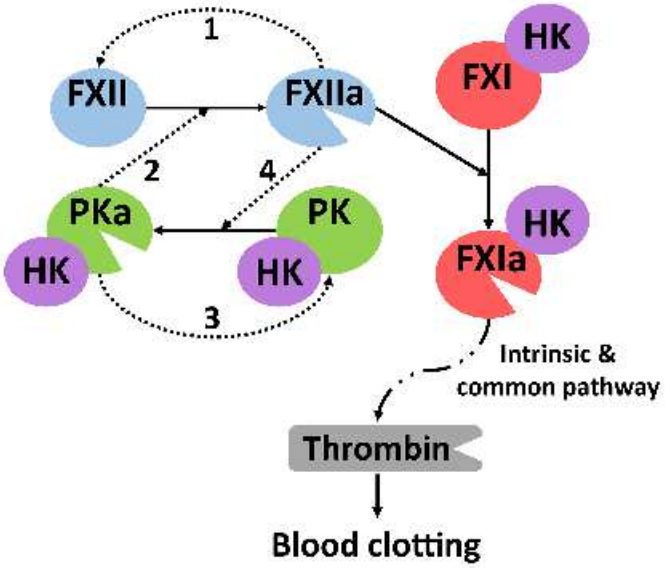
Figure 3. Zn^{2+} differentially influences the ability of polyP to accelerate individual reactions of the contact pathway. *A*, Zn^{2+} requirement for polyP-mediated FXII autoactivation. In an endpoint assay (stopped at 20 min), 100 nM FXII without HK was incubated with 10 μM $polyP_{1200}$ in the presence of either 2 mM 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 nM FXII and 100 pM PKa were incubated with 100 nM HK and 10 μM $polyP_{1200}$ in the presence of either 2 mM 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 nM PK and 100 pM FXIIa without HK were incubated with 10 μM $polyP_{1200}$ 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 nM PK and 100 pM FXIIa without HK were incubated with 10 μM $polyP_{1200}$ in the presence of varying EDTA concentrations. Data in all *panels* are mean ± S.E. ($n \geq 3$).

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 nM FXII with 10 μM $polyP_{1200}$ in the presence of 5 μM $ZnCl_2$ and 0 (●), 50 (△), 100 (■), 200 (▼), 300 (○), 500 (□), or 900 (▲) nM 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(FXII/FXIIa) values *versus* 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 nM FXII and 100 pM PKa with 10 μM $polyP_{1200}$ in the presence of 10 μM $ZnCl_2$ and varying HK concentrations. Initial FXII activation rates are plotted as nM FXII activated per min divided by nM 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 nM PK and 100 pM FXIIa with 10 μM $polyP_{1200}$ in the presence of 10 μM $ZnCl_2$ and varying HK concentrations. Initial PK activation rates were plotted as nM PK activated per min divided by nM FXIIa used. In *panel C-E*, blue rectangles represent the approximate concentration range of total HK in plasma. Data in all *panels* are mean ± S.E. ($n \geq 3$).

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 nM PK with 100 nM HK, 10 μM $polyP_{1200}$ and 10 μM $ZnCl_2$ in presence of 0 (●), 0.5 (◆) or 2.5 (▲) μM CTI. Data are mean ± S.E. ($n \geq 2$). *B*, In the presence of an inhibitory anti-FXII antibody, activation of PK-S559A was undetectable by western blot. *Left panel*: 200 nM PK-S559A without HK was incubated with or without 70 μM $polyP_{700}$ in the presence of 10 μM $ZnCl_2$ and 50 nM 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 nM 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 nM PK-S559A and 25 nM purified PKa were incubated without polyP or HK, in

the presence of 10 μM $ZnCl_2$ and 50 nM 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 μM long-chain $polyP_{700}$, with or without 200 nM 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.



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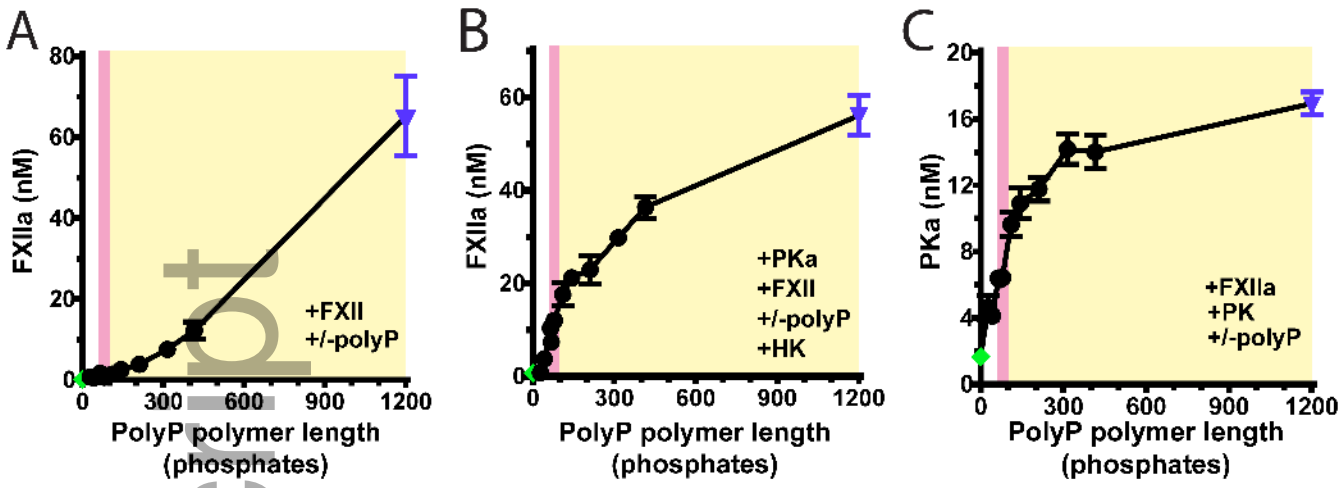

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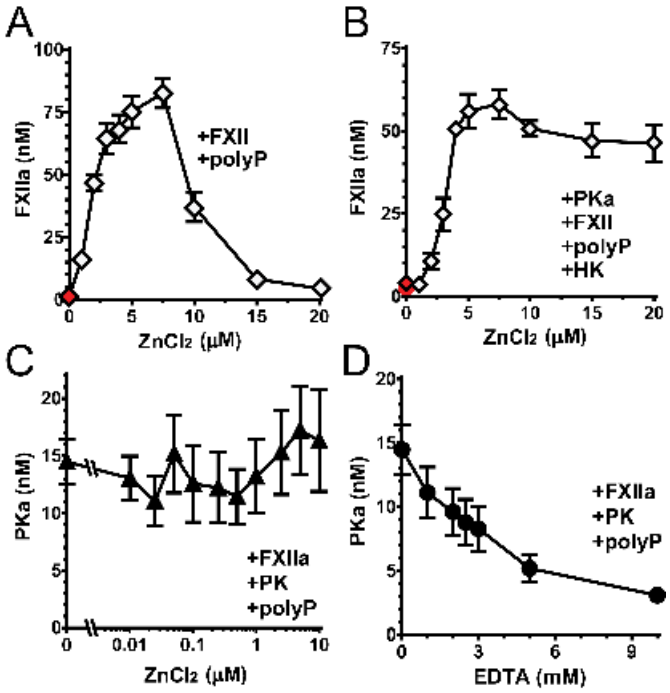

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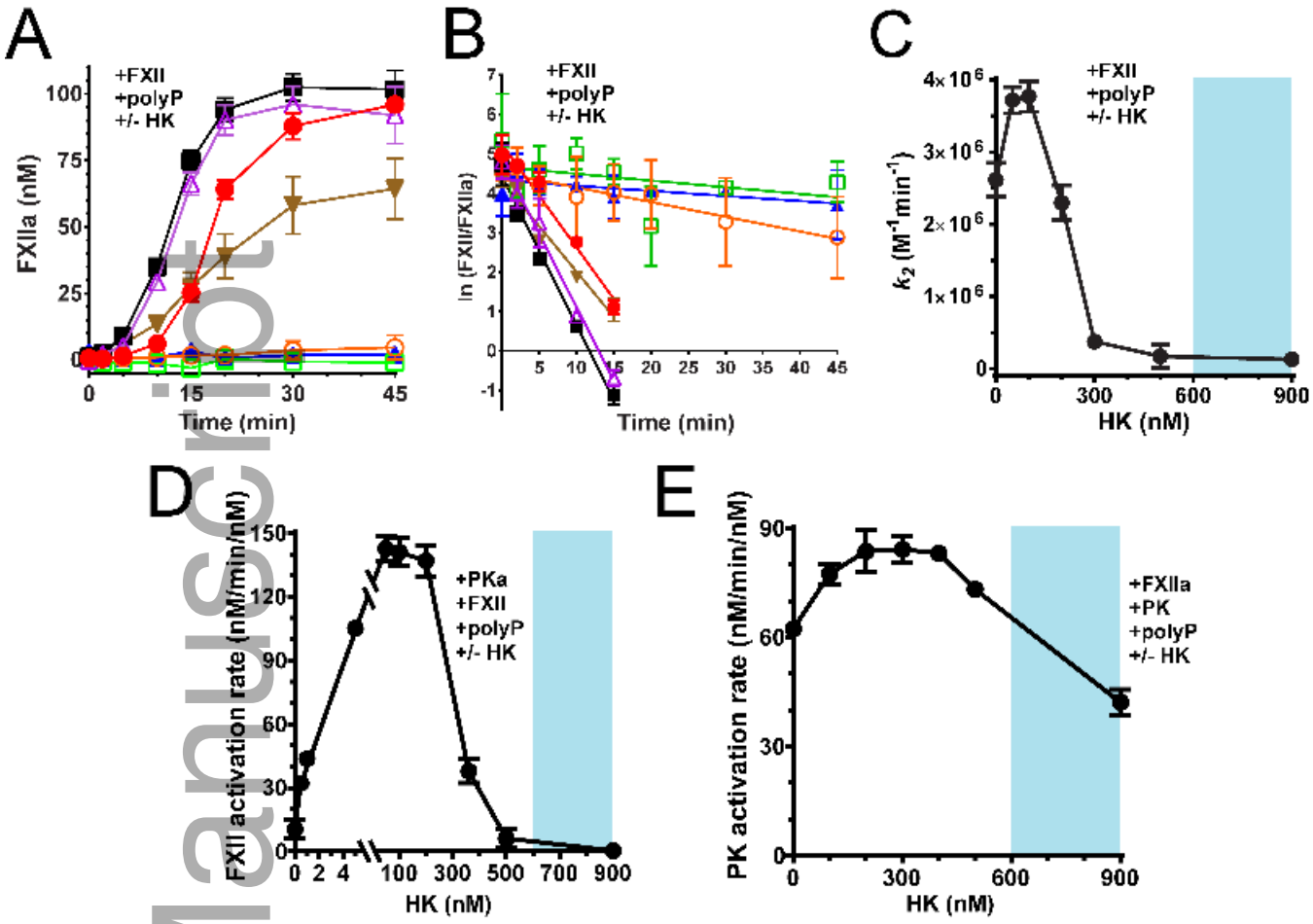

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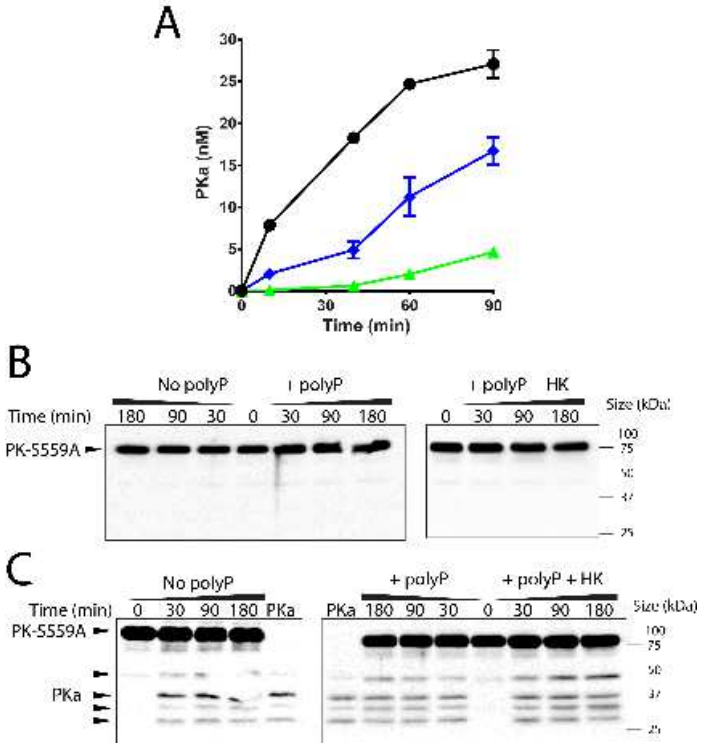

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