Polyphosphate and RNA Differentially Modulate the Contact Pathway of Blood Clotting*

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The contact pathway of the plasma clotting cascade is dispensable for normal hemostasis, but contributes to thrombosis and serves as a bridge between inflammation and coagulation. This pathway is triggered upon exposure of plasma to certain anionic polymers and artificial surfaces. Recently, extracellular nucleic acids and inorganic polyphosphate (polyP) have been implicated as being important (patho)physiologically relevant activators of this pathway. However, mechanistic details regarding how nucleic acids or polyP modulate the individual reactions of the contact pathway have been lacking. In this study, we investigate the ability of RNA homopolymers and polyP to bind the primary constituents of the contact pathway: factor XIa, factor XIIa, and plasma kallikrein, in the presence and absence of high molecular weight kininogen (HK), an important cofactor in this pathway. We examine seven proteolytic activation reactions within the contact pathway and report that polyP greatly enhances and supports these proteolytic activation reactions, depending on the specific reaction evaluated. Overall, we find that polyP is a potent mediator of contact pathway activation reactions in general, that RNA secondary structure may be important to its procoagulant activity, and that nucleic acids versus polyP may differentially modulate specific enzyme activation events within the contact pathway.

The contact pathway of blood clotting is initiated when plasma is exposed to anionic surfaces and substances such as glass and clay (1, 2). This mechanism for triggering clotting is irrelevant to normal hemostasis, since severe deficiencies in either of the two key enzymes that trigger this pathway, factor XII (FXII) (3) or plasma prekallikrein (PPK) (4), are not associated with bleeding tendencies in humans. However, recent findings have implicated the contact pathway in thrombosis, inflammation and innate immunity. Thus, in models of venous thrombosis, mice deficient in FXII (5–7) are protected, and pharmacologic targeting of either FXII or PPK is protective (8). Animal models of arterial thrombosis have also indicated a protective effect of FXII deficiency (7, 9, 10); FXII contributes to thrombus stability on atherosclerotic plaques (11); and several studies have reported a correlation between myocardial infarction and elevated plasma levels of contact pathway proteins (12–14). FXII also plays a central role in hereditary angioedema (15).

This renaissance in the contact pathway has led to renewed interest in understanding its true pathophysiologic triggers. Recently identified candidates include collagen (16) and misfolded proteins (17), as well as the anionic polymers, polyphosphate (polyP) (18–20), DNA (21–24), and RNA (25). Long-chain polyP and extracellular RNA appear to be particularly active at triggering the contact pathway and are implicated in thrombosis and inflammation (15, 20). The goal of the present study is to achieve better mechanistic insights into how polyP and RNA trigger the plasma clotting cascade via the contact pathway.

Fig. 1 is a schematic overview of how this pathway is initiated, with the present study examining how polyP and RNA influence the rates of each of the numbered proteolytic events in this figure. This pathway is initiated when zymogen factor FXII undergoes autoactivation and/or allosteric activation upon interaction with certain anionic surfaces or polymers (1, 2). The resulting active enzyme, FXIIa, converts PPK to PK, creating a positive feedback loop through reciprocal activation of PPK by FXIIa, and FXII by PK.

HK is not only an important cofactor for triggering the contact pathway, but it is also acted upon proteolytically by PK, resulting in the release of the vasodilator peptide, bradykinin (6, 26). Because bradykinin is a potent vasoactive mediator, it represents an important link between the coagulation cascade and inflammation. Binding of PPK and FXI to certain anionic surfaces is greatly enhanced by HK (27, 28), and HK is required in stoichiometric amounts for maximal enzymatic activity in vitro (28).

To date, limited studies have investigated the ability of polyP or RNA to accelerate the rates of the individual reactions of the contact pathway. In the present study, we show that polyP and RNA exert differential effects on these individual enzyme activation reactions, both in the presence and absence of HK.

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2 The abbreviations used are: FXII, factor XII; CTI, corn trypsin inhibitor; FXI, factor XI; HK, single-chain high molecular weight kininogen; PK, plasma kallikrein; polyanionic acid; polyc, polycytidylic acid; polyg, polyguanylic acid; polyl, polyinosinic acid; polyP, polyphosphate; PPK, plasma prekallikrein; STI, soybean trypsin inhibitor.

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Polyphosphate, RNA, and the Contact Pathway of Blood Clotting

Results

FXIa Binding and Rates of FXI Activation—Polyanions such as polyP (29, 30) and dextran sulfate (31) have been reported to facilitate FXI activation. To investigate the ability of FXI to bind to RNA, we utilized a microplate-based binding assay in which various RNA homopolymers or long-chain polyP in solution competed with immobilized polyP for binding to FXIa (Fig. 2). Because Zn$^{2+}$ enhances the binding affinities and activities of many proteins of the contact pathway (32, 33), we employed 10 μM ZnCl$_2$ in essentially all the assays in this study. PolyG, polyI, and polyP in solution were effective competitors for the binding of FXIa to immobilized polyP, while neither polyA nor polyC appreciably reduced FXIa binding. Addition of HK did not appreciably alter the outcome of this competition binding experiment with regard to any of the polymers tested.

FXI can be activated in three reactions, all of which are enhanced by polyanions; the relevant proteases, numbered as in Fig. 1, are: (5) FXIa (autoactivation), (6) FXIIa, and (7) thrombin. PolyP strongly enhanced the rate of FXIa autoactivation (confirming our previous reports, Refs. 34, 35), while polyI was enhanced by polyanions; the relevant proteases, numbered as in experiment with regard to any of the polymers tested.

HK circulated bound to FXI and PPK, so these seven reactions were examined in this study with and without HK. The common pathway represents the bulk of the clotting cascade, from factor IX activation through thrombin generation.

![FIGURE 1. Schematic overview of the contact pathway, in which the specific proteolytic activation events examined in this study are numbered: PPK can be activated by 1) PK (autoactivation) or 2) FXIIa; FXIa can be activated by 3) FXIIa (autoactivation) or 4) PK; and FXI can be activated by 5) FXIIa (autoactivation), 6) FXIIa, or 7) thrombin. HK circulates bound to FXI and PPK, so these seven reactions were examined in this study with and without HK.](image)

![FIGURE 2. FXI(a) interactions with RNA and polyP. A, ability of 50 μM solution-phase polyP or RNA homopolymers to compete with immobilized biotin-polyP for binding to 50 nm FXIa with (black bars) or without (white bars) 50 nm HK. Bound FXIa in microplate wells was normalized to the amount bound in the absence of any competing polyanion (None). B, reaction 5 from Fig. 1, second-order rate constants for FXI autoactivation without HK. 40 nm FXI was incubated with 100 pm FXIa and either 2.5 μM polyP, 1.25 μM polyG, 1.25 μM polyI, or no polymer (Vehicle). C, reaction 5, second-order rate constants for polyP- and polyI-mediated FXI autoactivation. 40 nm FXI plus 100 pm FXIa was incubated with (black bars) or without (white bars) 40 nm HK in the presence of either 2.5 to 10 μM polyP or 1.25 μM polyI. D, reaction 7, FXI activation by thrombin, plotted as initial rates of FXI activation divided by the thrombin concentration. 40 nm FXI plus 15 nm thrombin was incubated with (black bars) or without (white bars) 40 nm HK in the presence of either 15 μM polyP, 6 μM polyG, 10 μM polyI, or no polymer. E and F, reaction 6, activation of 40 nm FXI by 5 nm FXIa with (dotted lines) or without (solid lines) 40 nm HK. Data in panels C, D, and E are progress curves for 12 min in the presence of either 1.25 μM polyP (green ■), 1.25 μM polyG (purple ●), 1.25 μM polyI (blue ●), or no polymer (red ●). Data in panel D are progress curves over 30 min in the presence of the same concentrations of polyanions as in panel C, with the exception of polyP, which was 2.5 μM (green ■). Data in all panels are means ± S.E. (n = 3).](images)
Polyphosphate, RNA, and the Contact Pathway of Blood Clotting

FXIIa Binding and Rates of FXII Activation—PolyG, polyI, and polyP in solution were effective competitors of FXIIa binding to immobilized polyP, while polyA and polyC were ineffective (Fig. 3A). HK did not appreciably alter the competition binding results.

Autoactivation of single-chain FXII on anionic surfaces to the two-chain active enzyme, α-FXIIa, is thought to be a key initial enzymatic step in the contact pathway. However, a recent study reported significant enzymatic activity of single-chain FXII in complex with polyP, functioning as an allosteric activator of thiszymogen (36). In discontinuous assays of FXIIa generation in which time aliquots were quenched with polybrene (to disrupt FXII(a) binding to polyP), we found that polyP supported very little FXIIa generation via autoactivation in the presence or absence of HK (Fig. 3B), consistent with the findings of Engel et al. (36). On the other hand, both polyG and polyI supported generation of readily detectable FXIIa activity, although this was observed only in the presence of HK (Fig. 3B).

In a continuous assay in which a chromogenic substrate was included with zymogen FXII and polyP, we found that polyP led to robust FXII(a) amidolytic activity (Fig. 3C). Although polyP did not result in the generation of true FXIIa, its ability to allosterically increase FXII amidolytic activity was substantially greater than the ability of either polyG or polyI to enhance FXII activity (either through allosteric effects or by FXII autoactivation; Fig. 3C). In the presence of HK, polyG increased the second-order rate constant for FXII autoactivation by 8-fold over the vehicle control, while polyI mediated a 6.8-fold increase (Fig. 3D). In the absence of HK, polyG and polyI supported somewhat lower rates of FXII autoactivation than in the presence of HK. This reaction was undetectable in the absence of both HK and an anionic polymer (Fig. 3D).

We next evaluated the abilities of polyG, polyI, and polyP to support FXII activation by PK. In the absence of HK, all three anions enhanced the rate of FXIIa generation, with polyG and polyI yielding 5- and 27-fold greater activity, respectively, than with polyP (Fig. 3E). HK further enhanced the rates of FXII activation by PK in the presence of all three polyanions, but most profoundly for polyP (17-fold increase with HK relative to without HK; Fig. 3E).

PK Binding and Rates of PPK Activation—PK at 50 nM did not bind detectably to immobilized polyP in the absence of HK (not shown), but did bind in the presence of 50 nM HK (Fig. 4A). In the presence of HK, polyG, polyI, and polyP in solution were all effective competitors of PK binding to immobilized polyP, while polyA and polyC were ineffective.

In a continuous assay of PPK autoactivation, none of the polyanions supported appreciable substrate hydrolysis in the absence of HK (Fig. 4B). In the presence of HK, polyG, polyI, and polyP all supported robust PK enzyme activity. The second-order rate constants for PPK autoactivation in the presence of HK supported by polyG, polyI, or polyP are presented in Fig. 4C. The rate supported by polyP was ~40% higher than that with polyG or polyI.

We next examined the effect of polyanions and HK on the rate of activation of 50 nM PPK by 250 nM FXIIa. In the absence of HK, polyG, polyP, and polyI all substantially enhanced the rate of PPK activation, to a roughly equal extent. Adding 50 nM HK enhanced the rate of PPK activation even in the absence of anions. Adding polyG or polyI plus HK resulted in relatively little further enhancement of the rate compared with RNA alone, while adding polyP plus HK roughly doubled the rate compared with polyP alone (Fig. 4D).

Discussion

Recent work has identified a number of potential pathophysiological activators of the contact pathway, with particular attention to polyP (18) and extracellular RNA (25). However, systematic interrogation of the effects of polyP or nucleic acids on each step in the contact pathway has been lacking. We recently
found that, compared with long-chain polyP, whole-cell RNA or DNA isolated from mammalian cells is only weakly able to activate the contact pathway.\(^3\) On the other hand, previous work by Gansler \textit{et al.} (37) demonstrated the importance of secondary structure on the procoagulant ability of nucleic acids, finding that hairpin-forming RNA oligomers are not only more resistant to degradation but are also more procoagulant, and in particular, structure-forming RNA aptamers were shown to mediate PPK autoactivation. PolyG and polyl are known to form stable, non-canonical base-pairing structures such as G-quadruplexes (38, 39). We previously reported that polyG RNA has substantial procoagulant activity (40, 41). In the present study, we found that polyG and polyl RNAs competed effectively with polyP for binding to PK, FXIIa, and FXIa, while neither polyA nor polyC RNAs were effective competitors. (Neither polyA nor polyC exhibits measurable procoagulant activity; data not shown.) For these reasons, we focused on polyG and polyl as exemplars of procoagulant RNA, and undertook a detailed examination of the ability of these RNAs versus polyP to modulate the individual reactions that collectively trigger the plasma clotting cascade via the contact pathway.

\(^3\) J. Gajsiewicz, S. Smith, A. Mast, and J. Morrissey, manuscript submitted for publication.

Our findings are summarized visually in Fig. 5. PolyP and synthetic RNA homopolymers differed greatly in their ability to accelerate specific enzyme reactions, and furthermore, HK potentiated or suppressed polyP- or RNA-mediated activation of these enzymes, depending on the reaction. Thus, polyP and polyl supported all seven reactions tested, while polyG supported six of them (being essentially inactive toward FXI autoactivation and relatively poor at supporting FXI activation by thrombin). PolyP was superior to polyG or polyl at supporting six of the seven reactions tested, with polyG being superior to polyP only at supporting FXIIa activation by PK.

FXIIa activation is widely regarded to be a key step in triggering the contact pathway. Our findings here confirm those of Engel \textit{et al.} (36), who reported that polyP “activates” FXII by inducing reversible conformational changes in the single-chain protein. This mechanism of action appears to be unique to polyP, as polyG and polyl both induced FXIIa activity in the discontinuous FXII activation assay, consistent with a conventional mechanism of FXII autoactivation involving proteolytic cleavage and formation of two-chain FXIIa. Interestingly, FXII (auto)activation by either mechanism was enhanced in the presence of HK, despite the fact that HK is not generally thought to be an important cofactor for FXII autoactivation using artificial surfaces.

Interestingly, we found that PK bound to polyP, polyG or polyl only in the presence of HK. In two previous studies, we reported relatively high-affinity binding of PK to immobilized polyP in the absence of HK, but those studies were conducted at very low ionic strength (29, 40). The present binding and enzyme activation studies were conducted at ionic strengths more closely matching those of plasma, and also included 10 \(\mu\text{M}\) zinc. Under these conditions, which may be more (patho)-physiologically relevant, binding of PK to polyP was stimulated substantially by the presence of HK, and furthermore, HK was required for PPK autoactivation.

When examining FXIa activation by FXIIa, we note that FXIa levels plateaued relatively quickly in the presence of polyG or...
Polyphosphate, RNA, and the Contact Pathway of Blood Clotting

polyl. This effect is likely the combination of three reactions: FXI activation by FXIIa, FXI autoactivation, and FXIa autolysis. We previously found that polyp significantly accelerates FXIa autolysis (34), and now report that polyl also supported the time-dependent loss of FXIa activity.

FXI activation by FXIIa is the critical bridge linking the activation reactions of the contact pathway to the final common pathway of the plasma clotting cascade, and therefore is a critical link between the contact pathway and thrombosis (10). Our findings show that polyp mediates strong and sustained FXI activation catalyzed by both FXIIa and FXIa, whereas polyG and polyI only support an initial, small burst of FXIa generation. These differences may explain the substantially higher plasma procoagulant activity of long-chain polyp compared with nucleic acids.3 These findings also implicate down-regulation of FXIIa-mediated FXI activation through the inhibition of polyp as a potentially useful antithrombotic approach. Furthermore, reactions 1 through 4 in Fig. 5, which represent the initiation phase of the contact pathway, have also been called the plasma kallikrein/kinin system (42). A series of studies of this part of the contact pathway has shown that kallikrein activation and bradykinin generation can occur, at least under some circumstances but most particularly on certain cell surfaces, without significant FXIIa generation or propagation of the clotting cascade. It is tempting to speculate that, compared with long-chain polyp, nucleic acids may be much better at activating the plasma kallikrein/kinin portion of the contact pathway than at triggering clotting per se, although it would clearly require substantial further study to properly investigate this idea.

Experimental Procedures

Materials—Synthetic RNA homopolymers (polyguanylic acid, polyG; polynosinic acid, polyI; polyleadenic acid, polyA; and polycytidylic acid, polyC), hirudin, and soybean trypsin inhibitor (STI) were from Sigma. Human FXII, α-thrombin, FXI, FXIa, and corn trypsin inhibitor (CTI) were from Hematologic Technologies (Essex Junction); PPK, PK, α-FXIIa, and single-chain HK were from Enzyme Research Laboratories (South Bend, IN). Chromogenic substrates for FXIa (Pyr-Pro-Arg-pNA) and FXIIa or PK (H-d-Pro-Phe-Arg-pNA) were from Bachem (Bubendorf, Switzerland).

Polyp and RNA concentrations are given in terms of molar concentration of phosphate monomers: i.e. phosphate concentration for polyp and nucleotide concentration for nucleic acids. Throughout this study, polyp in solution was a preparation of narrowly size-fractionated, long-chain polyp with a polymer length range of 1110–1540 phosphates; while immobilized, biotinylated polyp had a polymer length range of 110–385 phosphates. Both were prepared as previously described (29, 43).

Competition Binding Assays—The ability of RNA homopolymers and polyp to bind to proteins of the contact pathway was investigated using a plate-based competition assay essentially as described (29, 40). Briefly, 10 μM biotin-polyp was captured on streptavidin-coated microplate wells. FXIa, FXIIa, or PK (at 50 nM) were incubated in the wells together with various competitors at ambient temperature for 1 h in 20 mM HEPES pH 7.4, 100 mM NaCl, 5 mM KCl, 1% polyethylene glycol (8000 MW), 10 μM ZnCl₂, 0.05% Tween-20. In some experiments, 50 nM HK was also included. Wells were washed and the bound enzyme was quantified via amidolytic activity (rate of cleavage of the appropriate chromogenic substrate), and normalized to wells without competitor added.

General Conditions for Enzyme-Substrate Reactions—Enzyme activation reactions (including autoactivation reactions) were generally carried out at 37 °C in 20 mM HEPES pH 7.4, 100 mM NaCl, 5 mM KCl, 1% polyethylene glycol (8000 MW), 10 μM ZnCl₂.

In discontinuous assays, the ice-cold quench buffer (HEP) was 20 mM HEPES pH 7.4, 5 mM EDTA, 15 μg/ml polybrene (as an inhibitor of the polyanions). Titration experiments were performed to determine the polyanion concentration (tested from 1.25 to 100 μM) that resulted in maximal enzyme activation rate for the specific reaction to be studied, and that polyanion concentration was used for further experiments. Amidolytic activities of the various enzymes were quantified in a Spectramax M2 96-well spectrophotometer ( Molecular Devices) by measuring the change in A405 for 1 h using 0.4 mM of the appropriate chromogenic substrate, typically at ambient temperature (except for PK, which was quantified at 37 °C to prevent temperature-dependent autoinhibition of this enzyme (44)).

FXI Activation by Thrombin or FXIIa—Rates of FXI activation were quantified using a discontinuous assay as previously described (34, 35), with minor modifications. Briefly, 40 nM FXI dimer was incubated with 15 nM thrombin or 5 nM FXIIa in the presence or absence of polyanion. In some experiments, 40 nM HK was also included. Timed 20-μl aliquots were removed and quenched on ice in 80 μL HEP plus an inhibitor of either thrombin (18 units/ml hirudin) or FXIIa (500 nM CTI) as appropriate. The FXIa concentration at each quenched time point was determined by amidolytic activity, with reference to a standard curve.

FXI Autoactivation—Second-order rate constants for FXI autoactivation (FXI activation by FXIa) were determined using a discontinuous chromogenic assay. 100 pM FXIa dimer was incubated with 40 nM FXI dimer in the presence or absence of polyanion, including 40 nM HK in some reaction mixtures. Timed aliquots were removed and quenched on ice in HEP, after which FXIa amidolytic activity was quantified. Data were analyzed according to a second-order reaction mechanism (FXIa + FXI → 2FXIa) as described previously for autoactivation reactions (45, 46).

FXIa Autolysis—Time-dependent loss of FXIa enzymatic activity (FXIa autolysis) was measured essentially as previously described (34). Briefly, 40 nM FXIa and 40 nM HK were incubated at 37 °C in the presence of 1.25 μM polyI or 5 μM polyp, and timed aliquots were removed and quenched with HEP buffer on ice. Residual FXIa activity was quantified using 0.4 mM FXIa chromogenic substrate with reference to a standard curve.

Autoactivation of FXII or PPK—A discontinuous assay was used to quantify FXII autoactivation similarly to that previously described (36). 200 nM FXII and 200 nM HK were incubated with polyanion. Timed aliquots (20 μl) were quenched on ice in 80 μl HEP, after which FXIIa concentrations were measured via...
amidolytic activity with reference to a standard curve. In separate experiments, continuous assays for FXII or PPK (autotactivation employed the relevant zymogen (200 nM FXII or 100 nM PPK, with or without equimolar concentrations of HK) pre-mixed with chromogenic substrate. After warming the solutions to 37 °C for 3 min, polyanion was added to initiate the reaction and rates of chromogenic substrate hydrolysis were measured at 37 °C. Enzyme concentrations were then calculated from the first derivative of \( A_{405} \) versus time; second-order rate constants were derived as described (45, 46).

FXII Activation by PK, and PPK Activation by FXIIa—In discontinuous assays, zymogen and enzyme were incubated with polyanions, with or without 200 nM HK. For PK activation of FXII, the concentrations were 1 nM and 200 nM, respectively. For FXIIa activation of PPK, the concentrations were 250 pm and 50 nM, respectively. Time aliquots were quenched on ice in HEP plus inhibitor for the activating enzyme (50 μM STI for PK, or 500 nM CTI for FXIIa). Enzyme concentrations were quantified via amidolytic activity, with reference to a standard curve.

Author Contributions—J. M. G. conducted most of the experiments, analyzed results, and wrote most of the report. S. A. S. prepared and characterized polyP, conducted experiments on FXI activation, analyzed results, and contributed to writing the report. J. H. M. conceived the idea for the project, helped design experiments and analyzed results, and wrote the report with J. M. G. and S. A. S. All authors reviewed the results and approved the final version of the manuscript.

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Polyphosphate, RNA, and the Contact Pathway of Blood Clotting

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