POLYPHOSPHATE MODULATES HEMOSTASIS, THROMBOSIS, AND INFLAMMATION

BY

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DISSERTATION

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ABSTRACT

Polyphosphate (polyP) is a linear polymer of inorganic phosphates that is secreted by activated platelets and is abundant in many pathogenic microorganisms. Our lab has recently shown that polyP from bacteria and human platelets play important roles in inflammation and coagulation \textit{in vitro} and \textit{in vivo}, supporting the paradigm of polyP as the long-sought (patho)physiologic activator of the contact pathway of blood clotting. These studies also implicate polyP in contributing to thrombosis and consumptive coagulopathies accompanying bacterial sepsis. PolyP polymer lengths are known to vary substantially among different organisms and cell types, with shorter polymers secreted by human platelets and much longer polymers accumulating in microorganisms, raising intriguing questions about the mechanisms by which various polyP sizes differentially modulate the blood clotting system. To accomplish this goal, I used bacterial and platelet-derived polyP, in addition to carefully size-fractionated synthetic polyP preparations, to investigate how polyP (dependent on polymer size) promotes each of the individual enzyme reactions that result in triggering and propagating blood clotting reactions. This data advances our understanding of the mechanisms by which polyP modulates blood clotting and inflammation, with a particular emphasis on the contact pathway and factor V activation. A serious impediment to progress in studying polyP in biological systems is the dearth of techniques for manipulating polyP to investigate polyP-protein interactions. To this end, I developed a novel method for covalently crosslinking polyP to solid supports or labeling polyP with primary amine-containing solid supports. This technique of crosslinking polyP has already facilitated studies on the ever-expanding role of polyP in blood clotting and other important biological processes.
DEDICATION

To my grandmother, who showed me that with hard work and determination, nothing is impossible
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My time in graduate school has been an extremely rewarding journey – I don’t think I could have asked for a better experience. I could not have completed this journey without the guidance and endless support of many people listed in the following paragraphs. I am most fortunate to have Dr. James (Jim) Morrissey as my thesis advisor. There are many great scientists at University of Illinois, but few can match Jim’s dedication to his students. Jim is always available for discussions both in person during work hours and e-mail (nobody replies more promptly than he does) after hours. While my thesis project got off to a slow start during the first couple years, Jim was continually positive about my project, providing often much-needed gentle pushes, and proving that perseverance does indeed pay off. His passion for science, attention to details, persistence, and undying patience have inspired me to become not only a better scientist, but also a better teacher.

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Polyphosphate

Inorganic polyphosphate (polyP) is structurally very simple, consisting of linear polymers of orthophosphate linked by high-energy phosphoanhydride bonds (Figure 1.1). At physiological pH, each internal phosphate unit of polyP carries a monovalent negative charge, making polyP an intensely anionic polymer. PolyP is ubiquitous in biology and can vary in polymer length from just a few phosphates to several thousand phosphate units long, depending on the organism and the tissue in which it is synthesized.\textsuperscript{1,2} PolyP is synthesized enzymatically from ATP; this reaction is fully reversible and may allow bacteria to synthesize ATP from stored polyP in times of starvation and environmental stress.\textsuperscript{3} PolyP can be degraded by endopolyphosphatases (which cleave within the polyP chain core) and exopolyphosphatases (which sequentially remove terminal phosphates from polyP). Mammalian alkaline phosphatase is a potent exopolyphosphatase.\textsuperscript{4} PolyP has a half-life of about 1.5 to 2 hours in human blood or plasma, presumably due to exo- and/or endopolyphosphatase digestion.\textsuperscript{5,6}

PolyP has been most extensively studied in prokaryotes and unicellular eukaryotes, but roles for polyP in mammalian systems are rapidly emerging. In mammalian cells, polyP has been identified in lysosomes,\textsuperscript{7} dense granules,\textsuperscript{8} mitochondria and nuclei.\textsuperscript{9} PolyP associated with poly-3-hydroxybutyrate has been reported to be a component of the Ca\textsuperscript{2+}-ATPase pump in human erythrocytes.\textsuperscript{10,11} PolyP has also been shown to induce proliferation and differentiation of mesenchymal stem cells via activation of the fibroblast growth factors.\textsuperscript{12} More recently, nucleoli of myeloma cells were shown to contain high levels of polyP compared to normal primary...
plasma cells. Other functions identified for polyP in mammalian cells include cell proliferation, angiogenesis, apoptosis, osteoblast function, bone mineralization, energy metabolism and tumor metastasis.

**PolyP in microorganisms**

Prokaryotic and eukaryotic microorganisms store high concentrations of polyP along with divalent metal ions such as Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$ in subcellular organelles termed acidocalcisomes (also known as volutin or metachromatic granules in some organisms). Microorganisms typically contain very long-chain polyP, ranging in length from hundreds to thousands of phosphate units, and furthermore, *Neisseria meningitidis* express long-chain polyP on their capsule. In the bacteria in which it has been studied, polyP kinase 1 (PPK1), a membrane-associated enzyme, synthesizes polyP from ATP; this reaction is fully reversible and may allow bacteria to resynthesize ATP from stored polyP in times of starvation. PolyP kinase is critical for cell viability in bacteria, as knocking out this enzyme results in defective motility and compromised survival during periods of environmental stress. PolyP activates the expression of *E. coli* RpoS, the sigma factor responsible for activation of more than 50 genes required for survival during starvation and exposure to UV radiation, oxidative damage, and osmotic stress.

*Pseudomonas aeruginosa* mutants lacking PPK1 are deficient in motility, quorum sensing, biofilm formation and virulence; however, the mutants still possess as much as 20% of the wild-type levels of polyP, which points to an additional polyphosphate kinase, PPK2. An interesting feature of PPK2 is that it kinetically favors GTP synthesis from polyP over polyP synthesis in contrast to PPK1, which strongly favors synthesis of polyP from ATP.
may contribute to the GTP-dependent synthesis of alginate in bacterium and its homologs have been identified in major bacterial pathogens.\textsuperscript{37} The importance of PPK1 and PPK2 in bacterial virulence and mucoid maintenance, respectively, suggest that the two polyP kinases may serve as possible targets for the discovery and design of novel antibiotics.

\textit{Dictyostelium discoideum}, a heavily-studied social slime mold, is one of a few eukaryotes known to possess a homolog of \textit{E. coli} PPK1.\textsuperscript{38} Similar to bacteria, \textit{D. discoideum} contains an additional PPK (termed DdPPK2), a tetramer that polymerizes into an actin-like filament concurrently with the synthesis of long-chain polyP.\textsuperscript{39,40} Interestingly, actin and polyP form a complex that masks the ends of the polyP chain, as the complex is susceptible to endopolyphosphatase, but not exopolyphosphatase.\textsuperscript{39}

\textbf{PolyP in platelets}

In 1958, Hermansky and Pudlak described two albino patients who presented with increased bruising and prolonged bleeding.\textsuperscript{41} Subsequent studies demonstrated bleeding tendencies in patients with congenital deficiencies in platelet dense granules (also called delta granules).\textsuperscript{42,43} Ruiz et al.\textsuperscript{8} noted that the dense granules of human platelets were strikingly similar in appearance to acidocalcisomes of unicellular organisms, as both are spherical, acidic,\textsuperscript{44} electron-dense,\textsuperscript{45} and contain divalent metal ions including Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and Zn\textsuperscript{2+}.\textsuperscript{46} Platelet dense granules were known to contain inorganic phosphate and pyrophosphate,\textsuperscript{46} but whether they contained polyP was unknown. In 2004, Ruiz et al.\textsuperscript{8} reported for the first time that platelet dense granules have abundant polyP, at a concentration inside dense granules of about 130 mM (expressed in terms of phosphate monomer, as polyP concentrations are typically reported in the literature). Consistent with this finding, Ruiz et al.\textsuperscript{47} subsequently reported that patients diagnosed with platelet dense
granule defects (and experiencing bleeding symptoms) have platelet polyP levels about ten times lower than normal. PolyP is efficiently secreted following platelet activation.\textsuperscript{8,48} Since human platelets contain 0.74 ± 0.08 μmol polyP/10\textsuperscript{11} platelets (expressed in terms of phosphate monomer),\textsuperscript{8} and given the normal range of platelets in human blood (1.5 to 4.5 × 10\textsuperscript{11} platelets/L), one can calculate that human blood will contain 1 to 3 μM polyP after full platelet activation. In platelet-rich thrombi, local polyP concentrations are likely to be orders of magnitude higher than this. Unlike microbial polyP, platelet polyP is smaller and much less heterodisperse, having polymer lengths about 60 to 100 phosphate units long.\textsuperscript{8,48}

**Initiation of blood clotting via the contact pathway: a critical role for polyP**

The classic blood clotting cascade can be triggered via either the tissue factor pathway or the contact pathway (Figure 1.2). While the tissue factor pathway is essential for hemostasis,\textsuperscript{49} the contact pathway is not required since humans and animals lacking factor XII have no bleeding tendencies.\textsuperscript{50} As outlined below, however, the contact pathway plays other important roles and is triggered when factor XII, prekallikrein and high molecular weight kininogen assemble on anionic polymers or surfaces. Factor XIIa then activates factor XI to XIa, which in turn activates factor IX, leading to propagation of the clotting cascade through the final common pathway.

Although the contact pathway is dispensable for hemostasis, it clearly participates in thrombosis. Clinical studies have associated elevated plasma factor XII with coronary heart disease, atherosclerosis\textsuperscript{51} and recurrent coronary events after acute myocardial infarction.\textsuperscript{52} The Study of Myocardial Infarction – Leiden (SMILE)\textsuperscript{53} reported that men in the highest quintile for factor XI had an approximately two-fold increased risk of myocardial infarction compared with the lowest quintile. Similarly, a Swiss case-control study showed a strong association between
plasma kallikrein and factor XI activity with a history of myocardial infarction. Another recent study found a significant reduction in the incidence of ischemic stroke in patients with severe factor XI deficiency compared with the general Israeli population. Activation of the contact pathway of blood clotting thus has clinical implications in pathologic thrombus formation in humans.

Animal models also support a role for the contact pathway in thrombosis. Mice deficient in factor XII are protected against thrombus formation in a variety of models of arterial and venous thrombosis. Factor XII gene knockout in mice also results in defective immune responses to infection, suggesting that the contact pathway participates in host responses to pathogens. Consistent with this concept, several microbial activators of the contact pathway have been identified, including bacterial surface proteins, lipopolysaccharide, teichoic/lipoteichoic acid, and, as discussed here, long-chain polyP.

The first reported role for polyP in blood clotting was a 2006 study from our lab that showed that polyP is strongly procoagulant, triggering clotting of plasma via the contact pathway as well as modulating downstream clotting reactions (Figure 1.2). For many years, the identity of the true (patho)physiologic activator(s) of the contact pathway has remained elusive, and in fact most published studies of this pathway have employed artificial activators such as glass, powdered clay (kaolin), diatomaceous earth, dextran sulfate, ellagic acid, or high concentrations of sulfatides. Most artificial contact activators are anionic surfaces or polymers. PolyP is also a highly anionic polymer, and we have shown that it binds tightly to the proteins responsible for initiating the contact pathway. Furthermore, activation of clotting via polyP has a bell-shaped concentration-dependence, consistent with the idea that polyP serves as a template for assembling multiple contact factors.
PolyP accelerates thrombin generation

As the essential cofactor for activation of prothrombin by factor Xa, factor Va occupies a central place in the clotting cascade (Figure 1.2). In 2006, our laboratory identified that polyP accelerates the proteolytic conversion of factor V to Va by both factor Xa and thrombin, resulting in an accelerated thrombin burst during plasma clotting reactions.\(^5\) Accelerating factor V activation has interesting consequences for blood clotting.\(^5\) When roughly platelet-sized polyP was added to plasma and triggered clotting with either tissue factor-liposomes or factor Xa plus phospholipids, the anticoagulant function of tissue factor pathway inhibitor (TFPI) was totally abrogated.\(^5,6²\) TFPI is a multifunctional serine protease inhibitor in plasma and platelets, and one of its most important targets is thought to be newly generated factor Xa.\(^6³\) Mast and Broze showed that TFPI is a poor factor Xa inhibitor once factor Xa is bound to factor Va, especially in the presence of plasma concentrations of prothrombin.\(^6⁴\) Consistent with this observation, spiking plasma with factor Va also abrogates TFPI’s anticoagulant activity.\(^5\) Interestingly, however, shorter polyP polymers are required to abrogate TFPI function than to enhance factor V activation, suggesting that polyP may be directly interacting with TFPI. Furthermore, platelet releasates strongly inhibit TFPI function and that almost all of this TFPI-abrogating activity is directly attributable to polyP.\(^5,4⁸\) And finally, platelets from Hermansky-Pudlak syndrome patients exhibit reduced plasma clotting activity, which could be restored by adding polyP.\(^4⁸\) Taken together, these studies show that polyP secreted by human platelets profoundly enhances blood clotting reactions.
PolyP enhances fibrin clot structure and stability

Thrombin converts fibrinogen into fibrin by limited proteolysis, and the resulting fibrin monomers spontaneously associate to form a three-dimensional clot. When fibrinogen was mixed with polyP plus plasma concentrations of Ca$^{2+}$, then added thrombin, the resulting fibrin clots were more turbid, had fibrils with higher mass/length ratios, were more resistant to elastic stretching, and were more resistant to fibrinolysis than were clots formed under identical conditions but without polyP. After such clots were washed extensively with buffer, they exhibited strong, metachromatic staining with toluidine blue, characteristic for the presence of polyP. Thus, polyP appears to become incorporated into fibrin clots, although how polyP alters fibrin clot structure is not known. Heparin, another highly anionic linear polymer, also increases fibrin clot turbidity but unlike the case with polyP, clots formed in the presence of heparin have increased susceptibility to fibrinolysis. PolyP therefore alters fibrin clot structure in a manner different from that of heparin or other anionic polymers. PolyP of the size secreted by activated platelets is just within the range necessary to enhance fibrin clot structure, although optimal fibrin enhancement requires longer polyP polymers.

Studies from our laboratory have also revealed that pyrophosphate abrogates the ability of polyP to enhance fibrin clot structure, while having no discernible effect on fibrin clots formed in the absence of polyP. It has been known for many years that activated platelets secrete pyrophosphate in relatively large amounts (comparable to the amount of polyP that they secrete) but to our knowledge, no convincing roles for platelet pyrophosphate have been proposed. These results suggest that pyrophosphate may be an overlooked regulator of fibrin clot structure.

Mutch et al. reported that polyP attenuates fibrinolysis by inhibiting the binding of fibrin to tissue-type plasminogen activator or plasminogen. PolyP also slows fibrinolysis in plasma clots.
in a manner that is dependent on the presence of thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase that removes C-terminal lysine residues from fibrin. This latter function may be a consequence of the earlier thrombin burst when plasma is clotted in the presence of polyP.

**PolyP drives inflammation and thrombosis**

The contact pathway plays important roles in inflammation. Activation of the contact pathway results in proteolytic liberation of bradykinin (a potent vasoactive peptide) from high MW kininogen. Furthermore, a body of work has shown that components of the contact pathway can be selectively activated on cell surfaces, with or without triggering of clotting via activation of factor XI by factor XIIa. Accordingly, the contact pathway is perhaps more accurately termed the kallikrein-kinin system, which is implicated in acute and chronic inflammation in a number of human diseases. The kallikrein-kinin system also represents a point of cross-talk between the clotting and complement systems. In addition to bradykinin generation, kallikrein has been shown to directly activate complement components C3 and C5, while factor XIIa also initiates the classical complement cascade. A tragic example of pathologic activation of the contact pathway in vivo occurred in 2008, when therapeutic heparin contaminated with oversulfated chondroitin sulfate entered the drug supply, resulting in 81 deaths. Follow-up studies confirmed that oversulfated chondroitin sulfate (a highly anionic glycosaminoglycan) triggers the contact system, resulting in bradykinin generation and activation of complement components C3 and C5 in a factor XII- and kallikrein-dependent manner.

Our studies have shown that long-chain polyP is an extremely potent trigger of the contact pathway, raising the possibility that polyP could drive inflammatory reactions. A recent study
from Renné and colleagues investigated in vivo roles for polyP using mouse models of inflammation and thrombosis. PolyP administered intravenously at high doses to wild-type mice led to lethal pulmonary embolism while factor XII-deficient mice survived, as did wild-type mice administered an inhibitor of factor XIIa (CSL829). In other experiments, platelets were activated in vivo in wild-type mice by intravenous injection of an agonist peptide that stimulates protease-activated receptors (PARs), resulting in death by pulmonary embolism. Factor XII-deficient mice were protected from this otherwise lethal challenge, as were wild-type mice given high doses of alkaline phosphatase intravenously prior to administration of agonist peptide (to digest polyP). These experiments demonstrate that polyP can be thrombogenic in vivo, in a factor XII-dependent manner.

As with other activators of the contact system, polyP promotes proteolysis of high MW kininogen with concomitant bradykinin release. In a mouse model of edema, mice were injected subcutaneously with polyP, which provoked localized capillary leak (quantified by extravasation of Evans blue dye). Mice lacking either factor XII or the bradykinin B2 receptor were protected from polyP-induced edema, as were wild-type mice that had been administered factor XIIa inhibitors. In another mouse model, intraperitoneal injection of E. coli-derived polyP into wild-type mice led to a rapid drop in systemic arterial blood pressure and death of 90% of the mice in 15 minutes, while mice lacking either factor XII or bradykinin B2 receptors survived this challenge. These experiments show that polyP can be strongly proinflammatory in vivo, in a manner that depends on factor XII activation and bradykinin generation.

The role of polyP in inflammation independent of the contact pathway has also received increasing attention in recent years. Extracellular histones have been shown to exhibit potent proinflammatory and procoagulant activities. Esmon and colleagues have shown that polyP
substantially augments the procoagulant activity of histones, resulting in enhanced platelet activation and thrombin generation independent of factor XII or tissue factor.\textsuperscript{80} The mechanism by which polyP enhances histones’ procoagulant activity is not known. Another recent study from Rezaie and coworkers reported that polyP exerts a proinflammatory effect via NF-κB activation.\textsuperscript{81} It is conceivable that polyP may also interact with pathogen-associated molecular pattern receptors on the cell surface or with chemokines to modulate inflammatory reactions. Future studies with pathogen-derived polyP are required to assess the multiple roles of polyP in host-pathogen responses.

**Methods for preparing and analyzing polyP**

An expanding body of research is investigating the roles of polyP in biological systems, but difficulties facing researchers in this field include the relative scarcity of commercial sources of size-fractionated polyP, as well as a dearth of techniques for manipulating and detecting polyP at µg and ng quantities (other than using radiolabeled polyP, which is not always practical). The late Dr. Arthur Kornberg, in his last paper on polyP biology,\textsuperscript{37} stated “for more widespread research on polyP, improvements in assay methods are needed. Among the reasons for the neglect of polyP research has been the lack of sensitive and facile analytical methods to assess its concentration in biological sources. . .” Recent studies from several groups, including our own, are starting to fill this gap.

PolyP is an industrial chemical with applications in water treatment, fertilizer production, food processing and the production of flame retardants.\textsuperscript{82} It can be chemically synthesized in very large quantities (metric tons) simply by heating sodium orthophosphate to a few hundred degrees Celsius, and then rapidly cooling.\textsuperscript{83} Commercial sources for heterodisperse polyP
preparations of various sizes include Sigma-Aldrich (St. Louis, MO) and BK Giulini (Ladenberg, Germany). A preparation of water-soluble, high MW polyP can be isolated from very high MW “insoluble” polyP by differential solubilization using LiCl solutions. Other size ranges of polyP can be prepared via partial alkaline or acidic hydrolysis of long-chain polyP, enzymatic degradation with endopolyphosphatases, or differential acetone precipitation under various salt conditions. Narrow size fractions of polyP are best prepared using preparative gel electrophoresis. Polymer lengths of short- to medium-chain polyP can be characterized using paper chromatography, ion-exchange chromatography (2-12mers), gel-filtration, or $^{31}$P-NMR spectroscopy. Larger and more heterodisperse polymers are best sized using analytical gel electrophoresis. PolyP may be detected in polyacrylamide gels using either toluidine blue or DAPI staining. PolyP can be quantified as monophosphate following complete hydrolysis by acid or by enzymatic digestion (with, for example, exopolyphosphatase from Saccharomyces cerevisiae or calf intestinal alkaline phosphatase). In solution and in cells and tissues, polyP can also be detected (and, to some extent, quantified) using the same dyes that are used to stain polyP in gels, including toluidine blue or DAPI, which exhibit metachromatic staining of polyP. Low levels of polyP can be accurately quantified using enzymatic methods, although these approaches are laborious and some of them require using radioisotopes ($^{32}$P) for high sensitivity.

The ubiquitous nature of polyP throughout biology, together with its simple structure, make it unlikely that specific blocking antibodies could be raised against polyP. Alternative methods for targeting polyP are being developed, however. For example, in the fifth and sixth chapters of this thesis, we successfully used the isolated polyP-binding domain of E. coli exopolyphosphatase (i.e., lacking the phosphatase catalytic domain) to block the procoagulant
function of polyP in platelet releasates,\textsuperscript{94} in a manner akin to using a blocking antibody. Alternatively, recombinant yeast exopolyphosphatase has been employed to digest polyP.\textsuperscript{13} Alkaline phosphatase (a highly active exopolyphosphatase), which also enzymatically degrades polyP, has been successfully used to destroy polyP procoagulant activity in vitro.\textsuperscript{5,48} In vivo, treatment of polyP with alkaline phosphatase abrogated activation of the contact system and bradykinin generation, abolished procoagulant platelet activity, and blocked platelet-induced thrombosis in mice.\textsuperscript{48} A potential drawback to using a relative nonspecific enzyme such as alkaline phosphatase is that it can also enzymatically degrade other phosphate-containing compounds such as ADP, an important platelet agonist. And finally, the isolated polyP binding domain of \textit{E. coli} exopolyphosphatase has been used successfully as a probe to localize polyP in yeast cells\textsuperscript{95} and in acidocalcisomes in eggs of sea urchins\textsuperscript{96} and insects.\textsuperscript{97}

\textbf{Scope of this thesis}

This first chapter serves as a brief introduction to polyP biology and the recently reported roles of polyP in individual blood clotting reactions. The second chapter of this thesis illustrates that polyP exerts differential effects on the contact pathway of blood clotting depending on polymer size. The third chapter identifies reaction conditions under which the terminal phosphates of polyP can be made to enter into stable phosphoramidate linkages with almost any primary amine-containing compound, using the zero-length coupling reagent, EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide). Using EDAC-mediated coupling chemistry, the terminal phosphates of polyP can be covalently attached to almost any primary amine-containing solid supports or probes such as biotin, fluorophores, and polyamines. The fourth chapter identifies a panel of polyphosphate inhibitors, including cationic proteins, polymers and small
molecules and compares their effectiveness in abrogating polyP-mediated procoagulant and proinflammatory reactions in vivo and in vitro. The fifth chapter shows that polyP of the size secreted by platelets strongly supports factor XI activation by thrombin and factor Xla (autoactivation). The sixth chapter demonstrates how polyP accelerates factor V procofactor activation by factor Xla. Finally, the seventh chapter will summarize the prohemostatic, prothrombotic and proinflammatory effects of polyP. This final section will also propose microbial polyP as a (patho)physiologic activator of the contact pathway and a key player in the web of host-pathogen interactions.

FIGURES

Figure 1.1. Structure of inorganic polyP. PolyP is a linear, highly anionic polymer of phosphates held together by high-energy phosphoanhydride bonds. Platelet polyP is approximately 60 to 100 phosphate units long, while microbial polyP can range up to thousands of phosphate units long.
Figure 1.2. The various effects of polyP in blood clotting. Before the beginning of this thesis project, polyP was identified to potently act at three points in the clotting cascade, indicated in red: 1, initiates the contact pathway of blood clotting;\textsuperscript{5,48} 2, accelerates factor V activation and abrogates TFPI function (the latter not shown explicitly);\textsuperscript{5} and 3, enhances fibrin polymerization.\textsuperscript{65,70}
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CHAPTER 2: POLYPHOSPHATE EXERTS DIFFERENTIAL EFFECTS ON THE CONTACT PATHWAY DEPENDENT ON POLYMER SIZE

ABSTRACT

Polyphosphate, a linear polymer of inorganic phosphate, is secreted by activated platelets and accumulates in many infectious microorganisms. We recently showed that polyphosphate modulates the blood coagulation cascade at three steps: it triggers the contact pathway, it accelerates factor V activation, and it enhances fibrin polymerization. We now report that polyphosphate exerts differential effects on blood clotting depending on polymer length. Very long polymers ($\geq 500$mers, such as those present in microorganisms) were required for optimal activation of the contact pathway, while shorter polymers ($\sim 100$mers, similar to the polymer lengths released by platelets) were sufficient to accelerate factor V activation and abrogate the anticoagulant function of tissue factor pathway inhibitor. Optimal enhancement of fibrin clot turbidity by polyphosphate required $\geq 250$mers. Pyrophosphate, which is also secreted by activated platelets, potently blocked polyphosphate-mediated enhancement of fibrin clot structure, suggesting that pyrophosphate is a novel regulator of fibrin function. In conclusion, polyphosphate of the size secreted by platelets is very efficient at accelerating blood clotting reactions but is less efficient at initiating them or at modulating clot structure. Microbial polyphosphate, which is highly procoagulant, may function in host responses to pathogens.

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INTRODUCTION

Polyphosphate (polyP) — a linear polymer of inorganic phosphate — accumulates in a variety of microorganisms\(^1\) and is secreted by activated human platelets.\(^2,3\) We recently showed that polyP is a potent modulator of the human blood clotting system.\(^3,4,6\) The polymer lengths of polyP are known to vary substantially among different organisms and cell types, with relatively short polymers being secreted by human platelets (approximately 60-100 phosphate units long)\(^2,3\) and very long polymers accumulating in microorganisms (many hundreds to over 1000 phosphate units long).\(^1\) In this study, we demonstrate that shorter versus longer polymers of polyP have differential effects on the blood clotting system, with important physiologic/pathophysiologic implications.

PolyP has been widely described in unicellular organisms such as bacteria, fungi, algae and protozoa, where it plays diverse physiologic roles, including regulating growth, stress responses, and virulence.\(^1,7\) Comparatively less is known about the metabolism or physiologic roles of polyP in mammalian cells,\(^8\) although polyP is reported to induce apoptosis in plasma cells,\(^9\) promote calcification in osteoblasts,\(^10\) block metastasis of melanoma cells in a mouse model,\(^11\) and possibly serve as a regulatory factor in proliferative signaling pathways.\(^12\)

PolyP is present at high concentrations in dense granules of human platelets and is secreted upon platelet activation.\(^2,3\) PolyP has a half-life in plasma of about ninety minutes, owing to degradation by phosphatases.\(^4,13\) We recently showed that polyP is a potent hemostatic regulator, acting at three points in the blood clotting cascade: it initiates the contact pathway of blood clotting;\(^3,4\) it accelerates the activation of factor V (FV) by thrombin and factor Xa (FXa);\(^4\) and it enhances the thickness of fibrin fibers.\(^5\) Our previous studies were conducted with heterodisperse polyP preparations, so the precise size-dependence of the actions of polyP on...
blood clotting was unknown. In the present study, we isolated polyP preparations of carefully defined polymer lengths and used them to investigate the effects of polyP on the blood clotting system. We now report that initiation of the contact phase of blood clotting, accelerating FV activation, and enhancing fibrin clot structure exhibited markedly different polyP size requirements. We further report that inorganic pyrophosphate (PP$_i$) — which is also secreted by activated human platelets — abrogated the polyP-mediated enhancement of fibrin clot structure. These findings have implications for the role of microbial versus endogenous (platelet-derived) polyP in modulating the blood clotting system in health and disease.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium phosphate, sodium PP$_i$, sodium triphosphate, kaolin, ADP, ATP, soluble polyP preparations of varying polymer size ranges (marketed as “sodium phosphate glass”), and high MW polyP (marketed as “phosphate glass, water insoluble”) were from Sigma-Aldrich (St. Louis, MO). In this paper, we use the naming convention of the supplier (Sigma) for the purchased polydisperse polyP preparations: type 25, type 45, type 65 (catalog number S6253), and type 75+ (catalog number S8262), with nominal mean polymer lengths of 25, 45, 65 and >75 phosphates, respectively. (In previous studies,$^4$–$^6$ we referred to polyP type 75+ as “polyP$_{75+}$”.) In this paper, we refer to size-fractionated polyP preparations of very narrow size distributions (described below) by their measured polymer length followed by “mer” (for example, 105mer).

Citrated, pooled normal human plasma was from George King Biomedical (Overland Park, KS) and FXII-deficient plasma was from Hematologic Technologies (Burlington, VT). Purified human fibrinogen, FXa, and α thrombin were from Enzyme Research Laboratories
Phospholipid vesicles consisting of 20% phosphatidylserine and 80% phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were made by sonication. Recombinant tissue factor pathway inhibitor (TFPI) was a kind gift from Dr. George Broze.

Isolation of polyP from bacteria

PolyP was extracted from an exopolyphosphatase-deficient strain of *Salmonella enterica* serovar Typhimurium provided by Dr. James Slauch. Extraction and purification of polyP from bacteria was adapted from Ault-Riche *et al.* as follows: Bacteria were grown at 37°C in LB in shaking cultures to mid-log phase ($A_{600} = 0.9$), collected by centrifugation, resuspended in MOPS medium containing 0.1 mM NaH$_2$PO$_4$ and 4 mg/mL glucose, and incubated with shaking at 37°C for an additional 1-2 hours to induce polyP accumulation. Bacteria were collected by centrifugation, resuspended in 10 mM Tris (pH 8.0), 2.7 kU/mL Ready-Lyse lysozyme (Epicentre, Madison, WI), 1.4 mM EDTA, and sonicated for 5 minutes. The lysate was supplemented with 3.5 mM MgCl$_2$ and 30 U/mL Benzonase nuclease (Novagen, San Diego, CA) and incubated for 30 minutes at 37°C, after which solid guanidinium isothiocyanate (4 M final) was dissolved in the lysate and the mixture heated for 5 minutes at 100°C. The mixture was cooled and the pH adjusted to 5.5 with sodium acetate, followed by addition of cold ethanol (30% final). Glass milk was added (2 μL of 50% glass milk slurry per mL of original culture) and polyP was allowed to bind for 10 minutes at 25°C with mixing. Glass milk was collected by centrifugation, then washed thrice with 5 mM Tris HCl pH 7.4, 50 mM NaCl, 50% ethanol. PolyP was eluted by resuspending the glass milk in 50 mM Tris pH 8.74 at 95°C for 5 minutes. Any contaminating nucleic acids were digested by incubating with 100 U/mL Benzonase, 0.2 mM ammonium molybdate for 30 minutes at 37°C, after which polyP was precipitated by adding
NaCl (50 mM final) followed by 2 volumes of ice-cold acetone. After incubating at -80°C for 30 min, the precipitate was collected by centrifugation at 10,000 × g, air dried for 10 min, and dissolved in purified water. Yield was 61 µg polyP per L of bacterial culture.

**Isolation of PolyP from Platelets**

PolyP was purified from human pheresis platelets (~3-4 x 10^{11} platelets/pheresis unit) obtained from the Regional Blood Center of Central Illinois. PolyP isolation was as described with these minor modifications: the lysis buffer contained 0.5% SDS; proteinase K digestion was conducted at 50°C; the pH of the solution following Dowex resolubilization was neutralized with 40 mM Tris base; and the final polyP preparation was dialyzed overnight, using 1000 MW cutoff dialysis tubing, against 20 mM Tris pH 7.4 plus Chelex 100 resin (Bio-Rad, Hercules, CA) to ensure removal of any bound metal ions.

**Size-fractionation of polyP**

PolyP was size-fractionated by preparative PAGE based on the method of Clark and Wood. 

For shorter polymers (<150mers), a mixture of 5 mg polyP type 25 plus 5 mg polyP type 75+ was resolved on 18 x 16 cm 15% polyacrylamide or 40 x 16 cm 20% polyacrylamide gels in TBE (90 mM Tris, 90 mM borate, 2.7 mM EDTA, pH 8.3) until xylene cyanol FF migrated 7 cm and bromophenol blue, 14 cm. For longer polymers (>150mers), “insoluble” high MW polyP was washed twice with purified water on a fritted glass funnel to deplete very short polymers, then resuspended in 250 mM LiCl and stirred for 2 hours at room temperature, after which residual insolubles were removed by centrifugation (5000 × g for 10 minutes). Dissolved polyP was precipitated from the supernatant by slowly adding 2.5 volumes of acetone, collected by
centrifugation (10,000 x g for 20 minutes), dried overnight, and dissolved in purified water, resulting in a highly disperse population of polyP polymer lengths. Approximately 20 mg of this preparation was resolved on 18 x 16 cm 4% or 6% polyacrylamide gels (in TBE) until xylene cyanol FF migrated 7 cm and bromophenol blue, 14 cm. Narrowly size-fractionated polyP preparations were eluted from individual 1 cm-wide slices of the 4%, 6%, 15% or 20% gels, while heterogeneous preparations containing very long polymers greater than either 400mer or 1000mer (termed polyP\textsubscript{400+} and polyP\textsubscript{1000+} respectively) were obtained by pooling several 1 cm-wide gel slices from 4% gels.

PolyP was eluted from gel slices by crushing and agitating overnight in 2.5 mL purified water. Fragments of polyacrylamide were removed by centrifugation using Handee centrifuge columns (Pierce, Rockford, IL), and the eluted polyP was dialyzed against purified water overnight using Snakeskin\textsuperscript{®} dialysis tubing (MW-cutoff, 3500) for polymers >100mers, or Slide-A-Lyzer mini dialysis units (MW-cutoff, 1000; both from Pierce, Rockford, IL) for polymers <100mers.

\textsuperscript{31}P NMR spectra of polyP preparations were acquired at 20°C with a Varian Unity INOVA 600 spectrometer using a 5mm Varian AutoTuneX \textsuperscript{1}H/X PFG Z probe, 13.5 \mu s (90°) pulse excitation, 16 kHz spectral width, and 5 second recycle time. Chemical shifts were referenced to 0 ppm using an external phosphoric acid standard. Spectra were signal averaged until the phosphate α peak (approximately -6 ppm) achieved a signal-to-noise ratio of at least 5:1 (typically 20,000 to 45,000 scans, depending on sample size and concentration) and processed with baseline correction and 25-Hz line broadening prior to Fourier transformation. Each spectrum showed a peak corresponding to external phosphates (α peak at about -6 ppm) and a second peak corresponding to internal phosphates (-22 to -24 ppm); for some samples, a third
peak was also observed, corresponding to phosphates neighboring the external residues (β peak at -21 to -22 ppm; see Figure 2.1 for a typical spectrum). Quantitation was performed by integrating the area under the curves for the each peak. Because each polyP polymer has two α residues, the number of internal phosphorus atoms, N, was determined using the equation, \( N/2 = t/\alpha \), where \( \alpha \) is the integration of the α peak and \( t \) the integration of the internal phosphate peak (plus the β peak, when present). PolyP polymer lengths equaled \( N+2 \).

PolyP preparations whose mean polymer lengths had been determined by NMR (and found to be 10, 34, 51, 60, 98, 110, 257 and 1350 phosphates long) were employed as size standards for estimating polymer lengths of the rest of the polyP preparations by analytical PAGE,\(^17\) using 5% or 15% polyacrylamide gels with 7 M urea in TBE (Ready Gels from Bio-Rad), and detecting polyP by toluidine blue staining or DAPI negative staining.\(^18\)

**PolyP concentrations**

PolyP concentrations were measured by quantifying inorganic phosphate\(^19\) after complete hydrolysis in 1 M HCl at 100°C for 30 minutes. Briefly, 50 µL hydrolyzed phosphate sample was mixed with 100 µL malachite green reagent (0.075% malachite green, 0.045% Tween-20, 4.2% ammonium molybdate, 5 M HCl) in polypropylene multiwell plates (Corning, Corning NY) and incubated for 20 minutes at room temperature, after which \( \text{A}_{660} \) was measured in a Spectramax microplate reader (Molecular Devices Corp, Sunnyvale, CA) and phosphate concentrations determined by reference to a standard curve. Note that all polyP concentrations reported in this study are given in terms of phosphate monomer concentration (monomer formula: \( \text{NaPO}_3 \)).
Clotting assays

Clotting times of citrated human plasma were quantified at 37°C using a STart4 coagulometer (Diagnostica Stago, France). Tests of the contact pathway of blood clotting were conducted using final concentrations of 33% plasma, 25 µM phospholipid, 0-40 µM polyP, 41.7 mM imidazole pH 7.0, and 8.33 mM CaCl$_2$. Prewarmed polyP in imidazole buffer was incubated in coagulometer cuvettes with prewarmed plasma and phospholipid for 3 minutes, after which clotting was initiated by addition of CaCl$_2$. For comparison, a standard curve was prepared using various concentrations of kaolin (0.04 to 3333 µg/mL), and no polyP, in the same clotting assay (data not shown). The specific activities of polyP preparations were then calculated, by reference to this standard curve, to yield kaolin equivalents (i.e., the kaolin concentration that yields the same clotting time as polyP, when both are plotted in terms of µg/mL).

Clotting reactions initiated by FXa contained 33% plasma, 33 µM phospholipid, 0-40 µM polyP, 16.7 mM Tris pH 7.4, 33.3 mM NaCl, 0.03% bovine serum albumin, 333 pM FXa, and 8.33 mM CaCl$_2$. Plasma was prewarmed separately from polyP and FXa components to minimize inhibition of FXa by plasma inhibitors and to prevent contact activation of plasma by polyP. Components were then mixed simultaneously in prewarmed coagulometer cuvettes to initiate clotting. As the ability of polyP to shorten FXa clotting times has been attributed to acceleration of FV activation, we converted these shortened FXa clotting times into polyP specific activities by comparison to a standard curve in which the FXa-initiated clotting reaction was conducted in the absence of polyP but with varying concentrations (0 to 6.7 nM) of FVa added to the plasma. The specific activities of polyP preparations were calculated, by reference to this standard curve, to yield FVa equivalents (i.e., comparing the FVa concentration that
would yield the same clotting time as polyP, when both are plotted in terms of molar concentrations).

In some experiments, the ability of polyP to antagonize the anticoagulant activity of TFPI was quantified by adding TFPI to plasma (400 ng/mL final), and initiating clotting with 2 nM FXa. For comparison, a standard curve was prepared in the absence of polyP but with varying TFPI concentrations (0-400 ng/mL) added to the plasma. This standard curve was then used to calculate the percent inhibition of TFPI activity.

**Fibrin clot turbidity**

Fibrin clots were formed in medium-binding, polystyrene 96-well plates as previously described. Briefly, mixtures of fibrinogen, polyP and calcium ions were preincubated for 15 minutes at room temperature, after which thrombin was added to trigger clot formation. Final (160 μL) reactions contained 2.6 mg/mL fibrinogen, 10 nM thrombin, 0-400 μM polyP, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% NaN₃, and 2.5 mM CaCl₂. Turbidities (A₄₀₅) of fibrin clots were quantified using a Spectramax microplate reader 60 minutes after thrombin addition.

**RESULTS**

We previously reported that heterodisperse polyP preparations are potent modulators of blood clotting. In particular, we showed that heterodisperse, commercially available polyP type 75+, whose polymer lengths encompass the polyP sizes secreted by human platelets, was more potent in triggering the contact pathway than was polyP type 25. However, both polyP type 25 and 75+ contain some polymers that are considerably longer (and shorter) than their nominal mean polymer lengths, so the minimum polyP polymer lengths required to modulate blood clotting
were not known. This is important because polyP secreted by platelets (60mers to 100mers)\textsuperscript{2} are considerably shorter than the polyP found in infectious microorganisms (frequently in excess of 1000 phosphate units long).\textsuperscript{1} We therefore tested the hypothesis that very long polyP polymers (500mers to 1500mers) exert differential effects on blood clotting compared to the much shorter polyP polymers that are secreted by platelets. In order to test this hypothesis we isolated polyP preparations with narrow size distributions.

**Size-fractionation of polyP and determination of polymer length**

PolyP was size-fractionated by preparative PAGE, and the polymer sizes of selected fractions were determined by NMR (an example is in Figure 2.1). We also used NMR analyses to determine the mean polymer lengths for polyP type 65 (67 phosphates) and type 75+ (158 phosphates). Figure 2.2 shows the size distributions of several commercial, heterodisperse polyP preparations, compared to narrowly size-fractionated polyP preparations used in this study. We also prepared heterodisperse polyP with polymer sizes \(\geq 400\)mer (polyP\textsubscript{400+}; Fig. 2.2A), and \(\geq 1000\)mer (polyP\textsubscript{1000+}; Fig. 2.2B). PolyP purified from \textit{S. Typhimurium} was very heterodisperse, with polymers ranging in size from about 100mers to about 1300mers (Fig. 2.2B) while platelet-derived polyP was fairly homogeneous in size (Fig 2.2C) and comparable to the size range reported previously.\textsuperscript{2,3}

**Very long polyP polymers are most efficient at initiating clotting via the contact pathway**

Using polyP preparations of narrowly defined polymer size, we found that polyP from monophosphate to 30mers did not shorten the clotting time of recalcified plasma, when tested at 20 \(\mu\)M polyP (data not shown). 53mers detectably accelerated plasma clotting, with clotting
times decreasing as polyP polymer length increased. In order to understand the relative potencies of these different polyP preparations, we converted their clotting times into specific activities based on “kaolin equivalents.” This was accomplished by determining the concentration, on a mass basis, of kaolin that would yield the same clotting time observed with a given concentration of polyP (data not shown). The results (Figure 2.3A) show that polyP specific activities increased as polymer length increased. Thus, polyP purified from human platelets (size range, approximately 60-100mer) had a specific activity in this clotting assay that was, on a mass basis, 4.9 ± 0.9 fold higher than kaolin. Note that polyP purified from human platelets had essentially the same specific activity as synthetic polyP of the same size (Figure 2.3A). Bacterial polyP had a very high specific activity, while the strongest procoagulant activity in this assay was exhibited by polyP_{1000+}, which had a specific activity >3000-fold higher than that of kaolin.

The procoagulant activity of polyP in this clotting assay was dependent on the contact pathway, since FXII-deficient plasma did not clot (all clotting times were >1000 seconds with 20 μM polyP; not shown).

Adding an excess of short-chain polyP of a variety of sizes inhibited plasma clotting initiated by long-chain polyP (Figure 2.3B). On the other hand, a similarly large excess of very short phosphates (monophosphate, PP_i and triphosphate) had no effect on polyP-initiated clotting via the contact pathway (Figure 2.3B).

**Shorter polyP polymers accelerate FXa-initiated clotting**

We previously reported that polyP type 75+ shortened plasma clotting times initiated by either tissue factor or FXa, an effect that we demonstrated was due to accelerating FV activation. In
the present study, we evaluated this aspect of polyP’s procoagulant effect using polyP polymers of defined lengths in clotting assays initiated by FXa. Short polymers did not appreciably shorten FXa-initiated plasma clotting times, but longer polyP polymers (>60mers) detectably accelerated plasma clotting (data not shown). We converted these clotting times into specific activity (FVa equivalents, on a molar basis) by reference to a standard curve in which plasma was spiked with varying FVa concentrations (standard curve not shown). The results (Figure 2.4) show that maximal specific activities in this clotting assay were achieved with polyP polymers that were about 125 to 200 phosphates long, while progressively longer polymers (especially ≥400mers, including bacterial polyP) had somewhat reduced specific activities compared to 125mers. A large excess of monophosphate, PPi, or triphosphate did not antagonize the procoagulant activity of longer-chain polyP (not shown). PolyP shortened FXa-initiated clotting times equally well in normal and FXII-deficient plasma (Figure 2.4B), indicating that this effect of polyP is independent of the contact pathway of blood clotting.

**Shorter polyP polymers abrogate TFPI anticoagulant activity**

We previously reported that polyP abrogated the anticoagulant function of TFPI, which was largely attributed to the ability of polyP to accelerate FV activation;\(^4\) FVa is known to protect FXa from TFPI inhibition,\(^{21}\) which we previously confirmed.\(^4\) When we tested the ability of polyP preparations of defined polymer lengths to abrogate TFPI anticoagulant activity, we found that polymers as short as 11mers detectably accelerated clotting in the presence of TFPI, while longer polymers were more potent in antagonizing TFPI anticoagulant function (data not shown). Maximal abrogation of TFPI anticoagulant activity was observed with polyP preparations in the size range of ~70mers to ~250mers (Figure 2.5). Platelet-derived polyP exhibited an intermediate
potency in antagonizing TFPI function, while bacterial polyP and polyP type 65 were very potent in this assay. Adding a large excess of monophosphate, PP$_i$, or triphosphate did not reverse the inhibition of TFPI anticoagulant activity by longer-chain polyP (data not shown).

**Longer polyP polymers enhance fibrin clot turbidity**

We recently described an additional procoagulant effect of polyP downstream of thrombin generation: when polyP was added to clotting reactions consisting of purified fibrinogen, thrombin and calcium ions, the resulting fibrin fibrils had markedly increased diameters.$^5$ Our previous study was performed with a heterodisperse polyP preparation (type 75+), so here we tested the ability of polyP preparations of defined polymer lengths to enhance fibrin clot structure, assessed by increased clot turbidity (Figure 2.6A). Shorter polymers (<100mers) did not influence fibrin clot turbidity, while progressively longer polymers increased clot turbidity, with ≥250mers supporting maximal turbidity increases. Bacterial polyP promoted similarly large increases in fibrin turbidity (Figure 2.6A). Notably, the magnitude of the increase in fibrin clot turbidity elicited by long-chain polyP (700mers and polyP$_{400+}$) was roughly double that elicited by polyP type 65 or 75+ (Figures 2.6A and 2.6B).

We investigated the ability of small phosphate-containing molecules to abrogate the enhancement of fibrin clot turbidity by long-chain polyP. Neither ADP nor ATP diminished the turbidity of fibrin clots formed in the presence of polyP$_{400+}$, but monophosphate, PP$_i$ and triphosphate — to varying extents — inhibited the ability of polyP$_{400+}$ to enhance fibrin clot turbidity (Figure 2.6C). In particular, PP$_i$ potently blocked the polyP-mediated enhancement of fibrin clot turbidity, with half-maximal inhibition at 58 µM and complete abrogation at 250 µM PP$_i$. Monophosphate and triphosphate were far less potent than PP$_i$; even at 1 mM they
decreased the polyP-mediated enhancement of fibrin clot turbidity by only 65% (Figure 2.6C). PPi had no measurable effect on fibrin clot turbidity in the absence of polyP (not shown). PolyP polymers from 23mers to 73mers (tested at 150 µM) did not detectably diminish the enhancement of fibrin clot turbidity induced by 100 µM polyP400+ (Figure 2.6D).

**DISCUSSION**

We previously reported that heterodisperse polyP preparations modulate the human blood clotting system by initiating the contact pathway of blood clotting,\(^3,^4\) accelerating the activation of FV by thrombin and FXa,\(^4\) and enhancing the thickness of fibrin fibers.\(^5\) Here we demonstrate that polyP polymers of different lengths exert differential effects on blood coagulation. In a separate study, we recently showed that polyP purified from human platelets triggered plasma clotting via the contact pathway, promoted activation of FXII and prekallikrein in plasma, promoted bradykinin formation in plasma, caused bradykinin-mediated edema when injected subcutaneously in mice, and triggered thrombosis when injected intravenously in mice.\(^3\) That study showed that FXII-deficient mice were protected from the lethal effects of injected polyP, and also that phosphatase treatment of activated platelets (to hydrolyze released polyP) abrogated their ability to trigger clotting via the contact pathway.\(^3\) In the present study, we found that polyP polymers of the sizes secreted by platelets (60mers to 100mers) initiated clotting via the contact pathway with a potency, on a mass basis, that was about fivefold greater than that of kaolin, a potent trigger of the contact pathway of blood clotting. Thus, the results in the present study are fully consistent with our previous demonstration that polyP secreted from platelets exerts procoagulant and proinflammatory activity *in vivo*.\(^3\) On the other hand, longer polyP polymers, including those isolated from bacteria, are even more potent at activating the contact pathway of
blood clotting — and in fact, these long polyP polymers are, on a mass basis, thousands of times more potent than kaolin in triggering blood clotting.

We now report that short polyP polymers antagonized the ability of very long polyP polymers to trigger blood clotting via the contact pathway. Long polyP polymers likely act as templates to assemble the multiple proteins necessary to fully activate the contact pathway, and in fact we recently showed that polyP binds to FXII, high MW kininogen, FXI and prekallikrein (with the tightest binding to FXII and high MW kininogen).\textsuperscript{3} We speculate that shorter polyP polymers also bind to these individual clotting proteins but are not long enough to bind multiple proteins simultaneously, thereby acting as competitors that keep these proteins from assembling effectively together on polymers long enough to promote the mutual activation of FXII and prekallikrein. Notably, polyP isolated from human platelets spans a narrow size range, and in particular, platelet polyP lacks polymers shorter than 60mers which could inhibit the activation of the contact pathway.\textsuperscript{2,3} On the other hand, commercial polyP preparations are typically very heterodisperse, with polyP type 65 and type 75+ containing significant quantities of very short polyP polymers. This may explain a recent report that polyP type 65 (from the same commercial source as in this study) was very effective at promoting FXII autoactivation in plasma, but much less effective at promoting FXI or prekallikrein activation.\textsuperscript{22} Enzymes of the contact pathway participate in a variety of (patho)physiologic processes other than triggering blood clotting, including blood pressure regulation as well as modulating fibrinolysis, angiogenesis and apoptosis; in fact, this system has been described as the plasma kallikrein-kinin system, since prekallikrein can be activated on cell surfaces independent of FXII.\textsuperscript{23} It is possible that shorter polyP polymers may promote activation of some of the enzymes of the contact pathway even when this does not lead to actual clot formation, an idea that we are investigating further.
In this study we showed that bacterial polyP was very potent at triggering the contact pathway of blood clotting. Damaged microorganisms might release intracellular polyP, and organisms such as *Neisseria meningitidis* and *Neisseria gonorrhoeae* express large amounts of long-chain polyP on the exterior of their cells. We previously showed that exposure of long-chain bacterial polyP to plasma also leads to elaboration of inflammatory mediators like bradykinin. We therefore propose that the response of the clotting system to very long chain polyP may be a part of the host response to pathogens.

Long-chain polyP may also come from endogenous sources in mammals; many tissues contain polyP with a size range of 50mers to 800mers, while brain contains primarily long chain polyP (approximately 800mers). Release of long-chain polyP molecules from intracellular compartments in association with ischemia-triggered cell injury might play a role in contact activation and subsequent thrombus formation, as well as contact pathway-mediated inflammatory responses. In mouse models of cerebral artery ischemia-reperfusion, deficiency of contact pathway clotting factors was neuroprotective.

PolyP enhances the turbidity of fibrin clots by causing the formation of thicker fibrin fibrils that appear to incorporate polyP into the clot. In the present study, we found that polyP polymers shorter than 100mers had no measurable effect on fibrin clot turbidity, suggesting that polyP of the size secreted by platelets will not modulate fibrin clot structure. On the other hand, polyP polymers longer than 250mers maximally enhanced fibrin clot turbidity, as did bacterial polyP. Interestingly, we found that PP₃ potently inhibited the enhancement of fibrin clot turbidity by long-chain polyP. Platelet dense granules contain abundant PP₃ (approximately 1.5 μmol PP₃ per 10¹¹ platelets), which is secreted in response to platelet agonists. PP₃ released from platelets could readily attain a concentration of 2-7 μM in whole blood, with orders of
magnitude higher concentrations possible within platelet-rich thrombi. A clear role for platelet-released PP$_i$ has not been previously defined, but we propose that PP$_i$ is a novel modulator of fibrin clot structure. Further studies of the interactions between PP$_i$ and fibrin(ogen), and the ability of PP$_i$ to block the effects of polyP on fibrin structure, are clearly warranted.

PolyP may have potential as a procoagulant agent to control bleeding. We previously reported that heterodisperse polyP type 75+ could reverse the anticoagulant effect of a number of anticoagulant drugs, as well as shorten the clotting times of hemophilic plasmas and plasmas from patients on coumadin therapy.$^6$ On the other hand, injection of heterodisperse polyP preparations intravenously in mice can elicit generalized activation of the contact pathway, resulting in release of vasoactive substances and inflammatory mediators leading to hypotension and edema.$^3$ The present study showed that short polyP polymers had limited ability to activate the contact pathway, but retained ability to accelerate FV activation and abrogate the anticoagulant activity of TFPI. It would therefore be interesting to investigate if suitably size-fractionated polyP preparations could be used pharmacologically to control bleeding while limiting the unwanted side effect of systemic activation of the contact pathway.
FIGURES

Figure 2.1. One-dimensional $^{31}$P NMR spectrum of 34mer polyP. Peaks corresponding to the two external phosphates ($\alpha$), phosphates adjacent to the external phosphates ($\beta$), and internal phosphates, as indicated.

Figure 2.2. Size distributions of polyP preparations used in this study. PolyP was resolved by electrophoresis on 5% polyacrylamide gels with 7 M urea (A, B) or on 15% polyacrylamide gels with 7 M urea (C) and detected by DAPI negative staining; or resolved on 15% polyacrylamide gels with 7 M urea and detected by toluidine blue staining (D). Lanes containing size-fractionated polyP preparations are labeled by estimated polymer length, while the heterodisperse polyP preparations are labeled polyP type 25, type 45, type 65, type 75+, polyP$_{400+}$, polyP$_{1000+}$, “Platelet” (human platelet-derived polyP), “Bact” (S. Typhimurium polyP),
and “Start” (the solubilized “insoluble” polyP preparation that was used as the starting material for obtaining very long-chain polyP). Note that polyP types 25, 45, 65, and 75+ refer to the nominal mean polymer lengths (given by the supplier), while the rest of the polyP sizes indicated in this figure were determined either by NMR analyses, or were estimated from the sample’s migration on analytical PAGE using polyP standards whose mean polymer lengths were determined by NMR (analyses not shown).

Figure 2.3. **Initiation of clotting via the contact pathway is most efficient with very long polyP polymers.** (A) Clotting was initiated by preincubating 5 μM polyP with citrated plasma for 3 minutes at 37ºC, after which calcium chloride was added and the time to clot formation recorded. PolyP specific activities were calculated by comparing polyP-initiated clotting times to
a standard curve in which clotting was initiated by varying kaolin concentrations (not shown), yielding “kaolin equivalents” on a mass basis. Activities of sized-fractionated polyP preparations (▼) are compared to platelet-derived polyP (△; also indicated with arrows), bacterial-derived polyP (“Bact”), polyP type 65, and PolyP_{1000+}. The inset focuses on polymers shorter than 200mer. The point for platelet polyP was plotted at its mean polymer length (80mer). (B) Inhibition of contact pathway-initiated clotting by an excess of small polyP polymers. Plasmas were preincubated for 3 minutes 37°C with a combination of 10 µM polyP_{1000+} and the indicated concentrations of shorter phosphate/polyP preparations (identified as “inhibitor” on the x axis), after which calcium chloride was added to allow clotting. The short phosphates were: monophosphate (▽), PP_i (▲), and triphosphate (□) (none of which had any effect); and 11mer polyP (▼), 16mer polyP (○), 35mer polyP (■), and 53mer polyP (△), and 83mer (●) (which prolonged the clotting times in a concentration-dependent manner). The dotted line represents the clotting time in the absence of polyP. Data in all panels are mean ± standard error (n = 3).
Figure 2.4. FXa-initiated clotting is accelerated by shorter polyP polymers than are required for initiating clotting via the contact pathway. PolyP (5 μM) was added simultaneously to normal plasma together with 333 pM FXa and calcium chloride, and time to clot formation was measured. (A) PolyP specific activities were calculated by comparing polyP clot times to a standard curve in the absence of polyP, but in which varying FVa concentrations were added to plasma (not shown). The FVa concentration (in nM FVa) that yielded the same clotting time as a given polyP preparation was then divided by the polyP concentration (5000 nM). SIZED-fractionated polyP preparations (▼) are compared to bacterial-derived polyP (“Bact”) and polyP type 65. The inset focuses on polymers shorter than 200mer. (B) Shortening of the FXa clotting time by polyP is independent of FXII. PolyP preparations of the indicated
polymer sizes (all at 20 μM phosphate) were added to FXa-initiated clotting reactions conducted with either pooled normal plasma (white bars) or FXII-deficient plasma (black bars). Clotting times with polyP were normalized to the clotting time of the respective plasma without polyP. Data in all panels are mean ± standard error (n = 3).

Figure 2.5. Accelerating FXa-initiated clotting requires even shorter polyP polymers in the presence of added TFPI. Clotting reactions were conducted as in Figure 2.4A except that plasma contained 400 ng/mL TFPI, clotting was initiated with 2 nM FXa and polyP, when included, was at 20 μM. Percent TFPI inhibition was calculated by comparing polyP clot times to a standard curve in the absence of polyP, but in which 0 to 400 ng/mL TFPI had been added to plasma (not shown). Sized-fractionated polyP preparations (▼) are compared to platelet-derived polyP (“Platelet”, at 150 μM), bacterial-derived polyP (“Bact”), and polyP type 65. The inset focuses on polymers shorter than 200mer. Data are mean ± standard error (n = 3).
Figure 2.6. Optimal enhancement of fibrin clot turbidity requires relatively long polyP polymers. Fibrin clots were prepared by adding thrombin to fibrinogen that had been preincubated with Ca$^{2+}$ and polyP, and clot turbidity ($A_{405}$) was quantified 60 minutes after thrombin addition. (A) Clot turbidity as a function of polyP polymer size (all at 150 $\mu$M phosphate). The value for $x = 0$ is in the absence of added polyP, while “Bact” indicates S. Typhimurium-derived polyP. The inset focuses on polyP preparations shorter than 180mer. (B) Clot turbidity as a function of polyP concentration, using the following polyP polymer lengths: 65mer ($\triangle$), 158mer ($\blacksquare$), 700mer ($\bigcirc$), polyP type 65 ($\bullet$), polyP type 75+ ($\square$), and polyP$_{400+}$ ($\blacktriangle$). (C) PP$_i$ abrogates the ability of polyP to enhance clot turbidity. Fibrinogen was preincubated with calcium ions, 150 $\mu$M polyP$_{400+}$ and the indicated concentrations of phosphate-containing substances, after which thrombin was added. The substances tested were: monophosphate ($\nabla$), PP$_i$ ($\blacktriangle$), triphosphate ($\square$), ADP ($\bigcirc$), and ATP ($\bullet$). The dotted line
represents clot turbidity in the absence of polyP. (D) PP$i$, but not other polyP polymers, abrogates the ability of polyP$_{400+}$ to enhance clot turbidity. Phosphate polymers of the indicated lengths (all at 150 μM phosphate) were included in fibrin clotting reactions as performed in panel C. The open bar with the asterisk represents clot turbidity in the absence of polyP, while the open bar with “0” represents clot turbidity with polyP$_{400+}$ but no added small phosphate polymer.

REFERENCES


CHAPTER 3: PHOSPHORAMIDATE END LABELING OF POLYPHOSPHATES

ABSTRACT

Polyphosphates, linear polymers of inorganic phosphates linked by phosphoanhydride bonds, are widely present among organisms and play diverse roles in biology, including functioning as potent natural modulators of the human blood clotting system. However, studies of protein-polyphosphate interactions are hampered by a dearth of methods for derivatizing polyphosphate or immobilizing it onto solid supports. We now report that EDAC (1-ethyl-3-[3-dimetylamlino-propyl]carbodiimide) efficiently promotes the covalent attachment of a variety of primary amine-containing labels and probes to the terminal phosphates of polyphosphates via stable phosphoramidate linkages. Using $^{31}$P NMR, we confirmed that EDAC-mediated reactions between primary amines and polyphosphate results in phosphoramidate linkages with the terminal phosphate groups. We show that polyphosphate can be biotinylated, labeled with fluorophores and immobilized onto solid supports; that immobilized polyphosphate can be readily used to quantify protein binding affinities; that covalently derivatized or immobilized polyphosphate retains its ability to trigger blood clotting; and that derivatizing the ends of polyphosphate with spermidine protects it from exopolyphosphatase degradation. Our findings open up essentially the entire armamentarium of protein chemistry to modifying polyphosphate, which should greatly facilitate studies of its biological roles.

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INTRODUCTION

Inorganic polyphosphate (polyP), a linear polymer of orthophosphate residues linked via phosphoanhydride bonds, is widely distributed in biology and plays important and diverse roles in nature.\textsuperscript{1,2} We recently showed that polyP is a potent modulator of the blood clotting cascade,\textsuperscript{3-5} and an expanding body of research is investigating its roles in other biological systems.\textsuperscript{6-11} Many technical obstacles remain, for investigating the biological roles of polyP and there is a real need for improved microscale methods for analyzing polyP. In particular, there is a dearth of approaches for covalently modifying polyP or attaching it to solid supports. One of the few published methods for immobilizing polyP onto surfaces is via Lewis acid/base interactions between polyP and zirconia beads.\textsuperscript{12} Although we have successfully used this method,\textsuperscript{13} it suffers from relatively high nonspecific binding of proteins to zirconia. Furthermore, this chemistry is not readily adaptable for attaching labels to polyP, or to immobilizing polyP onto the sorts of solid supports routinely used in analyses of protein interactions. The goal of the present study was therefore to develop conditions for routine covalent attachment of labels to the terminal phosphates of polyP.

The zero-length cross-linking reagent, EDAC (1-ethyl-3-[3-dimethylamino-propyl]carbodiimide), is widely used to couple primary amines to carboxylic acids via amide linkages. However, EDAC can also be used to couple primary amines to organic phosphates—including the 5’ phosphates of oligonucleotides—via stable phosphoramidate linkages.\textsuperscript{14} We now report that the terminal phosphates of polyP can be made to enter into covalent phosphoramidate linkages with primary amine-containing compounds via EDAC (Scheme 3.1). This finding essentially opens up the entire armamentarium of protein chemistry to modifying polyP, greatly facilitating investigations into polyP’s biological activities. In the present study, we
demonstrate conditions under which polyP can be biotinylated, labeled with fluorophores, and immobilized onto solid supports. We use the latter to quantify the binding affinities of three blood clotting proteins for polyP, and to demonstrate that covalently immobilized polyP retains its ability to trigger blood clotting. Furthermore, we also show that derivatizing the ends of polyP via phosphoramidate linkages protects it from exopolyphosphatase degradation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Amine Surface and Carbo-BIND (hydrazide) multiwell strips were from Corning (Corning, NY); Nunc Immobilizer Streptavidin multiwell strips and Covalink-NH plates were from Thermo-Fisher (Waltham, MA); amine-PEG₂-biotin was from Pierce (Rockford, IL, USA); polyethylenimine, spermidine, streptavidin, benzamidine and EDAC were from Sigma-Aldrich (St. Louis, MO); Cascade Blue-ethylenediamine was from Invitrogen (Carlsbad, CA); factor Xla, kallikrein, and thrombin were from Enzyme Research Laboratories (South Bend, IN); calf intestinal alkaline phosphatase was from Promega (Madison, WI); phospholipids were from Avanti Polar Lipids (Alabaster, AL); Biacore CM5 sensorchips were from GE Healthcare (Piscataway, NJ); chromogenic substrates S-2366 and S-2322 were from diaPharma (West Chester, OH); recombinant factor VIIa, and substrates Spectrozyme TH and Spectrozyme fVIIa were from American Diagnostica (Stamford, CT); and Sepabeads EC-HA were kindly provided by Resindion SRL (Milan, Italy). PolyP₅, polyP₂₅ and polyP₄₅ (nominal mean polymer lengths, 5, 25 and 45, respectively, marketed as “sodium phosphate glass, types 5, 25 and 45”), and a heterodisperse preparation of very high MW polyP (marketed as “phosphate glass, water
insoluble”) were from Sigma-Aldrich, as were sodium monophosphate, pyrophosphate, and triphosphate. A water-soluble fraction of relatively high MW polyP (here termed polyP$_{\text{HMW}}$) was obtained from “water insoluble phosphate glass” by stirring it in 250 mM LiCl and processing as described. PolyP$_{14}$, polyP$_{60}$ and polyP$_{130}$ (polymer lengths, 14, 60 and 130, respectively) were kindly provided by Regenetiss, Inc. (Tokyo, Japan). PolyP concentrations are given throughout this paper in terms of phosphate monomer (monomer formula: NaPO$_3$).

### Immobilization of polyP onto polystyrene microplate wells and coagulometer cuvettes

A variety of reaction conditions were tested in order to optimize EDAC-mediated covalent coupling of polyP$_{\text{HMW}}$ to primary amines displayed on Amine Surface stripwells. Parameters varied included the concentrations of EDAC, polyP, divalent metal ions and 2-(N-morpholino)ethanesulfonic acid (MES); pH; coupling time; and the presence or absence of 0.1 M imidazole. Optimal coupling conditions for immobilizing polyP on Amine Surface stripwells were to treat each well at 37°C for 3 h to overnight with 200 μl of a freshly-made solution of 10 to 100 μM polyP$_{\text{HMW}}$ in 25 mM EDAC and 77 mM MES pH 6.5. Unreacted polyP was then removed by two 10 min washes with 2 M LiCl followed by two 5 min water washes. When desired, immobilized polyP was quantified following hydrolysis in 1 M HCl at 100°C by malachite green assay. Briefly, 50 μL hydrolyzed phosphate sample was mixed with 100 μL malachite green reagent (0.1% malachite green, 4.2% ammonium molybdate, 4 M HCl) in Corning polypropylene multiwell plates and incubated for 20 min at room temperature, after which $A_{660}$ was measured and phosphate concentrations determined by reference to a standard curve.
Optimal conditions for immobilizing polyP_{HMW} onto polystyrene coagulometer cuvettes were to treat each well overnight at 37°C with 200 µl of 400 ng/mL polyethylenimine in 0.1 M carbonate buffer pH 9.2, wash 5 times in purified water, then incubate each well for 4 h with 200 µl of a freshly made solution of 245 µM polyP_{HMW} in 50 mM EDAC, 1 mM CaCl$_2$, and 77 mM MES pH 6.5. Wells were washed twice with 2 M LiCl, then twice with water.

Covalent coupling of biotin or fluorophores to polyP

For biotinylation of polyP, typical conditions were to incubate 10 mM polyP$_{HMW}$ overnight at 37°C with 0.5 mM amine-PEG$_2$-biotin, 100 mM EDAC, and 100 mM MES pH 6.5. For fluorescent labeling of polyP, typical reaction conditions were as for biotinylation except that 1 mM Cascade Blue-ethylenediamine replaced biotin and 1 mM CaCl$_2$ was added. Biotin-polyP and Cascade Blue-polyP adducts were purified by size-exclusion chromatography. PolyP and Cascade Blue-polyP preparations were resolved by polyacrylamide gel electrophoresis using 10% polyacrylamide gels in TBE (90 mM Tris, 90 mM borate, 2.7 mM EDTA, pH 8.3) and detected either by fluorescence (excitation at 365 nm) or by staining with toluidine blue as described.$^{15}$

**Binding of thrombin, kallikrein, factor Xla or factor VIIa to microplate-immobilized polyP**

PolyP$_{HMW}$ was immobilized on Amine Surface stripwells using EDAC-mediated coupling as described above. Alternatively, biotin-polyP$_{HMW}$ was immobilized by incubating 67 µM biotin-polyP$_{HMW}$ overnight at 4°C in streptavidin stripwells. Following washing, wells were blocked for 3 h with 50 mM Tris-HCl pH 7.4, 0.05% Tween-20 (Tris-Tween) plus 5% bovine serum albumin. Wells were then incubated with various concentrations of factor Xla, kallikrein,
thrombin or factor VIIa in Tris-Tween plus 0.6% bovine serum albumin, after which the wells were washed thrice with Tris-Tween. (In the case of factor VIIa, all solutions also contained 2.5 mM CaCl$_2$.) Bound factor XIa, kallikrein, thrombin or factor VIIa were detected by quantifying initial rates of hydrolysis of S-2366, S-2322, Spectrozyme TH or Spectrozyme fVIIa, respectively, and the single-site ligand binding equation was fitted to the data by nonlinear regression using Prism (GraphPad Software, La Jolla, CA).

**Clotting Assays**

Clotting times were quantified at 37°C on a Diagnostica Stago STart4 coagulometer by mixing, in coagulometer cuvettes, 50 µl prewarmed citrated human plasma (George King Biomedical, Overland Park, KS) with 50 µl prewarmed 20% phosphatidylserine/80% phosphatidylcholine vesicles (made by sonication) in imidazole buffer; incubating for 3 minutes; then initiating clotting by adding 50 µl prewarmed CaCl$_2$. Final concentrations were 33% plasma, 25 µM phospholipid, 41.7 mM imidazole pH 7.0, 8.33 mM CaCl$_2$ in 150 µl.

**NMR Analyses**

$^{31}$P NMR spectra of polyP preparations were acquired at 20°C as previously described,$^5$ with a Varian Unity INOVA 600 spectrometer using a 5 mm Varian AutoTuneX $^1$H/X PFG Z probe, 13.5 µs (90°) pulse excitation, 16 kHz spectral width, and 5 second recycle time. Chemical shifts were referenced to 0 ppm using an external phosphoric acid standard. Spectra were processed using 10 Hz line broadening.
**Immobilization of PolyP onto Polymethacrylate Beads**

PolyP was immobilized on primary amine-containing polymethacrylate beads (Sepabeads EC-HA) by gentle agitation of 100 mg (dry weight) of beads overnight at 37°C with 25 mM polyP\textsubscript{HMW} (or varying concentrations of other polyP polymer sizes) in 100 mM MES pH 6.5, 100 mM EDAC, and 1 mM CaCl\textsubscript{2}, then washing with a solution of 2 M LiCl and 10 mM EDTA followed by water. Immobilized polyP was quantified by malachite green assay following hydrolysis in 1 M HCl at 100°C.\textsuperscript{5} The typical yield of bound polyP\textsubscript{HMW} was 11 µg polyP per mg dry weight of Sepabeads.

For binding assays, polyP\textsubscript{HMW}-Sepabeads were blocked with 10% bovine serum albumin overnight at 4°C, washed twice with binding buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.1% bovine serum albumin), and incubated at room temperature for 30 min with thrombin, factor XIa, or kallikrein in binding buffer. The supernatants were collected by centrifugation using mini spin columns (Pierce), and beads were washed with binding buffer followed by elution buffer (50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.1% bovine serum albumin). Enzymes were quantified by measuring initial rates of chromogenic substrate hydrolysis as described above.

**Surface Plasmon Resonance (SPR) studies**

SPR analyses were conducted at 25°C using a Biacore 3000 instrument (Biacore, Columbia, MD). Streptavidin was covalently bound to CM5 sensorchips by the standard amine coupling method; after blocking and washing, biotin-polyP\textsubscript{HMW} was flowed over the surface until the signal reached 400 resonance units (RUs). Varying concentrations of thrombin in 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM benzamidine, 0.005% surfactant P20 were then flowed over the
chip surface at 50 μl/min using a 2 min association phase and 3 min dissociation phase, with background subtraction using a reference cell without polyP. Sensorchips were regenerated by washing with 1 M NaCl between runs.

**Preparation and Digestion of Spermidine-PolyP Adducts**

5 mM polyP_{130} was incubated for 6 h at 37°C with 70 mM spermidine, 100 mM MES pH 6.5, 300 mM EDAC, after which polyP was purified by size-exclusion chromatography in the presence of 1 M LiCl followed by acetone precipitation as previously described.\(^5\) To examine resistance to exopolyphosphatase digestion, 12 μM spermidine-polyP adduct was digested at 37°C with 5 U/ml calf intestinal alkaline phosphatase in 50 mM Tris-HCl pH 7.4, 1 mM MgCl\(_2\), 0.1 mM ZnCl\(_2\). Timed samples were removed and free monophosphate was quantified by malachite green assay.\(^5\) At the end of the experiment, an aliquot of the reaction was hydrolyzed for 1 h at 100°C in 1 M HCl and monophosphate was quantified.

**Abbreviations:** EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; MES, 2-(N-morpholino)ethanesulfonic acid; polyP, polyphosphate; polyP\(_{HMW}\), high molecular weight polyphosphate, SPR, surface plasmon resonance; RU, resonance units

**RESULTS**

**Covalent immobilization of polyP onto amine-derivatized polystyrene microplates and chromatography beads**

To optimize the reaction conditions for EDAC-mediated formation of phosphoramidate linkages between primary amines and the terminal phosphates of polyP, we found it convenient to employ
Amine Surface microplates as the source of primary amine. The degree of immobilization of polyP onto this surface was then used as the readout for optimizing conditions. (We found that noncovalently bound polyP was quantitatively removed from these plates by washing the wells with 2 M LiCl.) Figure 3.1A shows the results of a typical optimization study, in this case to optimize the polyP concentration. We obtained substantial covalent attachment of polyP to the amine-derivatized polystyrene surface when reactions were carried out in the presence EDAC (but not in its absence), with maximal coupling at \( \geq 2 \, \mu g/ml \) polyP. At 1 and 2 \( \mu g/ml \) polyP, the efficiency of coupling to the surface was 49\% and 27\%, respectively. When polyP was reacted with EDAC in secondary amine-modified (Covalink NH) or hydrazide-modified (Carbo-Bind) microplates under the same conditions, little or no bound polyP was detected over background (data not shown), suggesting that this reaction is much more efficient with primary amines.

Additional studies were undertaken to optimize the reaction conditions for covalently linking polyP to primary amines on Amine Surface microplates (data not shown, but the findings summarized in this paragraph): Optimal polyP immobilization was obtained when the EDAC concentration was 25 to 300 mM, when the pH was 6 to 7 (using 25 to 100 mM MES buffer), and when the reaction was allowed to proceed for 2 h to overnight. We also found that inclusion of 1 mM Ca\(^{2+}\), Mg\(^{2+}\), or Mn\(^{2+}\) increased the immobilization of polyP by about 1.5- to 2-fold relative to reactions in the absence of divalent metal ions.

EDAC-mediated formation of phosphoramidate linkages between primary amines and the 5’ phosphates of oligonucleotides is reported to be more efficient in the presence of imidazole, due to the formation of reactive phosphorimidazolide intermediates.\(^ {14} \) We found, however, that the efficiency of EDAC-mediated immobilization of polyP onto Amine Surface microplates was unaffected by the presence of up to 100 mM imidazole (not shown).
We also investigated the effect of polyP polymer length on efficiency of EDAC-mediated coupling to primary amines, using amine-containing polymethacrylate chromatography beads (Sepabeads EC-HA). To do this, we coupled polyP preparations of varying polymer lengths (holding the concentration of ends at a constant 1 mM) to the beads, then quantified the extent of covalent attachment of polyP. The results (Figure 3.1B) show that pentaphosphates and shorter coupled poorly to the beads, while 14mers and longer coupled relatively efficiently. Even at 50 mM, monophosphate still coupled inefficiently to the beads, demonstrating that the terminal phosphates of polyP are much more efficiently coupled to amines by EDAC than are small inorganic phosphates.

**31P NMR spectroscopy of a polyP-spermidine adduct**

NMR was used to obtain evidence for phosphoramidate linkages with the terminal phosphates of polyP. Figure 3.2 shows representative $^{31}$P NMR spectra of underivatized polyP$_{45}$ and of spermidine-labeled polyP$_{45}$. For underivatized polyP, the $^{31}$P signal for the terminal phosphates at approximately -5 ppm (α peak in Figure 3.2A) was well resolved from the much larger peak for internal phosphates at about -21 ppm. (In this particular spectrum, the penultimate phosphate residues—β peak—were also clearly resolved, although this is not always the case). For spermidine-derivatized polyP (Figure 3.2B), the signal at -5 ppm was greatly reduced and a new peak at about -0.5 ppm appeared, which we attribute to the presence of the P-N bond in the phosphoramidate-linked spermidine-polyP adduct.
Binding affinities of blood clotting proteases for immobilized polyP

We previously demonstrated that thrombin binds to polyP with relatively high affinity, via its anion-binding exosite II.\textsuperscript{13} We also showed that polyP is a potent triggering agent for the contact pathway of blood clotting,\textsuperscript{3} and that it binds to prekallikrein and factors XI and XII.\textsuperscript{4} As an example of the utility of immobilized polyP, we used it to quantify the binding of thrombin, factor Xla, kallikrein and factor VIIa to polyP. In Figure 3.3A-D, polyP was immobilized by EDAC-mediated covalent coupling to amine-derivatized polystyrene microplate wells. This was successfully used to quantify the binding affinities of thrombin, factor Xla and kallikrein for polyP, yielding $K_d$ values of 66, 32 and 92 nM, respectively. Factor VIIa, on the other hand, did not bind to immobilized polyP (Figure 3.3D). Alternatively, biotinylated polyP was immobilized via capture on streptavidin-coated microplate wells, and this presentation of polyP was also used to quantify thrombin binding. It yielded a $K_d$ value of 56 nM (Figure 3.3E), very similar to that obtained when polyP was covalently linked to amine-derivatized wells (Figure 3.3A).

In another experiment, thrombin, factor Xla, and kallikrein were incubated with polyP-derivatized, primary amine-containing chromatography beads, after which recovery of the enzyme was quantified in the flow-through and high-salt eluates (Figure 3.3F). These proteins bound quantitatively to polyP-derivatized beads and were eluted quantitatively by high salt concentration. There was negligible background binding to beads that had been treated with polyP in the absence of EDAC, or with EDAC in the absence of polyP (not shown). This demonstrates the utility of using polyP-derivatized beads to identify and isolate polyP binding proteins by pull-down assays, etc.

We also performed initial SPR analyses of thrombin binding to polyP by first immobilizing biotin-polyPHMW onto streptavidin sensorchips and then flowing varying
concentrations of thrombin over the surface. The results (Figure 3.4) demonstrate the utility of immobilizing biotin-polyP onto streptavidin-derivatized sensorchips in order to use SPR to study the kinetics of protein-polyP binding interactions.

**Fluorescently Labeled PolyP**

End-labeling of polyP with fluorophores would be highly advantageous for detecting polyP binding to proteins, cells and tissues, and for following polyP *in vivo*. Accordingly, we reacted the primary amine-containing fluorescent dye, Cascade Blue-ethylenediamine, with polyP$_{45}$ in the presence or absence of EDAC, purified the polyP and resolved it by polyacrylamide gel electrophoresis (Figure 3.5). PolyP that had been reacted with Cascade Blue-ethylenediamine in the presence of EDAC was intensely fluorescent (Figure 3.5B, lanes 1 and 2), whereas polyP incubated with the dye but without EDAC had no detectable fluorescence (Figure 3.5B, lane 3).

**Derivatizing the terminal phosphates of polyP confers resistance to exopolyphosphatase digestion**

Some polyP preparations isolated from biological sources are reported to be naturally resistant to exopolyphosphatase degradation, apparently due to an unidentified modification of the terminal phosphates.\(^1\) This prompted us to investigate the possibility that attaching primary amine-containing compounds to the terminal phosphates of polyP via phosphoramidate linkages might protect polyP from exopolyphosphatase degradation. Accordingly, we reacted polyP$_{130}$ with spermidine in the presence of EDAC, isolated the polyP, and then over-digested it with excess calf intestinal alkaline phosphatase (a very active exopolyphosphatase).\(^16\) As can be seen in
Figure 3.6, the polyP-spermidine adduct was highly resistant to phosphatase degradation, while underivatized polyP was rapidly digested to completion.

**Immobilized and derivatized polyP retains procoagulant activity**

We investigated whether immobilizing or end-labeling polyP would interfere with its procoagulant activity. EDAC was employed to covalently react long-chain polyP with polyethylenimine that had been coated onto polystyrene coagulometer cuvettes, after which the cuvettes were employed in plasma clotting assays. Immobilized polyP dramatically shortened the plasma clotting time, demonstrating that it retains significant ability to activate the contact pathway of blood clotting (Figure 3.7A). Similarly, in solution, 20 μM spermidine-labeled polyP was as active in triggering the clotting of human plasma as was 20 μM underivatized polyP (Figure 3.7B).

**DISCUSSION**

Studies of protein-polyP interactions have been hampered by a paucity of methods for derivatizing and immobilizing polyP. Here, we demonstrate that polyP preparations of varying chain lengths can be efficiently derivatized using the water-soluble carbodiimide, EDAC, to create phosphoramidate linkages between the terminal phosphates of polyP and several primary amines. We optimized the reaction conditions and provided NMR evidence for the presence of phosphoramidate linkages with the terminal phosphates of polyP. As examples of the utility of this approach, we quantified $K_d$ values for the binding of polyP to the blood clotting proteases, thrombin, factor XIa and kallikrein. Relatively low nonspecific background was observed using primary amine-containing solid supports, making this a very attractive method for immobilizing
polyP. We also demonstrated the utility of using biotinylated polyP in SPR studies to measure protein binding to polyP.

Carbodiimide-mediated crosslinking of polyP to labels, probes and solid supports should greatly facilitate studies on the ever-expanding role of polyP in important biological processes, including blood clotting. In addition to the examples provided in this study, the ability to covalently couple amine-containing compounds will also allow other types of labeling reactions with polyP, opening up essentially the entire armamentarium of protein chemistry. For example, polyP that has been end-labeled with a polyamine such as ethylenediamine, cadaverine or spermidine will have free primary amino groups available for further reactions, including coupling to succinimidyl ester derivatives of solid supports, biotin, fluorescent dyes or other probes, which are often more readily available commercially than are the same compounds with primary amines. Another example would be to couple a disulfide-containing primary amine such as cystamine to the ends of polyP; following reduction, this will provide free sulfhydryls tethered to the ends of polyP for reaction with maleimide- or iodoacetate-derivatives of biotin, fluorescent dyes or other labels.

We also found that modifying the ends of polyP by covalently attaching spermidine protected polyP from exopolyphosphatase degradation, suggesting that such end-labeled polyP derivatives may be more stable in biological systems. These end-labeled polyP adducts may also be useful in detecting the presence of endo- versus exopolyphosphatase enzyme activities, since the derivatized polyP preparations should be sensitive to digestion by the former but not the latter.

Previously, we demonstrated that soluble polyP can act as a general hemostatic agent, shortening the clotting time of plasma from patients with hemophilia and reversing the effect of several anticoagulant drugs. In this study, we found that covalently attaching amine-containing
compounds to the terminal phosphates of polyP did not interfere with polyP’s procoagulant activity, and polyP retained potent clotting activity when covalently attached to solid supports. This latter finding opens the possibility of covalently immobilizing polyP onto wound dressings, collagen sponges, etc., to create improved topical hemostatic agents to control bleeding.

FIGURES

Scheme 1

[Diagram showing the process of labeling polyP with primary amine and phosphoramidate linkages]
Figure 3.1. Attachment of polyP to solid supports. (A) Dose response for covalently immobilizing polyP onto Amine Surface microplates. Varying polyP_{HMW} concentrations were reacted overnight at 37°C in microplate wells in the absence (○) or presence (●) of 100 mM EDAC, after which unbound polyP was removed by washing with 2 M LiCl. Data are mean ± S.E.M. (n=3). (B) Influence of polymer length on coupling efficiency. PolyP preparations of varying mean polymer lengths were reacted overnight at 37°C with Sepabeads EC-HA and 100 mM EDAC with 1 mM CaCl₂, after which unbound polyP was removed by washing with 2 M LiCl and 10 mM EDTA. PolyP concentrations (given in terms of phosphate) were adjusted to yield a constant 1 mM ends: 1 mM monophosphate and pyrophosphate; 1.5 mM triphosphate; 2.5 mM polyP₅; 7 mM polyP₁₄; 12.5 mM polyP₂₅; 22.5 mM polyP₄₅; 30 mM polyP₆₀; and 65 mM polyP₁₃₀. Controls plotted on the right include 50 mM monophosphate as well as reactions without EDAC. Data are expressed as mean percent recoveries of offered polyP (± S.E.M.; n=3 to 5). In both panels, bound polyP was quantified as monophosphate following acid hydrolysis.
Figure 3.2. One-dimensional $^{31}$P NMR spectra of (A) underivatized polyP$_{45}$ and (B) spermidine-labeled polyP$_{45}$. Peaks corresponding to the external phosphates (α), penultimate phosphates (β), and internal phosphates (internal) are indicated.
Figure 3.3. Binding of clotting proteases to immobilized polyP. (A-C) PolyP_{HMW} was coupled via EDAC to Amine Surface microplate wells, which were then used to quantify the binding of (A) thrombin, (B) factor XIa, (C) kallikrein, or (D) factor VIIa. (E) Biotinylated polyP_{HMW} was immobilized on streptavidin-coated microplate wells, which were then used to quantify thrombin binding. For panels A-E, solid squares are binding data after background subtraction, while open circles are background binding (from wells without polyP); lines represent the single-site ligand binding equation fitted to the binding data, yielding the indicated $K_d$ values. (F) PolyP_{HMW} was coupled via EDAC to primary amine-containing Sepabeads. Thrombin (27 pmol), factor XIa (10 pmol), or kallikrein (10 pmol) were incubated with polyP-Sepabeads, after which the beads were collected and washed by centrifugation. Enzyme recovery was quantified in the flow-through (open bars) and high-salt eluates (closed bars), with recoveries calculated as percent of the starting material. Data are mean ± S.E.M. (n=3).
Figure 3.4. Representative SPR sensorgrams of thrombin binding to polyP. Biotin-polyP$_{HMW}$ was immobilized onto a streptavidin-derivatized sensorchip, after which varying concentrations of thrombin were flowed over the chip surface and RU values were measured using a Biacore 3000 instrument. Thrombin concentrations were 20 nM (lower curve), 60 nM (middle curve), and 120 nM (upper curve).
Figure 3.5. Fluorescently labeled polyP. The primary amine-containing fluorophore, Cascade Blue ethylenediamine, was reacted with polyP$_{45}$ in the presence of EDAC. PolyP was then purified, resolved by polyacrylamide gel electrophoresis, and the gels were either (A) stained with toluidine blue, or (B) photographed under illumination by 365 nm UV light. Reaction conditions were: lane 1, 1 mM fluorophore + EDAC; lane 2, 0.5 mM fluorophore + EDAC; lane 3, 1 mM fluorophore without EDAC.
Figure 3.6. Phosphoramidate derivatization of the terminal phosphates of polyP confers resistance to exopolyphosphatase digestion. PolyP\textsubscript{130} was either untreated (●) or reacted with spermidine in the presence (□) or absence (△) of EDAC, after which the polyP was purified and then digested at 37°C with 5 U/ml alkaline phosphatase. Levels of free monophosphate were quantified in timed samples, and at the end of the experiment, monophosphate was quantified following complete acid hydrolysis. Data are mean ± S.E.M. (n=3), although the error bars are smaller than the data points and therefore not visible.
Figure 3.7. Immobilized and derivatized polyP retains procoagulant activity. (A) PolyP$_{\text{HMW}}$ was immobilized via EDAC onto polyethyleneimine-coated polystyrene coagulometer cuvettes. Clotting was then initiated by incubating human plasma in the wells for 3 min at 37°C, after which CaCl$_2$ was added and the time to clot formation recorded. Control cuvettes included those untreated with polyethyleneimine, EDAC and/or polyP, as indicated. (B) Clotting assays were conducted as in panel A except that untreated cuvettes were employed and 20 μM polyP$_{\text{HMW}}$-spermidine in solution was preincubated with plasma for 3 min at 37°C, after which CaCl$_2$ was added and the time to clot formation recorded. Controls included polyP$_{\text{HMW}}$ that had been reacted without EDAC and/or spermidine, and also wells that received plasma but no polyP, as indicated. Data are mean ± S.E.M. (n=3).
REFERENCES


CHAPTER 4: INHIBITION OF POLYPHOSPHATE AS A NOVEL STRATEGY FOR PREVENTING THROMBOSIS AND INFLAMMATION

ABSTRACT

Inorganic polyphosphates are linear polymers of orthophosphate that modulate blood clotting and inflammation. Polyphosphate accumulates in infectious microorganisms and is secreted by activated platelets; long-chain polyphosphate in particular is an extremely potent initiator of the contact pathway, a limb of the clotting cascade important for thrombosis but dispensable for hemostasis. Polyphosphate inhibitors therefore might act as novel antithrombotic/anti-inflammatory agents with reduced bleeding side effects. Anti-polyphosphate antibodies are unlikely owing to polyphosphate’s ubiquity and simple structure, and while phosphatases such as alkaline phosphatase can digest polyphosphate, they take time and may degrade other biologically active molecules. We now identify a panel of polyphosphate inhibitors, including cationic proteins, polymers and small molecules, and report their effectiveness in vitro and in vivo. We also compare their effectiveness against the procoagulant activity of RNA. Polyphosphate inhibitors were antithrombotic in mouse models of venous and arterial thrombosis, and blocked the inflammatory effect of polyphosphate injected intradermally in mice. This study provides proof of principle for polyphosphate inhibitors as antithrombotic/anti-inflammatory agents in vitro and in vivo, with a novel mode of action compared to conventional anticoagulants.

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INTRODUCTION

Polyphosphate (polyP) is a linear polymer of inorganic phosphate residues that is widely present in biology. Of particular interest to hematology, polyP accumulates in many infectious microorganisms and is secreted by activated human platelets and mast cells. Work from our lab and others has shown that polyP is a potent procoagulant, prothrombotic, and pro-inflammatory molecule, acting at four points in the clotting cascade: It triggers clotting via the contact pathway; it accelerates factor V activation; it enhances fibrin clot structure; and it accelerates factor XI back-activation by thrombin.

The ability of polyP (especially, long-chain polyP of the type found in microorganisms) to trigger clotting via the contact pathway is interesting in light of an elegant series of studies that have shown that the contact pathway is important for thrombosis but dispensable for hemostasis. We therefore hypothesized that polyP inhibitors might act as novel antithrombotic/anti-inflammatory agents with reduced bleeding side effects. Raising antibodies against polyP is unlikely to be successful because of the ubiquity of polyP and its simple structure. Phosphatases such as alkaline phosphatase can digest polyP, but they take time to act and may degrade other phosphate-containing molecules in addition to polyP. In this study, identify a panel of polyP inhibitors including cationic proteins, polymers and small molecules. We report their effectiveness as anticoagulants in vitro and as antithrombotic and anti-inflammatory agents in vivo using mouse models. We also compare the effectiveness of these polyP inhibitors against the procoagulant activity of RNA and the anticoagulant activity of heparin. This study therefore provides proof of principle for polyP inhibitors as novel antithrombotic/anti-inflammatory agents that are directed against a unique target in the blood clotting system.
EXPERIMENTAL PROCEDURES

Materials

Reagents were from Sigma-Aldrich unless otherwise noted. Long-chain synthetic polyP (marketed by Sigma as “phosphate glass, water insoluble”) was differentially solubilized as previously described. Its polymer lengths ranged from about 50 to 1500 phosphates, with a modal length of about 650 phosphates, and its endotoxin content was $1.6 \times 10^{-3}$ units/μg polyP (by Limulus assay; Charles River Laboratories). Biotinylated long-chain polyP was prepared as described. All polyP concentrations in this paper are given in terms of the concentration of phosphate monomers (monomer formula: NaPO$_3$).

Other supplies included human platelet factor 4, antithrombin, plasma kallikrein, factor Xa and α-thrombin (Enzyme Research Laboratories); human factor XI (Haematologic Technologies); pooled normal plasma (George King Bio-Medical); and Sar-Pro-Arg-p-nitroanilide (Bachem). Recombinant polyP-binding domain from E. coli exopolyphosphatase (PPXbd) was produced as described. Liposomes made by sonication had 10% phosphatidylinositol, 40% phosphatidylethanolamine, 50% phosphatidylcholine (Avanti Polar Lipids). Recombinant human tissue factor was relipidated as described.

Inhibition of polyP binding to thrombin

Other than the high-throughput screens, thrombin binding to immobilized biotinylated polyP in streptavidin-coated, 96-well microplates was performed essentially as previously described. Briefly, 35 nM human α-thrombin was incubated with candidate inhibitor in 20 mM Hepes NaOH pH 7.4, 50 mM NaCl, 0.1% BSA, 0.05% Tween-20, 0.05% NaN$_3$ for 1 hour in wells
containing biotin-polyP. After washing, thrombin was quantified by cleavage of 400 μM Sar-Pro-Arg-p-nitroanilide (Bachem).

High-throughput screening assay

High-throughput screens were conducted at the High-Throughput Screening Facility (HTSF) at the University of Illinois. High-binding 384-well plates (Corning) were coated overnight at room temperature with 50 μL/well of 10 μg/mL avidin (Invitrogen) in water, then washed twice with 100 μL/well of TBS (50 mM Tris-HCl buffer pH 7.4, 100 mM NaCl, 0.05% NaN₃) containing 0.05% Tween-20. Biotinylated polyP was then immobilized on the wells (and, in the process, the wells were simultaneously blocked with BSA) by incubating the wells for 3 hours at room temperature with 50 μL/well of 20 μM biotin-polyP in TBS plus 1% BSA and 0.05% Tween-20. The wells were washed twice with 100 μL/well of 1 M LiCl, followed by two water washes. Each well then received 60 μL of storage buffer (50 mM Tris-HCl buffer pH 7.4 + 0.05% NaN₃) and the plates were stored at room temperature until needed. Thrombin-binding assays were performed by removing the storage buffer and dispensing 50 μL/well of 40 nM bovine thrombin (BioPharm Laboratories) in 20 mM Hepes-NaOH buffer pH 7.4, 50 mM NaCl, 1.4 mM CaCl₂, 0.5 mM MgCl₂, 0.05% Tween-20, 0.05% NaN₃, 0.1% BSA. The wells then received 100 nL aliquots of compounds to be tested. (To decrease the number of plates screened, five to seven compounds were pooled—always within libraries—and added per well, at 100 nL of each compound per well. Final concentrations in test wells were 1 μg/mL of each compound for the Chembridge compounds, or 2 μM of each compound for the NCI/Marvel/HTSF compounds.) Some wells received no compounds, which served as reference wells for the level of thrombin bound in the absence of any inhibitor. The plates were incubated for a minimum of
30 minutes (maximum of 3 hours) at room temperature, after which they were washed thrice. Each well then received 50 μL/well of 0.4 mM chromogenic thrombin substrate (Sar-Pro-Arg-pNA) diluted in 20 mM Hepes-NaOH buffer pH 7.4, 0.05% NaN₃. The wells were incubated for 1.8 hours at room temperature, after which the reaction was quenched with 25 μL/well of 0.1 N HCl and the absorbance at 405 nm was quantified. (Control experiments indicated that the rate of chromogenic substrate hydrolysis remained linear over the 1.8 hour time course, under the conditions tested.)

**Compound libraries screened**

Detailed descriptions of all four of the libraries screened in this study are available at HTSF web site (http://scs.illinois.edu/htsf/compound_collections.html), including comprehensive structural information files for the compounds in each library, readable by the ChemBioFinder program. The libraries screened in this study, which together included about 175,000 compounds, were:

1. ChemBridge MicroFormat Library (about 150,000 compounds); (2) HTSF House Library (about 4,700 compounds); (3) Marvel Library (about 10,000 compounds); and (4) NCI Library, comprising (a) Open Set (about 8,000 compounds), (b) Diversity Set (about 2,000 compounds), and (c) Natural Products and Challenge Set (about 300 compounds).

**Inhibition of heparin-catalyzed inactivation of factor Xa by antithrombin**

Antithrombin (120 nM), unfractionated heparin (1.5 × 10⁻² units/mL) and candidate inhibitor were incubated at room temperature for 2 minutes with 4.6 nM human factor Xa in 30 mM Hepes NaOH pH 7.4, 100 mM NaCl, 0.1% BSA. Factor Xa activity was quantified by
hydrolysis of 250 μM Spectrozyme Xa substrate (American Diagnostica) and converted to percent heparin activity by reference to a standard curve.

**Plasma clotting assays**

Plasma clotting times were quantified at 37°C using a STart4 coagulometer (Diagnostica Stago). Contact pathway tests used final concentrations of 33% plasma, 25 μM liposomes, 41.7 mM imidazole pH 7.0 and 8.33 mM CaCl\(_2\). Contact activator, inhibitor and liposomes were mixed with prewarmed plasma for 3 minutes, then clotting was initiated with CaCl\(_2\). Activator concentrations were selected to give 80-100 second clotting times: 10 μg/mL long-chain polyP; 10 μg/mL kaolin; 100 μg/mL diatomaceous earth; or 100 μg/mL polyguanylic acid (RNA). Tissue factor clotting tests used 30 pM relipidated tissue factor.

**Whole blood thromboelastometry**

Human blood studies were approved by the University of Illinois Institutional Review Board. Thromboelastometry was performed using ROTEM® (Pentapharm) with supplied software. Non-anticoagulated whole blood was collected via venipuncture (discarding the initial 3 mL) from normal human donors, then immediately transferred to the supplied plastic cups (280 μL per cup) and thoroughly mixed with 20 μL candidate inhibitor in TBS plus either 20 μL 1.7 mM long-chain polyP in TBS or tissue factor reagent (Ex-tem, Pentapharm). Final concentrations were 82% whole blood, 0 or 100 μM polyP, and inhibitor as indicated.
In vivo thrombus formation

Animal studies were approved by the IACUC of the Medical College of Wisconsin (venous thrombosis) or the University of Illinois (arterial thrombosis). For venous thrombosis, electrolytic injuries were induced on exposed femoral veins of pentobarbital-anesthetized C57BL/6 mice, as described. Three to five minutes before thrombus induction, rhodamine 6G-labeled platelets (up to $1 \times 10^7$ per mouse) and Alexa 647-labeled anti-fibrin antibodies (10-20 μg per mouse) were injected into the jugular vein at volumes up to 100 μL, followed by inhibitor, unfractionated heparin (APP Pharmaceuticals) or vehicle; fluorescence imaging of the thrombus induction site was then recorded for 60 minutes. Data among groups were analyzed by one-way ANOVA, with between-group comparisons using $p$ values calculated from post-hoc Tukey test.

For arterial thrombosis, C57/BL6 male mice (6-8 weeks old) were anesthetized using isoflurane, polyP inhibitors were injected retro-orbitally, the left carotid artery was exposed, and blood flow monitored with a Doppler vascular flow probe (Transonic, 0.5 PSB) connected to a perivascular flow meter (Transonic, TS420). To induce thrombosis, two pieces of 1 x 2 mm filter paper (Whatman GB003) saturated with freshly prepared 5% anhydrous FeCl$_3$ in 0.9% saline were applied to the deep and superficial surfaces of the artery. After 5 minutes, the filter papers were removed and the vessel irrigated with saline. Blood flow was monitored from FeCl$_3$ application for 30 minutes or until occlusion, defined as no detectable flow for one minute. Flow data were interpreted with LabScribe2 (iWorx Systems).

In vivo vascular leakage

Vascular leakage assays were employed to quantify polyP-induced extravasation of Evans blue dye in animal studies approved by the University of Illinois IACUC. Wild-type ICR mice
(Harlan Laboratories) anesthetized with isoflurane were injected retro-orbitally with 4% Evans blue in saline (1 μL/g body weight). PolyP inhibitors or saline were administered retro-orbitally (contralateral eye). After 40 minutes, three dorsal skin locations were injected intradermally with 25 μL of saline (negative control), 100 μM bradykinin (positive control), or 20 mM long-chain polyP. After 30 minutes, animals were euthanized, skins removed for punch biopsy (12 mm diameter), and Evans blue quantified as described. Data were compared between groups by Mann-Whitney Rank Sum test.

RESULTS

High-throughput screening for polyP inhibitors

We previously showed that polyP binds tightly to thrombin, a central protease in blood clotting. We used this interaction to develop a high-throughput screen for polyP inhibitors by first immobilizing biotinylated polyP on avidin-coated multiwell plates, then incubating wells with mixtures of thrombin and potential inhibitors, washing, and quantifying relative amounts of bound thrombin by chromogenic substrate hydrolysis. Candidate inhibitors were identified from high-throughput screening of a library of approximately 175,000 small molecules as well as a panel of 42 additional cationic compounds, polymers and proteins chosen for their possible association with a polyanion like polyP. For the latter panel (listed in Table 4.1), we hypothesized that cationic substances would bind to polyP, thereby competitively inhibiting its interaction with clotting proteins.

Nine of the tested wells from the initial high-throughput screen of about 175,000 compounds exhibited a ≥30% decrease in the rate of chromogenic substrate hydrolysis and were therefore flagged as containing potential inhibitors of thrombin-polyP binding. The 46 individual
compounds in these nine wells were re-tested singly in secondary screens to identify the actual inhibitors responsible for the reduction in thrombin binding. The secondary screens consisted of: (1) a repeat of the thrombin-polyP binding assay; (2) a kallikrein-polyP binding assay in which 100 nM human plasma kallikrein (Enzyme Research Laboratories) was substituted for thrombin and H-d-Pro-Phe-Arg-pNA substrate (Bachem) was used to detect bound kallikrein; (3) a test of direct thrombin inhibition, in which the compound was added directly to 1 nM thrombin in solution, and the rate of chromogenic substrate hydrolysis was quantified; and (4) a test of direct kallikrein inhibition, in which the compound was added directly to kallikrein in solution, and the rate of chromogenic substrate hydrolysis was quantified. The purpose of the parallel assays of both thrombin-polyP and kallikrein-polyP binding was to identify compounds that could inhibit the association of polyP with two different polyP-binding proteins, the idea being that this would identify compounds with a general ability to inhibit polyP-protein interactions (presumably via the compound binding to polyP). The purpose of the test of direct inhibition of thrombin and kallikrein was to identify compounds that decreased the measured signal, not by blocking polyP-thrombin or polyP-kallikrein binding, but by inhibiting the enzymatic activity of these proteases even in the absence of polyP.

Eight of the tested compounds showed reduced signal in one or more of these secondary assays, the results of which are summarized in Table 4.2. Four of the compounds (numbered A, B, D and G) exhibited less than 20% inhibition in the thrombin-polyP binding assay, and were therefore not promising leads to explore further. The other four compounds in Table 4.2 (numbered C, E, F and H) exhibited >90% inhibition in the thrombin-polyP binding assay. Of these latter four compounds, three (C, E and F) inhibited the enzymatic activity of free thrombin by >98%, one of which (C) also inhibited the enzymatic activity of free kallikrein by 97%. Thus,
these three compounds are direct protease inhibitors and apparently did not decrease the signal in the thrombin-polyP binding assay by actually interfering with thrombin binding to polyP. One of the compounds, surfen (compound H) decreased the signal in the thrombin-polyP binding assay by 95% and the signal in the kallikrein-polyP binding assay by 55%, while having a modest effect on the enzymatic activity of free thrombin. Thus surfen, which is also reported to be a small-molecule antagonist of heparin and heparan sulfate,\textsuperscript{21} was chosen for further analysis.

It was perhaps surprising that this relatively large screen identified just a single compound (surfen) that inhibited the binding of both thrombin and kallikrein to immobilized polyP under the conditions tested. A possible explanation is that the screen was performed at rather low concentrations of test compounds (typically, 1 μg/mL), so that only very potent inhibitors could be identified. Also, the libraries used in this study might not include simple polyamines and in any case they did not include polymers or proteins. Given the simple, repeating structure of polyP and its highly anionic nature, cationic polymers and proteins might be expected to bind tightly to polyP and thereby abrogate its procoagulant functions. Accordingly, we screened the additional panel of 42 cationic proteins, polymers and small molecules for ability to inhibit polyP-thrombin interactions. The majority of the cationic substances in this panel inhibited thrombin binding to polyP, with the results summarized in Table 4.1.

**In vitro potency of polyP inhibitors**

Potencies of prospective polyP inhibitors were determined using thrombin-polyP binding assays (example inhibition curves in Figure 4.1). IC\textsubscript{50} values for the 21 most potent inhibitors are plotted in Figure 4.2A on the basis of both mass and molarity. Of these, surfen was identified
from the high-throughput screen and the rest came from the panel of 42 cationic compounds, polymers and proteins. On a mass basis, low MW polyethyleneimine was the most potent (IC$_{50}$, 10 ng/mL) while the recombinant, isolated polyP-binding domain of *E. coli* exopolyphosphatase$^{22}$ (PPXbd) was the least potent (IC$_{50}$, 8.5 µg/mL). On a molar basis, poly-L-lysine was the most potent (IC$_{50}$, 0.49 nM) while spermidine was the least potent (IC$_{50}$, 11.7 µM). Four generations of cationic poly(amido amine) (PAMAM) dendrimers, with ethylenediamine cores and terminal NH$_2$ groups, all inhibited polyP binding to thrombin.

We chose eleven of the inhibitors for further scrutiny for the following reasons (with the compound numbers here in parentheses being the same as those in Figure 4.2A): low (inhibitor 1) and high (inhibitor 3) MW polyethyleneimine are among the most potent inhibitors; polybrene (inhibitor 2) is commonly included in Prothrombin Time clotting tests to inactivate heparin; spermine (inhibitor 4) is an endogenous polyamine that might modulate polyP function; polyP interacts with histone H1 (inhibitor 6) to modulate its procoagulant activities$^{23}$; surfen (inhibitor 7) is a small-molecule antagonist of heparin and heparan sulfate$^{21}$; intravenous protamine (inhibitor 8) is used clinically to reverse heparin anticoagulation$^{24}$; polymyxin B (inhibitor 9) is a clinical antibiotic that targets bacterial lipopolysaccharide (but perhaps polyP is an additional target of this drug, and furthermore, *Salmonella* lacking polyP kinase exhibit increased polymyxin B sensitivity$^{25}$); platelet factor 4 (inhibitor 10) is secreted from activated platelets and neutralizes heparin$^{26}$; and PPXbd (inhibitor 11) potently blocks polyP-mediated factor XI activation by thrombin.$^{11}$ In addition, cationic PAMAM dendrimers have received much recent attention for nanoparticle formation and drug delivery; we focused on generation 1.0 dendrimer (inhibitor 5; structure given in Figure 4.3) because lower generation cationic dendrimers are
reportedly less toxic than higher generation dendrimers or amine-functionalized linear polymers.\textsuperscript{27}

Heparin, like polyP, is a highly anionic linear polymer (although unlike polyP, heparin is strongly anticoagulant via binding to antithrombin). Cationic inhibitors of polyP might also bind to anionic glycosaminoglycans and thus exhibit complex in vivo activities. On the other hand, heparin and polyP have different anionic groups (sulfates versus phosphates) with different spacing and charge densities, so a given inhibitor might bind preferentially to one or the other polyanion. Figure 4.2B compares IC\textsubscript{50} values of the eleven selected polyP inhibitors, plotted on the y axis for potency for abrogating factor Xa inactivation by a mixture of heparin and antithrombin, and on the x axis for abrogating thrombin binding to polyP. Compounds interacting more potently with polyP than heparin lie above the dotted line, with the most potent and specific polyP inhibitors in the upper left. Spermine was 152 times more potent against polyP than against heparin/antithrombin, while polymyxin B, histone H1, polybrene, low MW polyethyleneimine and PPXbd were 4 to 7 times more potent against polyP than against heparin/antithrombin. On the other hand, platelet factor 4 was 18 times less potent against polyP than against heparin/antithrombin.

**Potency and specificity of polyP inhibitors in clotting assays**

We next evaluated the ability of these eleven inhibitors to block polyP-initiated clotting of human plasma. We expected at least some of these inhibitors to be considerably less effective in plasma clotting assays compared to assays using purified proteins, since plasma has many proteins and substances that might compete for binding to these candidate inhibitors. Also, to initiate plasma clotting, polyP must interact with multiple proteins in the contact pathway, which
differ in their affinities for polyP.\textsuperscript{11,17} To investigate inhibitor potency in human plasma, we therefore quantified the concentrations of inhibitors necessary to double the clotting time of plasma triggered by long-chain polyP, compared to clotting times triggered by other agents known to activate the contact pathway, including RNA,\textsuperscript{16} powdered kaolin, and diatomaceous earth. Cationic polyP inhibitors might also interfere with downstream clotting reactions (for example, by binding to the anionic lipid, phosphatidylserine); accordingly, we quantified the inhibitor concentration necessary to double the plasma clotting time triggered by relipidated tissue factor, the protein that initiates clotting in normal hemostasis.\textsuperscript{28} Figure 4.2C shows that, while low and high MW polyethyleneimine and protamine (inhibitors 1, 3 and 8) inhibited polyP-induced clotting, they also significantly prolonged the tissue factor clotting time (confirming the reported anticoagulant effect of protamine\textsuperscript{29}). The other eight inhibitors tested did not significantly prolong tissue factor clotting times at concentrations up to 100 µg/mL (Figure 4.2C); of these, generation 1.0 PAMAM dendrimer (inhibitor 5) was the most potent inhibitor of polyP-initiated clotting. Over the concentration range tested, PPXbd (inhibitor 11) inhibited clotting triggered by polyP but not by RNA, kaolin, diatomaceous earth or tissue factor. Spermine (inhibitor 4) was also a highly specific inhibitor of polyP-triggered plasma clotting, although it had some ability to attenuate clotting triggered by diatomaceous earth.

**Efficacy of polyP inhibitors in whole-blood thromboelastometry**

Coagulation of whole blood is more complex than plasma clotting, as it includes contributions from blood cells, including platelets. Consequently, we selected two polyP inhibitors (generation 1.0 dendrimer and the antibiotic, polymyxin B) to investigate their effects on polyP-triggered coagulation of whole blood in vitro, by measuring the viscoelastic properties of the
developing clot using thromboelastometry. When clotting was initiated by adding long-chain polyP to whole blood, both generation 1.0 dendrimer (Figure 4.4A) and polymyxin B (Figure 4.4B) prolonged clot formation in a concentration-dependent manner. On the other hand, neither inhibitor, over the same concentration ranges, significantly altered the thromboelastometry profiles of whole blood clotting initiated by tissue factor (Figure 4.4C and 4.4D).

**PolyP inhibitors abrogate the procoagulant activity of platelet polyP**

We next investigated the ability of polyP inhibitors to diminish the procoagulant effect of platelet polyP, since polyP is known to be secreted by activated platelets. In the first approach, we added polyP inhibitors to freshly drawn human blood, from which we prepared platelet-rich plasma. We then activated the platelets using a thrombin receptor agonist peptide (TRAP) and quantified thrombin generation in real time. In parallel experiments we triggered clotting by adding tissue factor to the platelet-rich plasma instead of TRAP. Both PPXbd (Figure 4.5A) and generation 1.0 dendrimer (Figure 4.5B) significantly delayed the onset of thrombin generation (lag time) as well as the time to peak thrombin, with only marginal effects on the peak thrombin levels themselves. In contrast, neither polyP inhibitor significantly altered thrombin generation when clotting was triggered with tissue factor.

In the second approach, we examined the ability of releases from activated human platelets to accelerate factor XI activation by thrombin, which we recently showed was entirely due to the presence of polyP. All five of the tested polyP inhibitors abrogated platelet releases-dependent factor XI activation by thrombin (Figure 4.6); IC$_{50}$ values were: low MW polyethyleneimine, 107 ± 1.7 ng/mL; generation 1.0 dendrimer, 360 ± 13 ng/mL; spermine, 17.1
± 1.4 μg/mL; PPXbd, 91.7 ± 2.5 μg/mL; and polymyxin B, 128 ± 2.8 μg/mL (mean ± standard error; n=4).

**Efficacy of polyP inhibitors in a mouse models of venous and arterial thrombosis**

PolyP contributes to thrombosis in mouse models.\(^6\) We therefore examined polyP inhibitors in a mouse venous thrombosis model driven by electrolytic injury to the femoral vein.\(^19\) Intravenous administration of generation 1.0 dendrimer or polymyxin B inhibited the accumulation of fibrin (Figure 4.7A) and platelets (Figure 4.7B) in the developing thrombus (as did heparin, provided for comparison). Peak accumulation of these thrombus markers (comparing mean relative intensities 26 minutes after injury) was significantly \((p<0.05)\) lower for animals treated with dendrimer, polymyxin B or heparin compared to animals receiving saline. We also examined polyP inhibitors in a mouse arterial thrombosis model driven by \(\text{FeCl}_3\) application to the carotid artery. Intravenous administration of generation 1.0 dendrimer, low MW polyethyleneimine or polymyxin B prior to \(\text{FeCl}_3\) application reduced or slowed vessel occlusion (Figure 4.7C). Log-rank analyses showed that median patency time was significantly prolonged in mice injected with generation 1.0 dendrimer \((p<0.01; n=8)\), polymyxin B sulfate \((p<0.01; n=10)\), or low MW polyethyleneimine \((p<0.01; n=8)\) versus mice injected with vehicle \((n=11)\). PolyP inhibitors therefore significantly attenuate both venous and arterial thrombosis.

**In vivo anti-inflammatory activity of polyP inhibitors**

PolyP injected intradermally in mice induces vascular leakage that is dependent upon localized activation of the contact pathway and concomitant bradykinin production.\(^6\) To evaluate whether
cationic molecules could inhibit these proinflammatory effects of polyP in vivo, we employed a modified Miles vascular leakage assay. Generation 1.0 dendrimer or polymyxin B was administered intravenously to mice along with Evans blue dye. After 40 minutes, polyP or bradykinin was injected intradermally and local vascular leakage detected by extravasation of Evans blue. Generation 1.0 dendrimer significantly abrogated vascular leakage induced by polyP (Figure 4.7D; \( p<0.001 \)) but not by bradykinin (Figure 4.7D; \( p=0.504 \)). Results obtained with polymyxin B were highly variable (Figure 4.7D) and not statistically significantly different from control animals (\( p=0.549 \)).

**DISCUSSION**

This study demonstrates proof of principle that inhibitors of polyP, including cationic small molecules, polymers and proteins, can block the procoagulant and pro-inflammatory effects of polyP, both in vitro and in vivo. Using in vitro clotting assays, the potencies of these inhibitors toward polyP varied considerably, as did their specificities toward polyP compared to heparin, RNA, minerals, or tissue factor. Spermine and PPXbd, in particular, were selective for inhibiting the procoagulant activity of polyP over that of other clotting activators. Spermine was also much more effective at blocking the procoagulant activity of polyP than the anticoagulant activity of heparin/antithrombin. On the other hand, both generation 1.0 dendrimer and polymyxin B—while more potent toward polyP—also significantly reduced the procoagulant activity of RNA and non-physiologic, mineral-based activators of the contact pathway. Inhibitors such as these may thus have utility as general inhibitors of the contact pathway of blood clotting triggered by anionic polymers and surfaces in vivo. In particular, the generation 1.0 dendrimer
was highly effective in vivo at reducing severity of venous thrombosis, arterial thrombosis, and polyP-mediated vascular leakage.

Our finding that spermine, norspermine and spermidine are potent polyP inhibitors is noteworthy since the naturally occurring polyamines, spermine, spermidine, and putrescine inhibit aggregation of human platelets,\textsuperscript{30-36} and systemic administration of polyamines is reported to prevent carotid artery occlusion in a canine thrombosis model.\textsuperscript{37} The latter investigators concluded that the antithrombotic activity of polyamines was due to inhibition of platelet aggregation, although it is tempting to speculate that inhibition of platelet-secreted polyP procoagulant activity may have also contributed to their protective effects. Direct tissue injection of spermine was effective in preventing footpad edema in a rodent model,\textsuperscript{38} and spermine administration was neuroprotective in an ischemic brain injury model in rats.\textsuperscript{39} Interestingly, brain contains long-chain polyP,\textsuperscript{40} and deficiency of contact pathway clotting factors is neuroprotective in mouse models of cerebral artery ischemia-reperfusion.\textsuperscript{13} It would therefore be interesting to examine the in vivo utility of polyP inhibitors in brain injury models.
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<th>No.</th>
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<tr>
<td>1,2-bis(3-aminopropylamino)ethane</td>
<td></td>
<td>(53% inhibition at 200 μg/mL)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
<td>(48% inhibition at 200 μg/mL)</td>
</tr>
<tr>
<td>DNase I</td>
<td></td>
<td>(36% inhibition at 200 μg/mL)</td>
</tr>
<tr>
<td>RNase A</td>
<td></td>
<td>(23% inhibition at 100 μg/mL)</td>
</tr>
<tr>
<td>Bacitracin</td>
<td></td>
<td>(12% inhibition at 200 μg/mL)</td>
</tr>
<tr>
<td>Methylenediamine</td>
<td></td>
<td>(73% inhibition at 6 mg/mL)</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td></td>
<td>(44% inhibition at 2.7 mg/mL)</td>
</tr>
<tr>
<td>Melamine</td>
<td></td>
<td>(0% inhibition at 200 μg/mL)</td>
</tr>
</tbody>
</table>
Each substance was tested at 200 μg/mL (or indicated concentrations) for inhibition of thrombin binding to immobilized biotin-polyP. Those exhibiting >70% inhibition were retested at varying inhibitor concentrations, from which IC_{50} values were derived (listed here in order of decreasing potency). Numbers in square brackets are the compound numbers used in Figure 4.2.

Table 4.2. Secondary screening of eight compounds identified from the high-throughput screen of approximately 175,000 compounds

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Library</th>
<th>Concentration tested</th>
<th>Thrombin binding to polyP</th>
<th>Kallikrein binding to polyP</th>
<th>Free thrombin activity</th>
<th>Free kallikrein activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-benzyl-2,2,6,6-tetramethyl-4-piperidinamine dihydrochloride</td>
<td>Chembridge</td>
<td>3 μg/mL</td>
<td>0%</td>
<td>0%</td>
<td>36.1%</td>
<td>---*</td>
</tr>
<tr>
<td>[(6-nitro-1,3-benzodioxol-5-yl)methylene]malononitrile</td>
<td>Chembridge</td>
<td>10 μg/mL</td>
<td>17.5%</td>
<td>0%</td>
<td>11.1%</td>
<td>2.5%</td>
</tr>
<tr>
<td>1-(3,4-dimethoxybenzoyl)-3-(2-furyl)-1H-1,2,4-triazol-5-amine</td>
<td>Chembridge</td>
<td>10 μg/mL</td>
<td>95.1%</td>
<td>81.4%</td>
<td>99.6%</td>
<td>97.4%</td>
</tr>
<tr>
<td>N-(2,4-dichlorophenyl)-N'-{5-[[4-(nitrophenoxy)methyl]-1,3,4-thiadiazol-2-yl]urea</td>
<td>Chembridge</td>
<td>10 μg/mL</td>
<td>0%</td>
<td>0%</td>
<td>5.3%</td>
<td>1.5%</td>
</tr>
<tr>
<td>2-(3-nitrophenoxy)-3,1-benzoxazin-4(4H)-one</td>
<td>NCI Open Plate Set</td>
<td>2 μM</td>
<td>98.6%</td>
<td>18.8%</td>
<td>98.9%</td>
<td>39.3%</td>
</tr>
<tr>
<td>5-(benzylthio)-1-butryl-3-phenyl-1H-1,2,4-triazole</td>
<td>Chembridge</td>
<td>10 μg/mL</td>
<td>92.8%</td>
<td>9.6%</td>
<td>99.5%</td>
<td>12.3%</td>
</tr>
<tr>
<td>Surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide)</td>
<td>NCI Diversity Set</td>
<td>2 μM</td>
<td>95.6%</td>
<td>55.3%</td>
<td>32.2%</td>
<td>---*</td>
</tr>
</tbody>
</table>

*not tested
Figure 4.1. Examples of plots of inhibition of thrombin binding to immobilized polyP for four selected inhibitors. The percent inhibition of thrombin binding to polyP is plotted for the following inhibitors that encompassed a range of IC$_{50}$ values: low MW polyethyleneimine (▽), generation 1.0 PAMAM dendrimer (●), polymyxin B (□), and spermidine (▲). The dotted line signifies 50% inhibition. Data are mean ± standard error (n=3).
Figure 4.2. Relative potencies of polyP inhibitors. (A) Inhibitor concentrations resulting in 50% reduction of thrombin binding to immobilized polyP (IC$_{50}$) are plotted for the 21 most potent substances tested, expressed in terms of mass (left) and molarity (right). Inhibitors that were also used in panels b and c are numbered in parentheses. Data are mean ± standard error (n=3). (B) Plot of IC$_{50}$ values of the 11 numbered substances from panel a for inhibition of heparin-mediated inactivation of factor Xa by antithrombin (y axis) versus inhibition of thrombin binding to immobilized polyP (x axis). Dotted line represents equivalent potency. Data are mean ± bidirectional standard error (although error bars are within the symbols; n=3). (C) Effectiveness of polyP inhibitors in prolonging clotting. Clotting of human plasma was initiated
by long-chain polyP (P), polyguanylic acid (RNA), kaolin (K), diatomaceous earth (Dia), or tissue factor (TF). Data are mean inhibitor concentrations that doubled the clotting time relative to no inhibitor (EC_{double}) ± standard error (n=4). Horizontal dotted lines indicate that the clotting time with that initiator was either unaffected by the inhibitor or was not prolonged sufficiently to reach a doubling point even at 100 µg/mL inhibitor.

Figure 4.3. Chemical structure of the generation 1.0 cationic PAMAM dendrimer used in this study.
Figure 4.4. Generation 1.0 dendrimer and polymyxin B inhibit clotting of whole human blood initiated by polyP but not by tissue factor. Thromboelastometry (ROTEM) profiles are given for clotting of freshly-drawn, non-anticoagulated whole human blood initiated by long-chain polyP (A,B) or tissue factor (C,D), in the presence of generation 1.0 dendrimer (A,C) or polymyxin B (B,D). The x axis is time from addition of clotting trigger; y axis is amplitude of clot strength.
Figure 4.5. (A) PPXbd and (B) generation 1.0 dendrimer delay thrombin generation in human plasma containing activated platelets. Real-time thrombin generation in plasma was quantified using Calibrated Automated Thrombogram assays (Thrombinoscope; Diagnostica Stago). Either 500 μg/mL PPXbd, 20 μg/mL dendrimer or saline were added to freshly drawn, citrated human blood, from which platelet-rich plasma was prepared and the platelet concentration adjusted to 150,000/μL. To some platelet-rich plasmas, TRAP was added at 10 μM to activate platelets. After 5 minutes, FluCa reagent (fluorogenic substrate + CaCl₂) was added and thrombin generation was quantified. Parallel assays were performed on the same platelet-rich plasmas not pre-treated with TRAP, but in which clotting was triggered using FluCa reagent that also contained 5 pM tissue factor (TF). Thrombin generation parameters are plotted as mean ± standard error (for 5 donors assayed in triplicate). Indicated p values are from paired t-tests with and without inhibitor.
Figure 4.6. PolyP inhibitors reverse the ability of platelet releasates to accelerate factor XI activation by thrombin. Initial rates of activation of 30 nM human factor XI by 20 nM human α-thrombin were determined in the presence of releasate prepared from TRAP-stimulated human platelets as described\textsuperscript{11}, normalized to the rate of factor XI activation without any added polyP inhibitor. Percent inhibition is plotted versus inhibitor concentration for the following: low MW polyethyleneimine (▲); generation 1.0 dendrimer (■); spermine (△); PPXbd (●); or polymyxin B (□). Data are mean ± standard error (n=4). IC\textsubscript{50} values calculated from these curves are given in the text. (In the second stage of the assay, factor Xla levels were quantified, as previously described,\textsuperscript{11} by monitoring the rate of cleavage of the chromogenic substrate, L-Pyr-Pro-Arg-\textit{p}-nitroanilide. At the concentrations used, none of the inhibitors altered the rate of hydrolysis of this substrate by factor Xla.)
Figure 4.7. In vivo antithrombotic and anti-inflammatory efficacies of polyP inhibitors.

(A,B) Murine model of venous thrombosis. Inhibitors were administered intravenously to mice prior to initiation of electrolytic injury of the femoral vein (time=0 in the graphs). Data are mean relative intensities for accumulation of fluorescently labeled fibrin-specific antibodies (A) or labeled platelets (B) in the developing thrombus for mice receiving: red circles, 4 μg/g generation 1.0 dendrimer (n=10); blue squares, 2 μg/g polymyxin B (n=8); orange inverted triangles, 100 units/kg unfractionated heparin (n=5); or open triangles, vehicle only (n=14). Bars represent one standard error. (C) Murine model of arterial thrombosis, with Kaplan-Meier curves showing percentage of mice with patent arteries. Inhibitors were injected retro-orbitally 10 minutes before ferric chloride injury to the carotid artery. Blood flow was monitored by Doppler, with occlusion defined as no flow for one minute. Log-rank analyses indicated that median patency time was significantly longer for mice injected with 8 μg/g generation 1.0 dendrimer (p<0.01, n=8), 4 μg/g polymyxin B (p<0.01, n=10), or 5 μg/g low MW polyethyleneimine (p<0.01, n=8) versus mice injected with vehicle (n=11). (D) Murine model of
polyP-induced vascular leakage. Mice were given separate retro-orbital injections with Evans Blue dye and either a polyP inhibitor (48 µg/g generation 1.0 dendrimer or 20 µg/g polymyxin B) or vehicle. After 40 minutes, saline, bradykinin, or polyP were injected intradermally at 3 sites on the back. After an additional 30 minutes, mice were euthanized and dye was extracted from skin biopsies for quantification. Plots show median (central horizontal lines), mean (triangles), 25-75th percentile (top and bottom of boxes), and 10-90th percentile (whiskers) concentrations of extracted dye. Dendrimer administration resulted in significantly less dye leakage at the site of polyP injection compared to control animals (p<0.001). Each group (no inhibitor, dendrimer and polymyxin B) contained 15 mice.

REFERENCES


CHAPTER 5: POLYPHOSPHATE AS A COFACTOR FOR FACTOR XI ACTIVATION BY THROMBIN AND FACTOR XIa$^5$

ABSTRACT

Factor XI deficiency is associated with a bleeding diathesis but factor XII deficiency is not, indicating that, in normal hemostasis, factor XI must be activated in vivo by a protease other than factor XIIa. Several groups have identified thrombin as the most likely activator of factor XI, although this reaction is slow in solution. While certain non-physiologic anionic polymers and surfaces have been shown to enhance factor XI activation by thrombin, the physiologic cofactor for this reaction is uncertain. Activated platelets secrete the highly anionic polymer, polyphosphate, and our previous studies have shown that polyphosphate has potent procoagulant activity. We now report that polyphosphate potently accelerates factor XI activation by α-thrombin, β-thrombin and factor XIa, and that these reactions are supported by polyphosphate polymers of the size secreted by activated human platelets. We therefore propose that polyphosphate is a natural cofactor for factor XI activation in plasma, which may help explain the role of factor XI in hemostasis and thrombosis.

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$^5$This chapter in its entirety has been published as Choi SH, Smith SA, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. Blood. 2011;118(26):6963-6970.
INTRODUCTION

In the original cascade/waterfall model of coagulation,\(^1\) factor XI (FXI) is activated by factor XIIa (FXIIa), a member of the contact pathway of blood clotting. Patients with severe FXI deficiency may exhibit bleeding tendencies,\(^2,3\) especially postoperative or posttraumatic bleeding in tissues with robust fibrinolytic activity.\(^4-6\) On the other hand, individuals with severe deficiencies in FXII, high-molecular-weight kininogen (HK) or prekallikrein do not exhibit bleeding diatheses at all, indicating that the proteins responsible for triggering the classical contact pathway of blood clotting are completely dispensable for hemostasis.\(^7\) Thus, in normal hemostasis, FXI must be activated \textit{in vivo} by a protease other than FXIIa. A solution to this conundrum was proposed in 1991 by Naito and Fujikawa\(^8\) and by Gailani and Broze\(^9\) who reported that thrombin up-regulates its own generation by feeding back to activate FXI, leading to a “revised model of coagulation.”\(^9-11\) More recently, Matafonov et al.\(^12\) identified that \(\beta\)-thrombin and \(\gamma\)-thrombin, proteolyzed derivatives of \(\alpha\)-thrombin, can also activate FXI in plasma.

The proposal that FXI activation by thrombin plays a significant role in blood clotting \textit{in vivo} is somewhat controversial.\(^13-15\) In solution, the rates both of FXI activation by thrombin and of FXI autoactivation are slow but are markedly enhanced in the presence of polyanions,\(^8,9,16,17\) although most studies have employed nonphysiologic cofactors such as dextran sulfate or high concentrations of sulfatides. The relevant physiologic cofactors for FXI activation by thrombin in plasma, if any, have yet to be definitely determined.

Polyphosphate (polyP) – a linear polymer of inorganic phosphate residues – accumulates in a variety of microorganisms\(^18\) and is secreted by activated human platelets.\(^19\) In the present study, we demonstrate that polyP potently accelerates FXI activation by \(\alpha\)-thrombin, \(\beta\)-thrombin and
FXIa. Using carefully defined polymer lengths, we report that polyP polymers of the size
secreted by activated human platelets are very active in stimulating FXI activation by thrombin
in both a purified system and in plasma. We further report that activated platelets and platelet
releasates promote FXI activation by thrombin. Together, these findings indicate that polyP is a
potent natural cofactor for FXI activation by thrombin, which may help explain the role of FXI
in normal hemostasis.

EXPERIMENTAL PROCEDURES

Materials
PolyP preparations of narrow size distributions were prepared as previously described, and are
indicated in this study by their polymer length followed by “mer” (e.g., 167mer). A
heterogeneous polyP preparation comprising 20 to 300mers was biotinylated on terminal
phosphates using amine-PEG₂-biotin from Pierce (Rockford, IL) as described. Note that all
polyP concentrations are reported in this study in terms of phosphate monomer concentration
(monomer formula: NaPO₃), except for Figure 3D which reports polyP polymer concentrations.

Purified FXI, FXIa, FVa, β-thrombin, corn trypsin inhibitor (CTI), mouse anti-human
FXI monoclonal antibody, and FXI- or FXII-deficient plasmas were from Hematologic
Technologies (Burlington, VT). α-thrombin was from Enzyme Research Laboratories (South
Bend, IN). Dextran sulfate with an average M_r of 500 kDa and protease-free bovine serum
albumin (BSA) were from Calbiochem (San Diego, CA). L-2145 (L-Pyr-Pro-Arg-p-nitroanilide)
was from Bachem (Torrance, CA). PCPSPE liposomes (20% phosphatidylserine, 40%
phosphatidylcholine, 40% phosphatidylethanolamine; Avanti Polar Lipids, Alabaster, AL) were
made by sonication. Polybrene, benzamidine, 4-(2-aminoethyl) benzenesulfonyl fluoride

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hydrochloride (AEBSF), phenylmethanesulfonyl fluoride (PMSF), streptavidin, theophylline, PGE₁, thrombin receptor agonist peptide (SFLLRN-NH₂, TRAP), and hirudin were from Sigma-Aldrich (St. Louis, MO). Biacore CM5 sensorchips were from GE Healthcare (Piscataway, NJ). EcPPXc, the recombinant polyP-binding domain of *Escherichia coli* exopolyphosphatase fused to maltose-binding protein and a His₆ tag, was produced as described.²² Recombinant *Saccharomyces cerevisiae* exopolyphosphatase fused to a His₆ tag (rPPX1) was produced as described.²³

**Preparation of stimulated platelet suspensions and platelet releasates**

Activated platelets and platelet releasates were prepared as follows. Fresh whole blood from normal, non-smoking donors not on medication was collected into 3.2% sodium citrate, 2 μM PGE₁, and 1 mM theophylline via atraumatic venipuncture. (All volunteer blood donors gave written informed consent under a blood-drawing protocol approved by the local Institutional Review Board.) Platelet-rich plasma was collected following centrifugation of blood at 37°C at 200 x g, after which the plasma was re-centrifuged at 1500 x g to collect the platelets. Pelleted platelets were washed by centrifugation once with Tyrode’s buffer (1 g/L D-glucose, 0.2 g/L CaCl₂, 0.1 g/L MgCl₂, 0.2 g/L KCl, 8 g/L NaCl, 0.05 g/L NaH₂PO₄, 1 g/L NaHCO₃) containing 3.2% citrate, 2 μM PGE₁, and 1 mM theophylline, once with Tyrode’s buffer containing 2 μM PGE₁ and 1 mM theophylline, and once in Tyrode’s buffer without additives. The resulting platelet pellets were resuspended at 5.3 x 10⁶/μL (approximately 17-fold their concentration in whole blood) or 1.6 x 10⁷/μL (approximately 50-fold whole blood concentration) in Tyrode’s buffer and stimulated with 4 μM TRAP for 10 minutes at 37°C with agitation. In some experiments, FXI activation was analyzed in the suspension of activated platelets, while in other
experiments FXI activation was analyzed using platelet releasates. To obtain cell-free releasate, activated platelets were pelleted by centrifugation at 2000 x g for 10 minutes, after which the supernatant was collected and re-centrifuged at 13,000 x g for 10 minutes to deplete residual platelets and particles. The resulting platelet releasates were stored frozen until use. Some platelet releasate samples were boiled for 30 minutes to denature proteins prior to being employed in FXI activation assays.

**Activation of FXI**

30 nM FXI and 5 nM α-thrombin were incubated with either polyP, stimulated platelet suspension, platelet releasate, or dextran sulfate at 37°C in 30 mM Hepes (pH 7.4), 50 mM NaCl, and 0.1% BSA. In some reactions, activated platelets or platelet releasates were treated with 70 μg/mL rPPX1 for 1 hour at 37°C or pre-incubated with 250 μg/mL EcPPXc immediately prior to FXI activation assays. Timed samples (0 to 20 minutes) were removed and quenched by addition of polybrene (6 μg/mL final) to neutralize polyP and hirudin (0.14 to 0.5 U/mL final) to inactivate thrombin, after which the generated FXIa was quantified by measuring rates of L-2145 hydrolysis using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). At the concentrations used, neither polyP nor polybrene affected L-2145 hydrolysis by FXIa.

FXI autoactivation reactions incubated with polyP or dextran sulfate and either 30 or 60 nM FXI were conducted similarly except that they lacked α-thrombin, and only polybrene (6 μg/mL final) was used to quench the timed samples. Rates of L-2145 hydrolysis were converted to FXIa concentrations using a standard curve. Second-order rate constants (k₂) for FXI autoactivation were calculated as described.²⁴
For SDS-PAGE analyses of FXI autoactivation, 60 nM FXI was incubated with polyP or
dextran sulfate in 30 mM Hepes (pH 7.4), 50 mM NaCl, and 0.1% polyethyleneglycol (Mr =
8000). At various time points, aliquots were removed into reducing SDS sample buffer, resolved
by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA), and proteins visualized by silver
staining.²⁵

**FXIa autolysis**

6 nM FXIa was incubated with polyP preparations of varying polymer lengths (whose
concentrations were adjusted to yield 3 nM polymer) at 37°C in 30 mM Hepes (pH 7.4), 50 mM
NaCl, and 0.1% BSA. Timed samples (0-2 minutes) were diluted and quenched with polybrene
(6 µg/mL final), and the residual FXIa concentration was determined by quantifying the rate of
L-2145 hydrolysis compared to a standard curve.

**Surface plasmon resonance (SPR) analyses**

SPR binding studies were performed at 25°C using a Biacore 3000 instrument (Biacore,
Columbia, MD). Streptavidin was bound to CM5 sensorships by standard amine coupling; after
blocking and washing, biotin-polyP was captured on the surface. Varying concentrations of α-
thrombin, β-thrombin, FXI, or FXIa in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 0.005%
surfactant P20 were flowed over the surface at 50 µL/min using a 2 minute association phase and
5 minute dissociation phase, with background subtraction using a streptavidin-coated reference
cell without polyP. The running buffer for α- or β-thrombin contained 5 mM benzamidine, while
FXI and FXIa were pretreated with 4 mM AEBSF for 30 minutes prior to use to block the active
sites of FXIa. Sensorchips were regenerated by washing with 1 M NaCl between runs. Binding
kinetics were analyzed according to the 1:1 Langmuir binding model. $K_d$ values were calculated from the quotient of the derived dissociation ($k_{diss}$) and association ($k_{ass}$) rate constants.

**Plasma clotting assay**

Clotting times of citrated human plasma were quantified at 37°C using a STart4 coagulometer (Diagnostica Stago, France). Prewarmed polyP in HBA (25 mM Hepes, pH 7.4, 1% BSA) was mixed in coagulometer cuvettes with prewarmed FXI- or FXII-deficient plasma to which CTI (100 µg/mL) and PCPSPE (60 µM) had previously been added. β-thrombin in HBA was then immediately added and the mixture was incubated for 1 min 37°C, after which clotting was initiated with addition of CaCl$_2$. Final concentrations were 33% plasma, 20 µM PCPSPE, 33 µg/mL CTI, 0-5 µM polyP, 8.33 mM Hepes (pH 7.4), 0.3% BSA, 12 nM β-thrombin and 8.33 mM CaCl$_2$.

**Thrombin generation assay**

Thrombin generation in human plasma was quantified at 37°C using a Calibrated Automated Thrombogram (CAT) system employing Thrombinscope software (Diagnostica Stago, France). Prewarmed polyP in HBA in microplate wells was mixed with prewarmed FXI- or FXII-deficient plasma to which CTI (100 µg/mL), FVa (20 nM), and PCPSPE (30 µM) had previously been added. The FluCa reagent (containing CaCl$_2$ and the thrombin fluorogenic substrate) was then added and thrombin generation profiles were collected. Final concentrations included 67% plasma, 20 µM PCPSPE, 13 nM FVa, 67 µg/mL CTI, 0-50 µM polyP, 8.33 mM Hepes (pH 7.4), 0.17% BSA, and 20 nM β-thrombin. For some control experiments, anti-FXI antibody (1 µg/mL) was preincubated with FXII-deficient plasma at 37°C for 30 minutes prior to use. For others, 5
μg/mL FXI was added to FXI-deficient plasma immediately after the FXI had been preincubated (at 500 μg/mL FXI in 25 mM Hepes pH 7.5) for 30 minutes with 0.5 mM PMSF to abolish any contaminating FXIa activity. As an additional control, 50 pM FXIa was added to FXI-deficient plasma and the rate of thrombin generation was measured. In some samples, polyP was digested with 40 μg/mL rPPX1 for 1 hour at 37°C, or pre-incubated with 250 μg/mL EcPPXc, prior to thrombin generation assays.

RESULTS

PolyP enhances FXI activation by thrombin

Previous studies have shown that FXI activation by thrombin is greatly accelerated in the presence of nonphysiologic polyanions such as dextran sulfate.\(^8,9\) We hypothesized that the highly anionic polymer, polyP, may serve as a physiologic cofactor for this reaction. Accordingly, we examined the rate of activation of 30 nM FXI by 5 nM α-thrombin over 20 minute time courses in the presence of varying concentrations of polyP of varying polymer lengths (Figure 5.1). In the absence of polyP, we observed very low rates of FXI activation, consistent with previous reports in the absence of polyanions (Figure 5.1A). Short-chain polyP (22mer) did not measurably stimulate FXI activation by α-thrombin at any tested polyP concentration, but longer polyP polymers (65mer, 167mer and 350mer) strongly enhanced FXI activation in a concentration-dependent manner (Figure 5.1A). The bell-shaped dose-response curve for polyP suggests a template mechanism, in which both thrombin and FXI, or FXI and FXIa, assemble on the same polyP molecule.

Optimal enhancement of FXI activation occurred at 4 μM polyP for the 167mer and 350mer preparations, so this polyP concentration was used to examine the relationship between
polyP polymer length and enhancement of FXI activation by α-thrombin. The results (Figure 5.1B) show that polyP preparations with polymer lengths >50 phosphate units significantly enhanced FXI activation by thrombin, with optimal rates observed with polymers that were 100mers or longer. We then compared the cofactor activity of polyP versus dextran sulfate and found that FXI activation by α-thrombin in the presence of 4 μM polyP (255mer) was significantly greater than that obtained in the presence of 1 μg/mL dextran sulfate (Figure 5.1C). The isolated polyP-binding domain of *E. coli* exopolyphosphatase (EcPPXc) has previously been utilized as a specific probe for localizing polyP in yeast cell walls,22,26 so we examined its ability to specifically inhibit the cofactor function of polyP. The inclusion of 30 μg/mL EcPPX profoundly inhibited FXI activation by α-thrombin in the presence of polyP, but had no effect on FXI activation by α-thrombin in the presence of dextran sulfate (Figure 5.1C).

**Activated platelets and platelet releasates enhance FXI activation**

Activated human platelets secrete polyP with polymer lengths about 60 to 100 phosphates long,19,27 which is in the size range that we found in Figure 5.1B would enhance FXI activation by α-thrombin. We therefore hypothesized that polyP secreted from activated platelets should serve as a cofactor for FXI activation by thrombin. Releasates from activated platelets markedly accelerated the rate of FXI activation in the presence of 5, 10, or 20 nM α-thrombin (Figure 5.2A). In addition to polyP, activated platelets release a host of granule contents including many proteins, so we sought to determine the contribution of polyP to the ability of the platelet releasate to accelerate thrombin-mediated FXI activation. Boiling will denature almost all proteins, while purified polyP’s cofactor activity is unaffected by boiling (not shown), so we examined if this cofactor effect in platelet releasates survived boiling. Figure 5.2A shows that
boiled platelet releasates augmented thrombin-mediated FXI activation slightly better than non-boiled releasates. This increase in cofactor function may be because boiling denatures proteins in platelet releasates that might compete with thrombin and FXI for binding to polyP. On the other hand, treatment of platelet releasates with EcPPXc, or digestion of releasates with recombinant exopolyphosphatase (rPPX1), abolished their ability to enhance FXI activation (Figure 5.2A), consistent with the idea that polyP secreted from activated platelets is the augmenting cofactor for FXI activation by α-thrombin.

We also investigated the effect of whole stimulated platelets (i.e., activated platelets plus their releasate) in supporting FXI activation by 20 nM α-thrombin. We found that whole activated platelets in suspension also strongly enhanced FXI activation by α-thrombin, although not quite as strongly as platelet releasate alone (Figure 5.2B). This reduction in FXI activation rate might be explained by thrombin, FXI and/or FXIa binding to the surface of activated platelets and therefore being less available for interaction with polyP. Nevertheless, our results demonstrate that suspensions of stimulated whole platelets efficiently promote FXI activation by thrombin. In a control experiment, incubating EcPPXc with activated platelets or releasate decreased the rate of thrombin-mediated FXI activation to approximately the same baseline rate of FX activation seen with thrombin but without platelets or releasate (Figure 5.2B). Furthermore, without added thrombin, very low rates of FXI were observed in the presence of platelets or releasates (Figure 5.2B).

**PolyP accelerates FXI autoactivation and FXIa autolysis**

In the experiments in Figure 5.1, we observed essentially linear initial rates of FXI activation in the presence of polyP when we included 5 nM α-thrombin as the FXI activator; however, in the
absence of thrombin but in the presence of polyP, we observed sigmoidal progress curves for FXIa generation, with a substantial lag phase before FXIa was detectable (Figure 5.3A). This behavior is typical of FXI autoactivation (i.e., FXIa-mediated FXI activation), which has been observed when purified FXI is incubated at appropriate concentrations with a variety of (typically non-physiologic) anionic polymers.\(^8,\,9,\,17\) The experiment in Figure 5.3A shows that polyP (77mer or 255mer) supported more robust autoactivation of 60 nM FXI than did an optimal concentration of dextran sulfate.

It is possible that FXI activation by thrombin in the presence of polyP actually consists of direct FXI activation by thrombin, plus FXI autoactivation once significant amounts of FXIa are generated. Therefore, we systematically examined the ability of polyP to enhance the rate of FXI autoactivation (i.e., FXI activation without thrombin), and determined the second-order rate constants for this reaction as a function of polyP polymer length. The results (Figure 5.3B) show that polyP polymers longer than 100mers maximally enhanced FXI autoactivation, while polymers in the range of 60-100 phosphate units long supported FXI autoactivation but at a slower rate. FXI autoactivation was not detectable with polyP polymers shorter than 60mers or in the absence of polyP.

Parallel samples from the FXI autoactivation reaction in Figure 5.3A were visualized on silver-stained SDS-PAGE (Figure 5.3C), showing that the 80 kDa FXI zymogen was converted to the 50 and 30 kDa heavy and light chains of FXIa, as expected.\(^8,\,9,\,16\) This experiment underscores that polyP (77mer or 255mer) supported much more rapid FXI autoactivation than did dextran sulfate (Figure 5.3C). (When 60 nM FXI was incubated in the absence of polyanions for 50 min, no FXIa was detectable by SDS-PAGE; not shown.)
FXIa autolysis exhibited a similar dependence on polyP polymer length (Figure 5.3D), consistent with studies showing that FXI autoactivation in the presence of dextran sulfate eventually leads to degradation of the heavy and light chains of FXIa, with concomitant loss in enzymatic activity.\(^9\)

**Thrombin, FXI and FXIa bind with high affinity to immobilized polyP**

We previously reported that polyP binds \(\alpha\)-thrombin with high affinity (\(K_d\) approximately 5 nM) when measured using SPR experiments in which thrombin was bound to the sensorchip and polyP was flowed over the surface.\(^29\) Using polyP bound to microplates, we have also reported high affinity polyP binding for \(\alpha\)-thrombin (\(K_d\) about 66 nM) and FXIa (\(K_d\) about 6 nM).\(^21\) In this study, we further evaluated the binding interaction between polyP and thrombin, FXI and FXIa utilizing SPR in which biotinylated polyP was immobilized on sensorchips and the proteins were flowed over the surface. FXI, FXIa, \(\alpha\)-thrombin and \(\beta\)-thrombin all bound tightly to immobilized polyP (Figure 5.4), yielding the following association (\(k_{ass}\)) and dissociation (\(k_{diss}\)) rate constants and \(K_d\) values: \(\alpha\)-thrombin, \(k_{ass} = 5.12 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), \(k_{diss} = 7.71 \times 10^{-2} \text{s}^{-1}\), \(K_d = 15.1\) nM; \(\beta\)-thrombin, \(k_{ass} = 3.85 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), \(k_{diss} = 3.12 \times 10^{-2} \text{s}^{-1}\), \(K_d = 8.1\) nM; FXI, \(k_{ass} = 1.64 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), \(k_{diss} = 1.05 \times 10^{-2} \text{s}^{-1}\), \(K_d = 6.4\) nM; and FXIa, \(k_{ass} = 1.92 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), \(k_{diss} = 2.91 \times 10^{-3} \text{s}^{-1}\), \(K_d = 1.5\) nM.

**PolyP can accelerate clotting of plasma, and enhance thrombin generation in plasma, in a thrombin- and FXI-dependent manner**

Although multiple studies have reported the activation of FXI by thrombin using purified proteins,\(^8\)\(^-\)\(^12\) some have questioned whether this reaction can proceed to any significant extent in
plasma.\textsuperscript{13-15} We therefore examined whether polyP could accelerate the clotting of plasma in a thrombin- and factor XI-dependent manner. To eliminate interference from the activation of FXI by FXIIa, we used FXII-deficient plasma and also added CTI to inhibit any remaining traces of FXIIa that might be generated. Also, since our previous studies had shown that polyP accelerates the conversion of FV to FVa, which itself can alter the kinetics of thrombin generation,\textsuperscript{30} we supplemented the FXII-deficient plasma with 20 nM FVa, in order to eliminate any contribution of polyP-mediated FV activation to thrombin generation. (FVa was not added to clotting assays performed with a mechanical coagulometer, as pilot studies indicated no impact of added FVa on clot times; data not shown.) And finally, because $\alpha$-thrombin will promptly clot fibrinogen on its own (obfuscating any effects on FXI activation), we added $\beta$-thrombin to the FXII-deficient plasma, since $\beta$-thrombin has greatly diminished ability to clot fibrinogen but retains the ability to activate FXI.\textsuperscript{12}

We performed clotting assays (in a coagulometer) using citrated FXII-deficient plasma containing CTI, to which we added $\beta$-thrombin and polyP, and then measured the time to clot formation following addition of CaCl\textsubscript{2}. Figure 5.5A shows that adding 22mer polyP preparations to such clotting assays does not alter the clotting time, while adding longer polyP polymers (65mer, 101mer, 211mer or 445mer) shortened the clotting times in a concentration-dependent manner. (In control experiments without $\beta$-thrombin, the clotting times were all >500 seconds; not shown.) FXI-deficient plasma (also containing CTI) exhibited prolonged clotting times in this assay, and the clotting times were essentially unaffected by the presence of polyP 445mer (Figure 5.5A). These results show that polyP shortens the $\beta$-thrombin clotting time of plasma in a manner that is dependent on FXI but independent of FXII. This is consistent with the notion that thrombin activates FXI in plasma in a polyP-mediated manner.
We also used the CAT system to examine the ability of polyP to enhance thrombin-mediated thrombin generation in plasma. Figure 5.5B shows mean thrombin generation profiles in FXII-deficient plasma to which 20 nM β-thrombin was added together with polyP (101mer). Increasing concentrations of polyP (up to 50 μM) yielded increased thrombin bursts, while essentially no thrombin generation was observed in the presence of polyP but in the absence of β-thrombin (not shown). In Figure 5.5C, we examined the ability of 50 μM polyP of varying polymer lengths to enhance thrombin generation, and found that polyP caused increased thrombin generation in a polymer size-dependent manner. Figure 5.5D summarizes the mean peak thrombin levels generated in experiments described in Figure 5.5B and C, showing that peak thrombin levels increased with polyP concentration (for the 101mer), and also showing the peak thrombin levels at 50 μM polyP for the 65mer and 445mer polymers. Furthermore, treatment of polyP 445mer with either EcPPXc or rPPX1 abrogated thrombin generation (Figure 5.5D). Figure 5.5E shows that adding a blocking antibody to FXI abolished polyP-mediated thrombin generation in FXII-deficient plasma. Also, there was no observable polyP-mediated thrombin generation in FXI-deficient plasma (Figure 5.5F), although this was restored when purified FXI was added back to the FXI-deficient plasma (Figure 5.5G). Lastly, polyP did not enhance thrombin generation in FXI-deficient plasma to which FXIa was added (Figure 5.5H).

DISCUSSION

Deficiencies of FXII, prekallikrein or high MW kininogen are not associated with bleeding tendencies, while individuals with severe FXI deficiency can exhibit mild to moderate bleeding diatheses, most particularly injury-induced bleeding in tissues with high fibrinolytic activity. This has led to the proposal that the primary role of FXI is not to participate in the
initiation of blood coagulation, but to further thrombin generation, possibly for activation of thrombin-activatable fibrinolysis inhibitor (TAFI) to protect and consolidate the clot.\textsuperscript{31-33} A body of work supports a model in which FXI is activated by thrombin, a process that is accelerated by anionic molecules and surfaces. Furthermore, previous studies have shown that FXI activation by thrombin is enhanced in the presence of activated platelets.\textsuperscript{34-36} We recently showed that polyP of the size secreted by human platelets accelerates blood clotting reactions\textsuperscript{20,30} and binds with high affinity to $\alpha$-thrombin and FXIIa.\textsuperscript{21,29} Taken together, these findings led us to formulate and test the hypothesis that polyP, a natural polyanion secreted by activated platelets,\textsuperscript{19,27} mediates FXI activation by $\alpha$-thrombin.

Using purified proteins, we found that polyP polymers of the sizes secreted by platelets (60-100mers)—and larger—potently accelerated FXI activation by $\alpha$-thrombin, possibly by a template mechanism, with polyP being more active than the nonphysiologic polyanion, dextran sulfate. We also showed that polyP-mediated FXI activation by $\alpha$-thrombin was specifically abrogated by EcPPXc, the isolated polyP-binding domain of \textit{E. coli} exopolyphosphatase, indicating that this recombinant protein may be used to interrupt the procoagulant activity of polyP. Consistent with reports of anionic polymers accelerating FXI autoactivation \textsuperscript{8,17} and autolysis,\textsuperscript{9} we found that polyP polymers >50 phosphate units long strongly enhanced FXI autoactivation. These results suggest that the combination of thrombin and polyP in plasma could result in the generation of additional thrombin through a combination of polyP-mediated FXI activation by thrombin and polyP-mediated FXI autoactivation.

Oliver et al.\textsuperscript{36} demonstrated that FXII-independent activation of FXI by thrombin was augmented in the presence of activated platelets while Wielders et al.\textsuperscript{35} showed that thrombin initiates and augments FXI-dependent thrombin generation in platelet-rich plasma. To date,
however, the platelet-derived cofactor for FXI activation by thrombin, and the underlying mechanism, has not been well defined. In this study, we showed that platelet releasates enhanced FXI activation by α-thrombin, an activity that was not diminished by boiling (to denature potentially confounding proteins, including platelet-derived FXI\(^{34}\)), but that was abrogated by EcPPXc treatment or rPPX1 digestion. Furthermore, suspensions of activated platelets also strongly enhanced thrombin-mediated FXI activation, albeit at rate approximately 2-fold lower than that of platelet releasates. It is possible that binding of some of the thrombin, FXI, and/or FXIa to the surface activated platelets\(^{28}\) could therefore reduce their interaction with polyP, and that this might explain the somewhat reduced rate of FXI activation in the presence of platelets compared to the same concentration of platelet releasate. Taken together, our findings indicate that platelet polyP may be a natural, physiological cofactor for the activation of FXI by thrombin.

The physiological relevance of FXI activation by thrombin in a plasma environment has been questioned.\(^{13,14,37}\) Some criticisms include excessively diluting the plasma in FXI-dependent clotting assays and inadvertent FXI activation by the contact system during blood drawing and isolation of plasma. We addressed these issues using FXII-deficient plasma supplemented with CTI to greatly reduce the possibility of FXI activation by FXIIa. Thrombin generation using CAT assays in minimally diluted FXII-deficient plasma demonstrated substantial thrombin-mediated thrombin generation in the presence of polyP. This thrombin generation required FXI but not FXII.

A recent report suggests that FVa can promote FXI activation by α-thrombin.\(^{10}\) We did not address this question directly in the present study, but we did find that adding 20 nM FVa to FXII-deficient plasma in clotting assays initiated with β-thrombin in the presence of polyP
resulted in no further shortening of clotting time compared to FXII-deficient plasma not spiked with FVa (not shown). In further studies, it will be interesting to examine if polyP can synergize with FV or FVa to accelerate thrombin-mediated FXI activation.

Our previous studies established that polyP acts at three points in the blood clotting cascade, while the present study adds a fourth point of action (summarized in Figure 5.6). Using carefully size-fractionated polyP preparations, we previously established that polyP of the size range that accumulates in many infectious microorganisms (i.e., hundreds to thousands of phosphate units long) potently triggers the contact pathway, accelerates FV activation, and enhances fibrin polymerization (Figure 5.6A). On the other hand, shorter polyP polymers, of the size secreted by activated human platelets (i.e., 60 to 100mers) were far less potent than long-chain polyP in triggering contact activation or in enhancing fibrin polymerization, while retaining full ability to promote FV activation (Figure 5.6B). The present study now shows that polyP preparations of the size secreted by human platelets—as well as longer polymers—potently stimulate the activation of FXI by thrombin as well as FXI autoactivation (Figure 5.6B). Taken together, these findings support the notion that platelet polyP primarily functions to accelerate and enhance thrombin generation, but not to trigger it. On the other hand, microbial polyP can potently trigger the blood clotting cascade via the contact pathway, together with enhancing thrombin generation and fibrin polymerization, possibly as part of the host response to pathogens.

In addition to activated platelets, various injured tissues may also release polyP, as mammalian tissues have been reported to contain polyP in sizes ranging from 50mers to 800mers, with brain containing primarily very long-chain polyP (~800mers). Interestingly, a recent study described a significant reduction in the incidence of ischemic stroke in patients with
severe FXI deficiency,\textsuperscript{39} while another found that homozygous FXI knockout mice are protected against ischemic brain injury in an experimental stroke model.\textsuperscript{40} Our demonstration that polyP is a potent cofactor for FXI activation by thrombin offers a potentially important piece of the puzzle regarding the role of FXI in hemostasis and thrombosis.
Figure 5.1. PolyP enhances FXI activation by α-thrombin. In all panels, initial rates of FXI activation were quantified at 37°C in reactions containing 30 nM FXI, 5 nM α-thrombin, and polyP or dextran sulfate. (Data are mean ± standard error; n=4.) (A) Concentration-dependence of polyP-mediated enhancement of FXI activation by α-thrombin, tested with four different polyP polymer lengths: 22mer (●), 65mer (▲), 167mer (■), and 350mer (◇). (B) PolyP polymer length-dependence of the enhancement of FXI activation by α-thrombin, using size-fractionated polyP preparations at 4 µM phosphate. (“0” indicates no polyP.) (C) EcPPXc
abrogates the ability of polyP, but not dextran sulfate, to enhance FXI activation by \(\alpha\)-thrombin. Rates of FXI activation were quantified in the absence (open bars) or presence (solid bars) of EcPPXc. Reaction conditions included: no polyanion (control), with 1 \(\mu\)g/mL dextran sulfate (DS), or with 4 \(\mu\)M polyP (80mer or 255mer, as indicated).

Figure 5.2. Activated platelets and platelet releasates enhance the rate of FXI activation by \(\alpha\)-thrombin. In all panels, initial rates of FXI activation were quantified at 37°C in reactions containing 30 nM FXI, 5 to 20 nM \(\alpha\)-thrombin, and activated platelets or platelet releasate. (A) Dose-response for platelet releasates from donor A in supporting FXI activation by 5 nM (\(\upDelta\)), 10 nM (\(\Box\)), or 20 nM (\(\bullet\)) \(\alpha\)-thrombin. Controls included: boiled releasate (\(\odot\)) incubated with FXI and 20 nM \(\alpha\)-thrombin; and releasate pre-incubated with 250 \(\mu\)g/mL EcPPXc (\(\bigcirc\)) or predigested with 70 \(\mu\)g/mL rPPX1 (\(\phi\)), then allowed to react with FXI and 20 nM \(\alpha\)-thrombin. (B)
Activated platelets and releasates enhance FXI activation by thrombin. Initial rates of FXI activation by 20 nM α-thrombin were quantified in the presence of 10-fold diluted platelet releasate (solid bars) or the same dilution of activated platelets + releasate (open bars) from donor B, with or without pre-incubation with 250 μg/mL EcPPXc. Controls include FXI incubated with activated platelets (or releasate) from donor B but without α-thrombin, and FXI incubated with α-thrombin but without platelets or releasate. Data are mean ± standard error (n=3-4) from two separate donors. Platelets from donor A were activated at a concentration of 1.6 x 10^7/μL, while platelets from donor B were activated at a concentration of 5.3 x 10^6/μL.

Figure 5.3. PolyP accelerates FXI autoactivation and FXIa autolysis. (A) Progress curves of FXI autoactivation in which 60 nM FXI was incubated with 4 μM polyP 77mer (○), 4 μM polyP 255mer (■), or 2 μg/mL dextran sulfate (□). (B) PolyP polymer length-dependence of the
enhancement of FXI autoactivation. Second-order rate constants for FXI autoactivation ($k_2$) were determined in reactions containing 30 nM FXI and 4 µM polyP of the indicated polymer lengths. (‘0’ indicates the absence of polyP.) (C) SDS-PAGE analyses of FXI autoactivation in the presence of polyP (77mer or 225mer) or dextran sulfate (DS). Parallel timed samples from the experiment in panel B were resolved on reducing SDS-PAGE and silver stained. The position of FXI (Z) and the FXIa heavy chain (HC) and light chain (LC) are indicated. The lane labeled FXIa contained purified FXIa. (D) PolyP polymer length-dependence of the enhancement of FXIa autolysis. Initial rates of loss of FXIa enzymatic activity were quantified in reactions containing 6 nM FXIa and polyP preparations of varying polymer lengths, whose concentrations were adjusted to yield 3 nM polymer. (“0” indicates the absence of polyP.) Data in panels A, B, and D are mean ± standard error (n=3).

Figure 5.4. α-Thrombin, β-thrombin, FXI, and FXIa bind with high affinity to immobilized polyP. Binding of α-thrombin, β-thrombin, FXI, or active-site-inhibited FXIa to polyP was quantified using surface plasmon resonance, with biotinylated polyP bound to
streptavidin sensorchips, over which varying protein concentrations were flowed. Panels are representative sensorgrams for: (A) 2.5 to 20 nM α-thrombin; (B) 5 to 80 nM β-thrombin (C) 1.25 to 40 nM FXI; and (D) 2.5 to 20 nM active-site-inhibited FXIa. $K_d$ values were derived as described in Methods.

**Figure 5.5. PolyP plus β-thrombin accelerates plasma clotting and enhances thrombin generation.** (A) PolyP shortens plasma clotting times triggered by β-thrombin (measured using a mechanical coagulometer). Citrated FXII- or FXI-deficient plasmas containing 100 μg/mL corn trypsin inhibitor and 20 μM PCPSPE were incubated for 1 minute at 37°C with 12 nM β-thrombin and varying polyP concentrations, after which CaCl$_2$ was added and the time to clot formation was measured. PolyP tested with FXII-deficient plasma included: 22mer (●), 65mer (▼), 101mer (◆), 211mer (●), or 445mer (▲). FXI-deficient plasma was tested with 445mer
polyP (△). Data are mean ± standard error (n=5). Panels B and C are mean thrombin generation (CAT) curves for FXII-deficient plasmas containing 100 μg/mL corn trypsin inhibitor, 20 μM PCPSPE and 20 nM FVa (4 experiments of triplicate wells). (B) Concentration-dependence of polyP’s ability to enhance thrombin generation in the presence of 20 nM β-thrombin and varying polyP (101mer at 0-50 μM phosphate). (C) Effect of polyP polymer length on thrombin generation in the presence of 20 nM β-thrombin and with or without 50 μM polyP (65mer, 101mer or 445mer). Panels D-H report mean peak thrombin levels from experiments represented in panels B and C (± standard error; n=4) obtained with 20 nM β-thrombin and varying polyP. (D) Peak thrombin levels in FXII-deficient plasma at the indicated concentrations of polyP 65mer (▽), 101mer (◆), or 445mer (△). In control experiments, polyP 445mer was pre-incubated with 250 μg/mL EcPPXc (◇), or predigested with 40 μg/mL rPPX1 (□). (E) Peak thrombin levels in FXII-deficient plasma preincubated with anti-FXI antibody ± 50 μM polyP 101mer. (F) Peak thrombin levels in FXI-deficient plasma ± 50 μM polyP 101mer. (G) Peak thrombin levels in FXI-deficient plasma to which 4 μg/mL FXI had been added ± 50 μM polyP 101mer. (H) Peak thrombin levels in FXI-deficient plasma to which 50 pM FXIa had been added ± 50 μM polyP 101mer.
Figure 5.6. Summary of the roles of polyP in blood clotting. (A) Long-chain polyP (hundreds to thousands of phosphate units long) acts at four points in the clotting cascade, indicated in red: a, initiates the contact pathway of blood clotting;\textsuperscript{20,30} b, accelerates FV activation;\textsuperscript{20,30} c, enhances fibrin polymerization;\textsuperscript{20,41} and d, accelerates FXI activation by thrombin (this study).

(B) Platelet-size polyP (60 to 100mers) acts most potently at two points in the clotting cascade, indicated in red: b, accelerates FV activation;\textsuperscript{20} and d, accelerates FXI activation by thrombin (this study).

REFERENCES


CHAPTER 6: POLYPHOSPHATE ACCELERATES FACTOR V ACTIVATION BY FACTOR XIa

ABSTRACT

The factor Xa-factor Va prothrombinase complex increases the rate at which prothrombin is activated by approximately 5 orders of magnitude compared to the rate of the reaction by factor Xa alone. Generation of initiating levels of factor Va from factor V, the inactive precursor of factor Va, is a critical event early in hemostasis, as factor V exhibits little or no procoagulant cofactor activity. Alpha-thrombin is the most potent physiological activator of factor V; however, the prothrombinase complexes formed before thrombin generation require a source of factor Va that precedes the initial thrombin formed by the factor Xa-factor Va complex. The alternative route by which initial levels of factor Va are formed has yet to be definitely determined. Although phospholipid-bound factor Xa is an effective activator of factor V, the low concentration of factor Xa during the early phase of coagulation may be insufficient to provide the initiating source of factor Va.

A recent study identified factor XIa as a possible source of the initial thrombin-independent factor V activation prior to recalcification in the widely used activated partial thromboplastin time (aPTT) assay. Activated platelets secrete the highly anionic polymer, polyphosphate (polyP), and our previous studies have shown that polyP has potent procoagulant activity. More recently, we observed that polyP (of the size secreted by human platelets) binds with high affinity to factor XIa and potently accelerates factor XI autoactivation and activation by thrombin. We now present evidence that polyP potently accelerates factor V activation by factor XIa, and that this reaction is supported by polyP polymers of the size secreted by activated human platelets.
INTRODUCTION

Factor V (FV) was discovered by Paul Owren, a Norwegian physician, in 1943 while encountering a patient with a congenital bleeding disorder and a prolonged prothrombin time.¹ The patient’s symptoms could not be ameliorated by the then known four coagulation factors necessary for hemostasis. This deduction allowed Dr. Owren to identify a fifth component required for coagulation that he identified as “factor V.” Subsequent work identified FV as one of the essential components of the complex required for the rapid conversion of prothrombin to thrombin.²⁻⁵ FV is the inactive precursor of FVa that contributes to the blood clotting reaction by binding with factor Xa (FXa) on a membrane surface to form the prothrombinase complex. Removal of FVa from the prothrombinase complex reduces the rate of thrombin generation by four orders of magnitude.³⁻⁴

Human plasma FV circulates at a concentration of 20 nM, as a single-chain procofactor with a molecular weight of 330 kDa.⁶⁻⁷ Approximately 20% of the total human FV found in whole blood is contained in the platelet alpha granules.⁸ The clinical significance of platelet FV is underscored by patients with a deficiency in platelet FV (FV-Quebec⁹ or FV-New York¹⁰) who exhibit a bleeding diathesis or by asymptomatic individuals with potent circulating inhibitory FV antibodies that cannot access platelet FV.¹¹

Congenital FV deficiency (<2% FV clotting activity) in humans is a rare disorder with a reported prevalence of 1 in 1 million. Furthermore, the severity of bleeding in FV-deficient individuals is poorly correlated by FV antigen and activity levels.¹² However, several reports describe FV-deficient patients with mild to severe bleeding disorders consistent of ecchymosis, epistaxis, menorrhagia, and bleeding in the mucosal tracts.⁹,¹³⁻¹⁶ Excessive bleeding has also been reported following trauma, surgery, or dental extraction in FV-deficient patients.¹⁰,¹⁷⁻¹⁹
Single-chain procofactor FV comprises three A domains, a long B domain and two C domains, organized in an A1-A2-B-A3-C1-C2 structure. FV is cleaved by FXa or thrombin at Arg709, Arg1018, and Arg1545. Proteolysis at these sites removes the B domain and releases FVa, which consists of an N-terminal heavy chain (A1-A2, 105 kDa) associated via Ca$^{2+}$ ions to the C-terminal light chain (A3-C1-C2, 74 kDa). Generation of initiating levels of FVa from FV, the inactive precursor of FVa, is a critical event early in hemostasis, as FV is unable to function as a cofactor for FXa catalysis of the conversion of prothrombin to thrombin.

A few proteases with potential physiological significance have been reported to cleave FV including calpain, neutrophil elastase, tissue factor-factor VIIa complex, and platelet-localized proteases. However, not all of the FV cleavage products derived from these proteases are functional in FVa cofactor assays. The alternative route by which initial levels of FVa are formed has yet to be definitely determined.

Recently, Whelihan et al. identified that FXIa can generate a FVa species that exerts cofactor activity in human plasma. Our laboratory has shown that polyphosphate (polyP) accelerates the activation of FV by α-thrombin and FXa. More recently, we have demonstrated that polyP released from activated platelets accelerates FXI activation by FXIa and α-thrombin. In the current study, we demonstrate that polyP potently accelerates FV activation by FXIa. Furthermore, we report that polyP polymers of the size secreted by activated human platelets, as well as releasates from activated platelets, are very active in stimulating FV activation by FXIa. We also observed that FVa species derived from FXIa and polyP exhibit cofactor activity in a purified system. Results from these studies not only provide insight into FV structure and function but also provide an alternative route by which initial levels of FVa are formed during the early phase of coagulation.
EXPERIMENTAL PROCEDURES

Materials

Size-fractionated polyP preparations of very narrow size distributions were prepared as previously described, and referred to by their polymer length followed by “mer” (for example, 167mer). Note that all polyP concentrations reported in this study are given in terms of phosphate monomer concentration (monomer formula: NaPO$_3$). Platelet releasates were prepared as described previously.

Purified FXIa, FV, FVa, and corn trypsin inhibitor (CTI), dansylarginine \(N\)-(3-ethyl-1,5-pentanediyl)amide (DAPA) and anti-human FV heavy chain AHV-5146 monoclonal antibody were from Hematologic Technologies (Burlington, VT). \(\alpha\)-thrombin and FXa were from Enzyme Research Laboratories (South Bend, IN). Rivaroxaban was from Selleckchem (Houston, TX). Peroxidase-conjugated goat anti-mouse immunoglobulin (IgG) and enhanced chemiluminescence (ECL)-plus blotting substrate were from Pierce (Rockford, IL).

Phospholipid vesicles consisting of 20% phosphatidylserine and 80% phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were made by sonication. Spermine and hirudin were from Sigma-Aldrich (St. Louis, MO). Aprotinin was from Calbiochem (San Diego, CA).

Recombinant polyP-binding C-terminus of the \textit{Escherichia coli} exopolyphosphatase (EcPPXc) fused to maltose-binding protein and His$_6$ tag was expressed in \textit{E. coli} BL21 cells and purified as described. Human prothrombin was a kind gift from Dr. Craig Jackson (San Diego, CA).
Western blot analysis of FV activation in the presence of polyP or platelet releasates

Hirudin-treated human plasma containing 100 µg/mL CTI and 50 µg/mL rivaroxaban and varying concentrations of FXIa (0 – 3 nM) were incubated with polyP at 37°C in 30 mM Hepes (pH 7.4), 50 mM NaCl and 0.25% bovine serum albumin. In a purified system, we incubated 20 nM FV and varying concentrations of FXIa (0 – 900 pM) with polyP or platelet releasates at 37°C in 30 mM Hepes (pH 7.4), 50 mM NaCl, 1.2 mM CaCl₂ and 2% BSA. Some platelet releasate samples were boiled for 20 minutes to denature proteins before being used in FV activation assays. Control reactions samples included 500 µg/mL EcPPXc. At various time points, aliquots were removed into SDS sample buffer and resolved by SDS-PAGE (Bio-Rad Laboratories). Proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes and probed with anti-FVa heavy chain antibodies followed by peroxidase-conjugated anti-mouse IgG. Antibody binding was visualized using enhanced chemiluminescence (ECL).

Activation of FV and FVa prothrombinase assay

We incubated 6 nM FV with varying FXIa concentrations (0 – 10 nM) with polyP in 30 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM CaCl₂ and 0.5% BSA at 37°C. After 15 minutes, the reactions were quenched by addition of spermine (200 µg/mL final concentration) to neutralize polyP and aprotinin (10 µM final concentration) to inhibit FXIa. The cofactor activity of the FXIa-derived FV(a) species was measured via its ability to stimulate FXa-catalyzed thrombin generation. FXIa-treated FV was added to mixtures containing PCPS vesicles (400 nM), prothrombin (900 nM), and DAPA (9 µM) in 30 mM Hepes (pH 7.4), 100 mM NaCl, 3 mM CaCl₂, and 0.2% BSA. The reaction was initiated with human FXa (200 pM final) and thrombin generation was measured by the fluorescence intensity caused by the binding of DAPA to
thrombin (excitation at 280nm, emission at 545 nm, and cutoff at 515 nm) using a SpectraMax microplate fluorometer (Molecular Devices). DAPA inhibits thrombin activity, thereby allowing quantitation of FVa-dependent thrombin generation without feedback activation of any unactivated FV. The rates of increase in fluorescence were converted to FVa concentrations using a standard curve. Control experiments indicated that at the concentrations used, neither spermine nor aprotinin affected the prothrombinase assays.

RESULTS

PolyP supports FV cleavage by FXIa

Previous studies have shown that FXIa activates FV although the kinetics are slow.\textsuperscript{29} We hypothesized that polyP may serve as a physiologic cofactor for this reaction. Accordingly, we measured FV cleavage at physiologically relevant concentrations of FV (20 nM) and FXIa (<1 nM) in the presence or absence of polyP. The fragment profile of the purified-protein reactions was visualized by using Western blotting with an antibody directed against the FVa heavy chain. Figure 6.1 shows that the cleavage of intact FV and appearance of the newly-generated FVa heavy chain (105 kDa) was dependent on the presence of both FXIa and polyP (85mer or 211mer). Using SDS-PAGE analysis, the cleaved fragments of FXIa/polyP-treated FV comigrated in an identical fashion with the heavy and light chains of thrombin-derived FVa (data not shown). Additional control experiments in which FXIa was omitted from the reaction showed no cleavage of FV, indicating that the observed activity was not due to protease contamination of the FV preparation.

We next wanted to investigate the physiologic relevance of polyP-accelerated FV activation by FXIa by performing this reaction with endogenous FV in human plasma. To
eliminate interference from feedback activation of FV by $\alpha$-thrombin or FXa, plasma samples were pretreated with rivaroxaban and hirudin. Plasma samples also contained CTI to prevent polyP-mediated contact activation. Consistent with the results in the purified protein assay, the presence of both FXIa and polyP (211mer) in plasma strongly enhanced FV cleavage based on the appearance of the FVα heavy chain (Figure 6.2). These results show that polyP accelerates the activation of FV by FXIa in plasma in a manner that is dependent of FXIa but independent of thrombin, FXa, and FXII. This is consistent with the notion that FXIa activates FV in plasma in a polyP-mediated manner.

**Activated platelet releasates enhance FV activation**

Activated human platelets release polyP with polymer lengths ~ 60 to 100 phosphates,\textsuperscript{34,35} similar to the size range that we found in Figure 6.1 would enhance FV activation by FXIa. We therefore hypothesized that polyP secreted from activated platelets should serve as a cofactor for FV activation by FXIa. Releasates from activated platelets markedly accelerated FV activation in the presence of 4.8 nm FXIa (Figure 6.3). In addition to polyP, activated platelets release a host of granule contents including heat-labile platelet-derived proteases,\textsuperscript{24} so we examined whether this cofactor effect in platelet releasates survived boiling. Figure 6.3 shows boiled platelet releasates augmented FXIa-mediated FV activation as well as nonboiled releasates. On the other hand, treatment of platelet releasates with EcPPXc abolished their ability to enhance FV activation (Figure 6.3) consistent with the idea that polyP secreted from activated platelets is the augmenting cofactor for FV activation by FXIa.
FXIa/polyP-derived FVa exerts cofactor activity in a purified system

To compare the amount of FVa generated by FXIa in the absence or presence of polyP (96mer or 130mer), we employed a purified prothrombinase assay with limiting amounts of FXa (200 pM). The presence of polyP markedly enhanced FVa generation by FXIa, with optimal FV activation observed with at least 1 nM FXIa in the presence of 10 μM of 130mer and at least 3 nM FXIa in the presence of 10 μM 96mer (of the size secreted by activated platelets). In the absence of polyP, FXIa-generated FVa was not detectable with FXIa concentrations lower than 4 nM.

DISCUSSION

Results from this study confirm and extend previous observations of FXIa-mediated activation of FV. The use of DAPA-based prothrombinase assay provides evidence that polyP markedly accelerates FXIa-mediated generation of functional FVa cofactor species that effectively converts prothrombin to thrombin in the presence of FXa and phospholipid. Furthermore, we tested the capacity of polyP to augment FXIa-mediated activation of FV in both a purified system and in plasma using Western blot analysis. The cleavage pattern of FXIa-derived FVa was similar to that generated by α-thrombin, evident by the appearance of the FVa light and heavy chain.

The generation of an active FVa cofactor species which effectively functions in the prothrombinase complex is a critical component of the coagulation cascade. The physiologic significance of FVa for clot formation is clearly demonstrated in mice with complete deficiency of FV results in massive hemorrhage and death during mid-embryogenesis. Furthermore, FV-deficient patients suffer moderate to severe bleeding, though residual FV activity is detectable in nearly all cases.
The B-domain of procofactor FV is thought to prevent premature procoagulant activity prior to proteolytic processing by sterically blocking binding sites on intact FV.\textsuperscript{39,40} Evidence for this notion came from studies using a B-domain-truncated FV derivative that exhibit constitutive cofactor activity without intentional proteolysis.\textsuperscript{39,41} Subsequent work by Zhu et al. revealed a highly basic region within the B-domain that is conserved among most vertebrates whose deletion yielded derivatives with cofactor-like properties in the absence of proteolysis.\textsuperscript{42} These findings imply that the basic region of the B-domain serves as an inhibitory function, which, under normal physiological conditions, is efficiently removed upon proteolytic processing.\textsuperscript{43,44}

We previously demonstrated that polyP accelerates FV activation by thrombin and FXa.\textsuperscript{30} Taken together with our current findings that polyP and activated platelets augment FV activation by FXIa, we speculate that platelet polyP may interact tightly with FV. It is tempting to propose that polyP is involved in this release-from-inhibition mechanism by interacting with or facilitating the removal of the highly-basic inhibitory sequences within the B-domain. Additionally, platelet polyP may explain an explanation for reports that platelet FV is stored in a partially activated form which already expresses FVa cofactor activity prior to exposure to FXa or thrombin, stored in alpha granules.\textsuperscript{8,45} Future work addressing these hypotheses would provide new insights into the mechanism by which polyP accelerates FV procofactor activation at the onset of contact pathway-initiated blood coagulation.
Figure 6.1. FV cleavage by FXIa in the presence of polyP. 20 nM FV was incubated with 10-900 pM FXIa for 10 minutes at 37°C in the absence or presence of polyP of 2 different polymer lengths: 85mer and 211mer (indicated at the top of the panel). Reaction samples were subject to SDS-PAGE and Western blot analysis. FVa was detected with a monoclonal anti-FV heavy chain antibody. The position of the factor Va heavy chain (HC) is indicated.
Figure 6.2. **FV cleavage by FXIa in the presence of polyP.** Hirudin-treated plasma containing 100 μg/ml corn trypsin inhibitor and 50 μg/ml rivaroxaban was incubated with 0.25-3 nM FXIa for 10 minutes at 37°C in the absence or presence of polyP (211mer). Reaction samples were subject to SDS-PAGE and Western blot analysis. FVa was detected with a monoclonal anti-FV heavy chain antibody. The position of the factor Va heavy chain (HC) is indicated.

Figure 6.3. **FV cleavage by FXIa in the presence of platelet releasates.** 20 nM FV was incubated with 4.8 nM FXIa for 10 minutes at 37°C in the absence or presence of varying ratios of platelet releasate (PR) in FV activation reaction. Controls include boiled releasate and treatment of platelet releasates with EcPPXc (the isolated polyP-binding domain of E. coli exopolyphosphatase).
Figure 6.4. Prothrombinase activity of FVa generated by FXIa and polyP. 6 nM FV was incubated with varying concentrations of FXIa in the absence (red circles) or presence of polyP (96mer or 130mer) for 15 minutes at 37°C. Reactions were stopped by addition of spermine to neutralize polyP and aprotinin to inhibit FXIa. Generated FVa was measured via its ability to stimulate FXa-catalyzed thrombin generation in the presence of DAPA.

REFERENCES


CHAPTER 7: CONCLUSIONS AND PERSPECTIVES

Similar to other activators of the contact pathway, polyphosphate (polyP) is a highly anionic polymer, a property which likely facilitates its binding to the anion-binding sites of high molecular weight kininogen (HK) and factor XII (FXII). PolyP accumulates in a variety of pathogenic microorganisms and is secreted by activated human platelets. The findings reported in this thesis add to our knowledge of how polyP modulates blood clotting. To date, we have identified that polyP is a potent modulator of the human blood clotting system, acting at 5 points: It initiates the contact pathway of blood clotting in a FXII-dependent manner (requiring very long polyP polymers for optimal activity); it accelerates the activation of factor V (FV) by thrombin and factor Xa (FXa); it enhances the thickness of fibrin fibrils; it accelerates factor XI (FXI) activation by thrombin and FXIa; and it accelerates factor V (FV) activation by FXIa (reported in chapter 6 of this thesis). Figure 7.1 summarizes the roles of polyP depending on polyP length. These findings therefore have the potential to explain previously unexplained abilities of activated platelets to enhance blood clotting reactions. It is likely that polyP has other, as-yet undiscovered, roles in blood clotting.

PolyP’s ability to trigger the contact pathway exhibits a profound dependence on polymer length, with optimal specific activities requiring very long polyP polymers (Chapter 2). Consistent with this finding, polyP purified from Salmonella is extremely potent in triggering the contact pathway. We also found that while platelet-derived polyP—and synthetic polyP of the

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same size (i.e., 60-100mers)—could trigger clotting via the contact pathway,\textsuperscript{7,9} it is thousands of times less potent than very long-chain polyP (1000-2000mers).\textsuperscript{8} These findings provide an explanation for reports, dating back to the 1960s, that activated human platelets express a weak but measurable ability to trigger the contact pathway in a FXII-dependent manner (reviewed by Caen and Wu\textsuperscript{12}). However, the low specific activity of platelet polyP toward the contact pathway is consistent with the idea that platelets are much more effective at accelerating clotting reactions than they are at initiating clotting.

Chapter 5 reports our finding that polyP potently accelerates FXI activation by both thrombin and FXIa (i.e., FXI autoactivation).\textsuperscript{11} PolyP of the size secreted by platelets (~60 to ~100 phosphate units long) strongly supports these reactions. We also found that platelet releasates strongly promote FXI activation by thrombin, and we showed that this activity is due to polyP.\textsuperscript{11} PolyP binds to both thrombin and FXI, and FXI activation by thrombin exhibits a bell-shaped concentration dependence on polyP, consistent with a template-based mechanism.\textsuperscript{7,11,13} Chapter 6 demonstrates that platelet-size polyP also supports FV procofactor activation by FXIa. These studies therefore show that platelet polyP modulates normal hemostasis independent of FXII.

In addition to promoting and enhancing clot formation, we recently demonstrated that polyP induces bradykinin-mediated capillary leakage,\textsuperscript{9} which is a hallmark of inflammatory reactions. The clear contributions of polyP to thrombus formation and inflammation suggest that targeting polyP may be an attractive approach for identifying novel antithrombotic/anti-inflammatory agents. In fact, digesting polyP with phosphatases, which enzymatically degrade polyP, abrogated activation of the contact system and bradykinin generation, abolished procoagulant platelet activity, and blocked platelet-induced thrombosis in mice.\textsuperscript{9} Employing
phosphatase as an antithrombotic or anti-inflammatory agent is limited by the relatively slow reaction rates and high doses of phosphatase necessary to efficiently digest polyP. Therefore, it would be highly advantageous to have a small-molecule inhibitor that rapidly and tightly binds to polyP, thereby blocking its procoagulant and proinflammatory functions.

In chapter 4, we characterized small-molecule and protein-based polyP inhibitors with respect to their ability to specifically inhibit polyP binding to clotting factors and abrogate polyP-mediated clotting reactions in vitro and ex vivo. We identified that the isolated polyP binding domains of *E. coli* exopolyphosphatase (EcPPXc) specifically abrogates the procoagulant function of polyP. We further demonstrated that gen. 1 dendrimer and polymyxin B are both effective at reducing thrombus growth in mice and that gen. 1 dendrimer attenuates the proinflammatory effects of polyP in vivo. These observations illustrate the ability of a small molecule or protein to directly interfere with polyP-mediated inflammatory and thrombotic reactions by targeting specific polyP-protein interactions.

It would be advantageous to extend the studies from chapter 4 and engineer polyP-binding proteins with increased affinity for polyP. This can be explored by creating dimers or multimers of the isolated polyP-binding domains of *E. coli* EcPPXc, as a way of increasing their effective binding affinity for polyP. These dimeric proteins can be produced recombinantly by linking their coding sequences with an oligopeptide spacer or by chemical crosslinking. The binding affinities of oligomeric forms of polyP-binding proteins will be evaluated by Biacore analyses, and also by their potencies in reversing the procoagulant activities of polyP in clotting assays and in abrogating thrombin binding to polyP. If this approach alone does not result in sufficiently enhanced polyP binding affinities, phage display technique (using immobilized
polyP during the screening phase) can be employed to select mutants of these binding proteins with enhanced affinity for polyP.

For a variety of experiments, it would be desirable to be able to covalently attach biotin, epitope tags, dyes, fluorophores, etc., to polyP, and also to covalently immobilize polyP onto solid supports such as magnetic beads and multiwell plates. PolyP can be immobilized onto zirconia beads via Lewis acid/base interactions — a method the Morrissey lab has used to quantify exosite II-mediated thrombin binding to immobilized polyP. Unfortunately, this coupling chemistry is not suitable for attaching other sorts of probes to polyP. Chapter 3 identifies reaction conditions under which the terminal phosphates of polyP can be made to enter into stable phosphoramidate linkages with almost any primary amine-containing compound, using the zero-length coupling reagent, EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide). We have successfully used this coupling chemistry to covalently attach probes such as biotin, fluorophores, and polyamines to the terminal phosphates of polyP, and we have successfully coupled polyP to a variety of solid supports. This opens up a number of possibilities, including: identifying polyP-binding proteins in biological samples; developing high-throughput screens for polyP inhibitors; using biotin- or fluorophore-tagged polyP to visualize polyP in vitro and in vivo (including flow cytometry); and developing therapeutics based on coupling polyP to solid supports (such as wound dressings) or to targeting molecules or nanoparticles.

Our laboratory recently reported that polyP of approximately the size released by activated platelets can reverse the anticoagulant activity of a variety of anticoagulants, including unfractionated and low MW heparins, as well as direct inhibitors of thrombin and FXa. PolyP can also shorten the clotting times of plasma from patients with hemophilia A or B, or patients
taking vitamin K antagonists.\textsuperscript{17} It is thus tempting to speculate that polyP of the size secreted by human platelets – or suitable polyP derivatives – might be useful as injectable hemostatic agents. Long-chain polyP may have utility as a topical hemostatic agent, and in fact, adding polyP to chitosan-based wound dressings made them substantially more procoagulant.\textsuperscript{18}

Oscar Ratnoff, the late discoverer of FXII, proposed the concept that blood coagulation, fibrinolysis and inflammation are intimately related via surface contact, which he called “a seamless web of host defense reactions.”\textsuperscript{19} Together, the studies reviewed above provide evidence that polyP is a key player in the web of host-pathogen interactions. Indeed, long-chain microbial polyP is a potent activator of the blood clotting system via the contact pathway and can trigger both thrombosis and inflammation (the latter via bradykinin generation and possibly complement activation). The clear contributions of polyP to thrombus formation and inflammation suggest that antagonizing polyP function in vivo may be an attractive approach for identifying novel antithrombotic/anti-inflammatory agents, perhaps with reduced bleeding side effects compared with conventional anticoagulant/antithrombotic drugs. Finally, the detailed molecular mechanisms by which polyP acts as such a potent modulator of blood clotting and inflammation are still largely unknown, so this will be a fruitful and interesting topic for much future research.
Figure 7.1. The roles of polyP in blood clotting vary depending on polymer length. (A) Microbial long-chain polyP (ranging from less than a hundred phosphates to several thousand phosphate units long), acts at five points in the clotting cascade, indicated in red: 1, initiates the contact pathway of blood clotting; 2, accelerates FV activation and abrogates TFPI function (the latter not shown explicitly); 3, enhances fibrin polymerization; 4, accelerates FXI back-activation by thrombin; and 5, supports FV activation by FXIa (Chapter 6). (B) Platelet-size polyP (~60 to ~100 phosphate units long) acts most potently at three points in the clotting cascade, indicated in red: 2, abrogates TFPI function (and overlaps the minimal size necessary to accelerate FV activation); 3, overlaps the minimal size necessary to enhance fibrin polymerization; 4, accelerates FXI back-activation by thrombin; and 5, supports FV activation by FXIa.
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