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Polyphosphate: a link between platelets, coagulation and inflammation

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Abstract

Inorganic polyphosphate (polyP) is abundant in biological organisms. PolyP is a major component of dense granules of human platelets and is secreted upon platelet activation. Studies from our lab and others have shown that polyP is a potent modulator of the blood clotting cascade, acting as a pro-hemostatic, prothrombotic and proinflammatory agent depending on its polymer size and location. PolyP may represent at least one of the long-sought (patho)physiologic activators of the contact pathway of blood clotting, and its actions may also help to explain previously unexplained abilities of activated platelets to enhance plasma clotting reactions. PolyP may have utility as a hemostatic agent to control bleeding, and conversely, polyP antagonists might have utility as antithrombotic/anti-inflammatory agents with reduced bleeding side effects. The detailed molecular mechanisms by which polyP modulates blood clotting reactions still remain to be elucidated.

1 Introduction

Polyphosphate (polyP) has a very simple structure, consisting of a linear polymer of inorganic phosphates linked together via high energy phosphoanhydride bonds (Fig. 1). PolyP is ubiquitous in biology, being found in all kingdoms of life and possibly even predating life on this planet [1]. PolyP can vary in length from just a few phosphates to several thousand phosphate units long, depending on the organism and the tissue in which it is synthesized [1, 2]. The most extensive studies of polyP biology have been conducted on prokaryotes and unicellular eukaryotes. PolyP is synthesized enzymatically by transferring the γ phosphate from ATP to the end of the growing polyP chain, and in the prokaryotes in which it has been studied, this reaction is fully reversible and may allow the bacteria to synthesize ATP from stored polyP in times of starvation and environmental stress [3]. PolyP is degraded by both endopolyphosphatases and exopolyphosphatases, and in fact, mammalian alkaline phosphatase is a very active exopolyphosphatase [4]. In human blood or plasma, polyP has a half-life of about 1.5 to 2 hours, owing to the action of phosphatases [5, 6]. Biological roles of polyP have been studied most extensively in prokaryotes and unicellular eukaryotes, in which it has been shown to be important for responses to environmental stresses and virulence in some infectious microorganisms [1]. Unfortunately, in spite of its ubiquity, much less is known about roles of polyP in mammals. This is now changing, with recent studies implicating polyP in cell proliferation [7], angiogenesis [8], apoptosis [9], osteoblast function [10] and, as outlined in this review, blood clotting and inflammation [5, 11–18]. Indeed, recent studies from our lab and others have shown that polyP acts at the beginning, middle, and end of the blood clotting cascade (Fig. 2), with pro-hemostatic, prothrombotic and proinflammatory effects.

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2 PolyP and platelets

In both prokaryotes and unicellular eukaryotes, polyP is typically packaged along with divalent metal ions such as Ca^{2+}, Mg^{2+} and Zn^{2+} in subcellular organelles termed acidocalcisomes (also called volutin or metachromatic granules in some organisms) [19]. In 2004, Ruiz et al. [20] noted striking similarities between acidocalcisomes and platelet dense granules, as both are spherical, acidic [21], electron-dense [22], and contain relatively large quantities of Ca^{2+}, Mg^{2+} and Zn^{2+} [23]. While platelet dense granules (sometimes also called delta granules) were already known to contain inorganic phosphate (P_i) and pyrophosphate (PP_i) [23], no one had previously examined them for polyP. This prompted Ruiz et al. [20] to purify dense granules from human platelets, discovering that these granules contain abundant levels of polyP, at a concentration of about 130 mM polyP inside the granules (expressed as concentration of phosphate monomer). Platelet polyP has a rather narrow size distribution, ranging from about 60 to 100 phosphate units long [13, 20]. (Microbial polyP, on the other hand, is much more heterodisperse and can be hundreds or even thousands of phosphate units long [3, 24].) When platelets are activated, they secrete polyP along with the other contents of their dense granules [13, 20], in amounts that could easily reach micromolar concentrations in thrombi.

3 The beginning: PolyP triggers the contact pathway of blood clotting

Given that activated platelets secrete polyP, we hypothesized that polyP might contribute in some way to blood coagulation. Accordingly, we obtained synthetic polyP of roughly the size range secreted by human platelets and examined its ability to modulate blood clotting. We immediately found that polyP is strongly procoagulant, dramatically shortening the “recalcification” clotting time of citrated human plasma in the absence of any other triggering agent of blood clotting [5]. Our detailed studies showed that polyP did this by activating what is called the contact pathway of blood coagulation (step 1 in Fig. 2a).

There are two major mechanisms for initiating the blood clotting cascade: the tissue factor (or “extrinsic”) pathway and the contact (or “intrinsic”) pathway. The tissue factor pathway, which is essential for normal hemostasis, is triggered when blood is exposed to tissue factor, an integral membrane protein expressed by many cell types outside the vasculature [25]. The contact pathway, on the other hand, is dispensable for hemostasis since humans and animals with genetic deficiencies in the proteins that trigger this pathway do not exhibit bleeding tendencies [26]. The contact pathway is so named because it is triggered when factor XII, prekallikrein and high molecular weight kininogen come into contact with certain types of negatively charged surfaces or polymers, including glass, powdered clay, diatomaceous earth or dextran sulfate. Interaction of the contact factors with such polymers/surfaces initially triggers factor XII autoactivation, which in turn sets off a series of reactions that include the reciprocal activation of zymogens factor XII and prekallikrein, as well as cleavage of high molecular weight kininogen to release bradykinin (a potent vasoactive peptide). Once factor XIIa (the enzymatically active form of factor XII) is formed in sufficient amounts, it converts factor XI to XIa, which in turn activates factor IX, leading to propagation of blood clotting through the final common pathway. Interestingly, although the contact pathway is not required for hemostasis, it has nevertheless been shown to participate in thrombotic diseases, since mice deficient in factor XII are protected against thrombus formation in a variety of models of arterial and venous thrombosis [27, 28]. Furthermore, deletion of the factor XII gene in mice results in defective immune responses to infection [29], suggesting that the contact pathway’s main physiologic role may be in host responses to pathogens. Consistent with this concept, several microbial activators of the contact pathway have been identified, including certain bacterial surface proteins [30, 31] as well as lipopolysaccharide [32]. Our studies would add long-chain microbial polyP to this list [5,

15]. Components of the contact pathway have also been implicated in fibrinolysis [33], complement activation [34], angiogenesis [35], and kinin formation [36]. These findings are consistent with the idea that the contact pathway of blood clotting is much more important for inflammation and host responses to pathogens than it is for normal hemostasis.

Similar to other known activators of the contact pathway, polyP is a highly anionic polymer, and we have shown that polyP binds tightly to the proteins responsible for initiating the contact pathway [5, 15, 37]. Furthermore, activation of clotting by polyP exhibits a bell-shaped concentration-dependence, consistent with its functioning as a template for the assembly of contact factors [5, 15]. We have carefully investigated the polymer length-dependence of polyP's procoagulant activities and found that optimal triggering of the contact pathway requires very long polyP polymers (> 1000 phosphate units long; see Fig. 3) [15]. These are the sorts of polymer lengths of polyP that accumulate in microorganisms, and indeed, we found that polyP purified from *Salmonella* was extremely potent in triggering blood clotting via the contact pathway [15]. (Interestingly, bacteria can alter the size and abundance of their polyP based on environmental cues [24].) Thus, while platelet-derived polyP — and synthetic polyP of the same size (i.e., 60–100mers) — can trigger clotting via the contact pathway [5, 13], it is thousands of times less potent at doing this compared to very long-chain polyP (1000–2000mers) [15]. These findings are consistent with the idea that platelets (and polyP secreted from platelets) are much more effective at accelerating blood clotting reactions than at initiating clotting, an idea that is developed further in the next section (see also Fig. 2).

4 The middle: PolyP accelerates thrombin generation

Like most clotting proteins, coagulation factor V circulates in plasma as an inert precursor (in the case of factor V, as an inert protein procofactor). When converted to the active cofactor, factor Va occupies a central place in the blood clotting cascade, serving as the essential cofactor for prothrombin activation by factor Xa. We found that polyP accelerates the proteolytic conversion of factor V to Va by both factor Xa and thrombin (a type of feedback activation) [5], although the mechanism by which polyP does this is currently not known. Studies that have addressed the relative abilities of factor Xa and thrombin to back-activate factor V to Va have generally concluded that factor Xa is not a significant activator of factor V at low levels of tissue factor [38] and at physiologic concentrations of clotting factors [39]. When we examined the polymer-length dependence of polyP's ability to accelerate factor V activation, we found that it required much shorter polymers than are required to trigger the contact pathway, and in fact, platelet-size polyP can strongly support this reaction (Fig. 3). Thus, polyP secreted from activated platelets might play an important role in accelerating factor V activation by factor Xa and/or thrombin during clotting in vivo.

PolyP's ability to accelerate the rate of factor V activation has some interesting consequences for blood clotting reactions [5]. When platelet-sized polyP is added to plasma, and clotting is initiated with tissue factor, we observed an earlier thrombin burst than in the absence of polyP [5], and we also found that the anticoagulant function of tissue factor pathway inhibitor (TFPI) was totally abrogated (Fig. 3) [5, 15]. TFPI, a multifunctional serine protease inhibitor, is the primary inhibitor of tissue factor-initiated blood clotting, with one of its most important targets thought to be newly generated factor Xa that has not yet dissociated from the tissue factor/factor VIIa complex [40]. Furthermore, previous studies have shown that factor Xa is protected from inhibition by TFPI when factor Xa is bound to its cofactor, factor Va, and especially when in the presence of its substrate, prothrombin [41]. In fact, we showed that just adding exogenous factor Va to plasma abrogates TFPI anticoagulant activity [5]. We also showed that platelet releasates effectively inhibit TFPI function and that this activity is due to the presence of polyP in the releasates

[5, 13]. Furthermore, polyP polymers of the size secreted by platelets are optimal at blocking TFPI activity (Fig. 3) [15]. And finally, we showed that platelets from patients with Hermansky-Pudlak syndrome (which lack dense granules) supported reduced plasma clotting but that this activity could be restored by adding exogenous polyP to mixtures of such activated platelets and plasma [13].

Recently, extracellular histones have been shown to have potent proinflammatory and procoagulant activities. PolyP substantially augmented the procoagulant activity of histones, resulting in enhanced platelet activation and thrombin generation in manner that is independent of factor XII or tissue factor [16]. The mechanism by which polyP augments this activity of histones is not known.

Taken together, these studies show that polyP secreted by platelets can profoundly alter the regulation of the blood clotting system.

5 The end: PolyP alters the structure and stability of fibrin clots

Adding polyP to clotting reactions results in alterations to the physical structure of fibrin clots, generating thicker fibrin fibrils that are more resistant to fibrinolysis (Fig. 3) [12, 18]. Clots can be generated and studied in vitro simply by cleaving purified fibrinogen with thrombin; the resulting fibrin monomers then spontaneously associate to form a three-dimensional fibrin clot. We found that, when we also included polyP and plasma concentrations of Ca^{2+}, the resulting fibrin clots were more turbid, had fibrils with higher mass/length ratios, and were more resistant to elastic stretching and fibrinolysis than clots formed under identical conditions but without polyP [12]. We only observed this effect on fibrin clots if we preincubated the polyP, fibrinogen, and Ca^{2+} together before adding thrombin, suggesting that polyP interacts directly with fibrin(ogen) in a Ca^{2+}-dependent manner. When we prepared such clots and then washed them extensively with buffer, they still stained strongly with toluidine blue (a dye that binds tightly to polyP), and exhibited the metachromatic staining with toluidine blue that is characteristic for polyP [12]. This indicates that polyP is incorporated in some way into the fibrin clot, although the mechanism by which polyP alters clot structure is not known. Heparin – another highly anionic polymer — also increases fibrin clot turbidity [42], but unlike the case with polyP, clots formed in the presence of heparin are *more* susceptible to fibrinolysis [43, 44]. Thus, polyP alters fibrin clot structure in a manner that is very different from that of heparin or other anionic polymers that have been tested [45]. When we examined the polyP size-dependence for enhancing fibrin clot structure, we found that it was optimal with polyP polymer sizes that were larger than those secreted by platelets, but well within the range of polyP polymers that accumulate in infectious microorganisms (Fig. 3) [15].

In studies using purified fibrinogen, polyP and thrombin, Mutch et al. [18] showed that polyP attenuates fibrinolysis by inhibiting the interaction of tissue-type plasminogen activator and plasminogen with fibrin, possibly as a consequence of polyP binding to C-terminal lysine residues in fibrin and thereby blocking their association with fibrinolytic proteins. We also showed that polyP 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 [5].

6 From the end to the beginning again: Platelet polyP enhances factor XI activation by thrombin

In the classic model of blood clotting, factor XI is activated by factor XIIa. Severe factor XII deficiency is not associated with bleeding tendencies in humans or mice. On the other hand,

humans with severe congenital factor XI deficiency may exhibit significant bleeding diatheses, particularly after surgery or trauma [46]. Therefore, factor XI must be activated in vivo by a protease other than factor XIIa. This mystery was potentially solved by the finding that thrombin — the last protease in the clotting cascade — can up-regulate its own generation by feeding back to activate factor XI [47, 48], possibly leading to sustained thrombin generation and decreased fibrinolysis via activation of TAFI. A problem with this proposal is that rates of factor XI activation by either thrombin or factor XIa (i.e., factor XI autoactivation) are extremely slow unless nonphysiologic polyanions such as dextran sulfate, heparin or high levels of sulfatides are employed [47–50]. This had led some to question the physiologic significance of back-activation of factor XI by thrombin in vivo [51]. We were able to offer a solution to this conundrum by finding that polyP very potently accelerates factor XI activation by both thrombin and factor XIa [17]. Furthermore, polyP polymers of the size secreted by activated human platelets are highly active in stimulating factor XI activation, both in purified systems and in human plasma (Figs. 2 and 3). We also showed that activated platelets and platelet releasates strongly promote factor XI activation by thrombin, and that this activity is attributable to the presence of polyP in the releasates [17]. PolyP binds with high affinity to both thrombin and factor XI, and the ability of polyP to promote factor XI activation by thrombin exhibits a bell-shaped concentration dependence, indicative of a template-based mechanism [5, 14, 17, 37]. Thus, polyP is a natural cofactor for factor XI activation by thrombin and factor XIa, which may provide a key missing piece to the puzzle of how factor XI contributes to normal hemostasis independent of factor XII.

7 Contributions of polyP to inflammation and thrombosis

A recent study from the Renné laboratory and colleagues (including our lab) investigated in vivo roles for polyP using mouse models of inflammation and thrombosis [13]. Administering large quantities of polyP intravenously to wild-type mice led to lethal pulmonary embolism, but the majority of factor XII-deficient mice survived the challenge, as did wild-type mice pretreated with a protein-based inhibitor of factor XII (CSL829). In other experiments, platelets were systemically activated in vivo in wild-type mice by intravenous injection of an agonist peptide that stimulates platelets via protease-activated receptors (PARs), causing the death of most of the mice by pulmonary embolism. Factor XII-deficient mice were protected from this otherwise lethal challenge, and wild-type mice were also largely protected when large amounts of alkaline phosphatase were infused prior to administration of agonist peptide. (Alkaline phosphatase is also a potent exopolyphosphatase [4].) These experiments demonstrate that polyP can be thrombogenic in a factor XII-dependent manner [13].

Similar to many other activators of the contact system, adding polyP to plasma in vitro led to proteolysis of high MW kininogen with concomitant release of the highly vasoactive peptide, bradykinin [13]. In a Miles edema model, wild-type mice were injected subcutaneously with polyP, which provoked substantial (localized) capillary leak as visualized by extravasation of Evans blue dye. Mice that were homozygous for knockout of the genes for either factor XII or the bradykinin B_2 receptor were largely protected from polyP-induced edema, indicating that injected polyP induces capillary leak via localized factor XII-dependent bradykinin generation. Pharmacologic inhibitors of factor XIIa also protected wild-type mice from polyP-induced edema. In a different model, intraperitoneal injection of *E. coli*-derived polyP into wild-type mice led to a rapid drop in systemic arterial blood pressure (from 109 ± 17 to 57 ± 39 mm Hg) and death of 90% of the mice in 15 minutes. Mice homozygous for knockout of genes for either factor XII or bradykinin B_2 receptors mostly survived this challenge [13]. These experiments show that polyP —

especially long-chain polyP derived from bacteria — can be strongly proinflammatory in vivo, in a manner that depends on factor XII activation and bradykinin generation.

8 Conclusions and future prospects

The recent studies discussed in this review have shown that polyP secreted by activated platelets can play important roles in normal hemostasis, largely by accelerating clotting reactions at the level of the activation of factors V and XI (Fig. 2b). These findings with polyP therefore have the potential to explain previously unexplained abilities of activated platelets to enhance blood clotting reactions [52]. Furthermore, we have found that polyP of approximately the size released by activated platelets can reverse the anticoagulant activity of a variety of anticoagulants, including heparins as well as direct inhibitors of thrombin and factor Xa, and they can also shorten the plasma clotting times of patients with hemophilia A or B, and patients taking vitamin K antagonists [11]. This raises the possibility that polyP of the size secreted by human platelets could possibly be used as an injectable hemostatic agent. On the other hand, very long-chain polyP is a potent activator of the blood clotting system via the contact pathway (Fig. 2a), and can trigger both thrombosis and inflammation (the latter via bradykinin generation). These findings suggest that microbial polyP may play a role in host responses to pathogens. It is intriguing to think that long-chain polyP, perhaps immobilized covalently [37] onto wound dressings, might have utility as a topical hemostatic agent to control bleeding. Indeed, Ong et al. [53] have reported that adding polyP to chitosan-based wound dressings resulted in dressings that were considerably more hemostatically active. PolyP may therefore have promise as a therapeutic agent to treat bleeding. On the other hand, drugs or other agents that specifically block polyP's procoagulant and proinflammatory effects may have utility as improved antithrombotic agents, possibly with reduced bleeding side effects relative to conventional anticoagulant drugs. And finally, the molecular mechanisms by which polyP acts as such a potent modulator of the blood clotting system are still largely unknown, so this should be a fruitful and interesting topic for further research.

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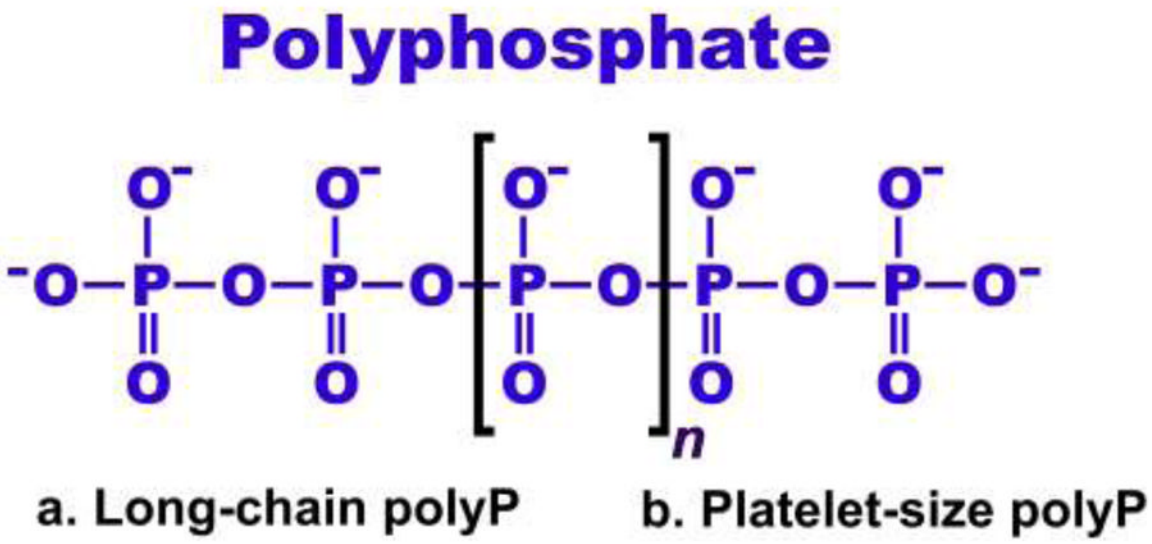

Fig. 1.
Structure of inorganic polyP. PolyP from biological sources can range in length from just a few phosphates to thousands of phosphate units long [3, 24].

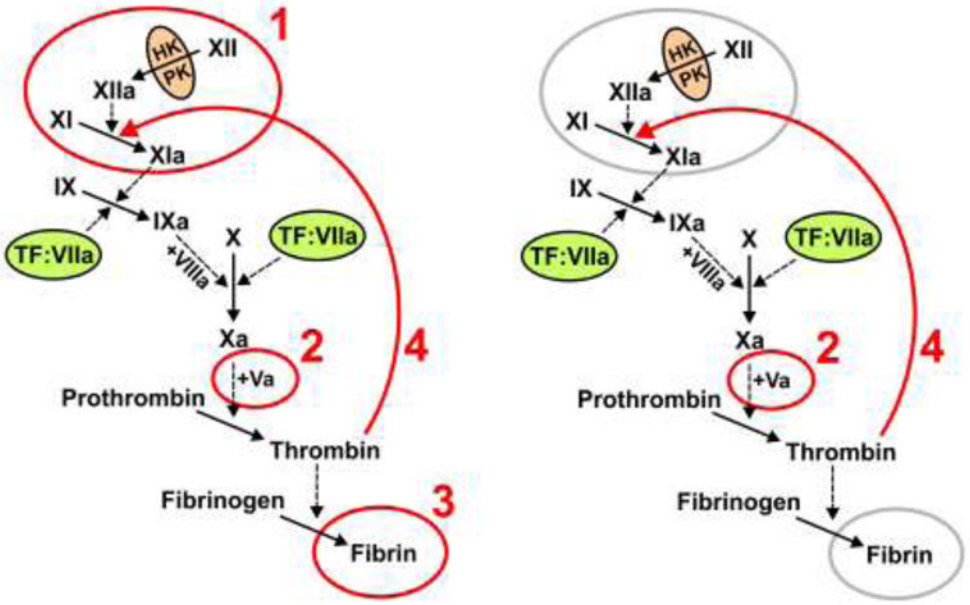

Fig. 2.

Differential roles of long-chain polyP and platelet-size polyP in blood clotting. **a** Long-chain polyP, such as can be found in microorganisms (i.e., hundreds to thousands of phosphate units long), acts at four points in the clotting cascade, indicated in red: 1, initiates the contact pathway of blood clotting [5, 13, 15]; 2, accelerates factor V activation (and abrogates TFPI function) [5, 15]; 3, enhances fibrin polymerization [12, 15, 18]; and 4, accelerates factor XI back-activation by thrombin [17]. **b** Platelet-size polyP (~60 to 100 phosphate units long) acts most potently at two points in the clotting cascade, indicated in red: 2, accelerates factor V activation (and abrogates TFPI function) [5, 15]; and 4, accelerates factor XI back-activation by thrombin [17]. (This research was originally published in *Blood*. S.H. Choi, S.A. Smith, and J. H. Morrissey. Polyphosphate is a cofactor for the activation of factor XI by thrombin. *Blood*. 2011;118:6963–6970. © the American Society of Hematology.)

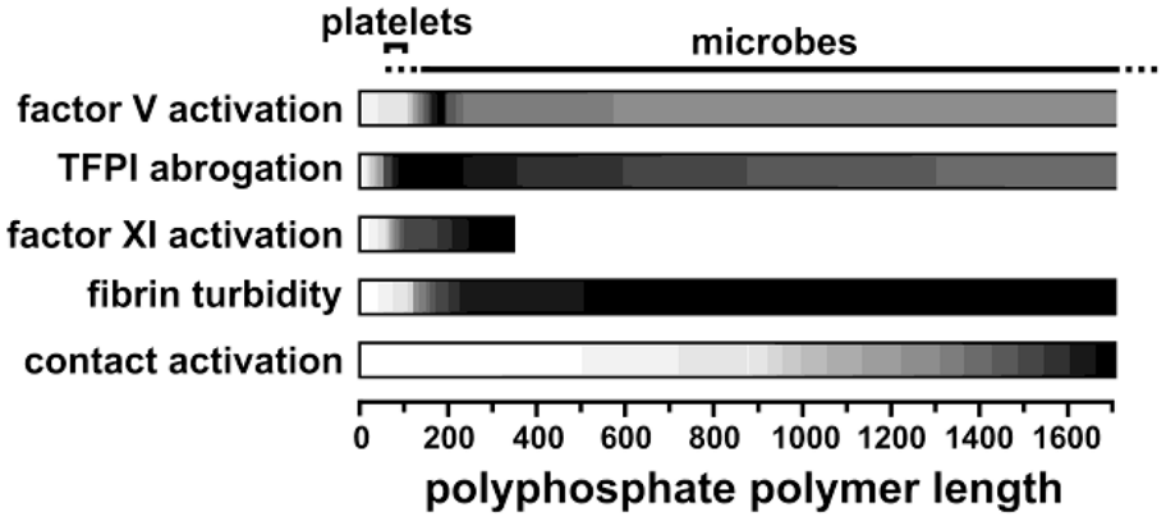

Fig. 3.

Graphical summary of the polymer length-dependence of the procoagulant activities of polyP. The normalized specific activities of polyP of the indicated polymer lengths are depicted by the darkness of the bar, with completely black indicating 100% maximal activity and white indicating 0% activity. Data are replotted from Smith et al. [15] for factor V activation, TFPI abrogation, fibrin turbidity and contact activation; and from Choi et al. [17] for factor XI activation. (For factor XI, polyP polymer lengths greater than 350mers were not tested). The indicators at the top of the figure labeled “platelets” and “microbes” reflect the approximate polymer length distributions of polyP isolated from these sources [15].