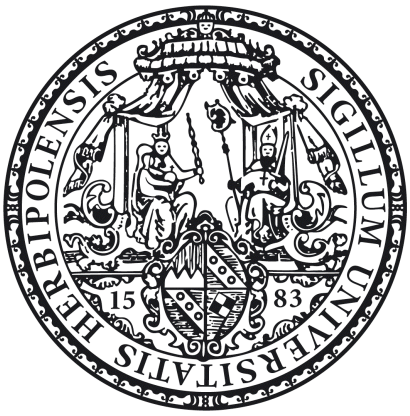

Analysis of the factor XII-driven contact system activation *in vivo*

Charakterisierung der Faktor XII-vermittelten Aktivierung des Kontaktsystems *in vivo*

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1 SUMMARY

Platelets play a central role in thrombosis, hemostasis, and inflammation. Here, we show that activated platelets release inorganic polyphosphate (polyP), a polymer of 60-100 phosphate residues that directly bound to and activated the plasma protease factor XII. PolyP-driven factor XII-activation triggered release of the inflammatory mediator bradykinin by plasma kallikrein-mediated kininogen processing. PolyP increased vascular permeability and induced fluid extravasation in skin microvessels of mice. Mice deficient in factor XII or bradykinin receptors were resistant to polyP-induced leakage. PolyP initiated clotting of plasma via the contact pathway. Ablation of intrinsic coagulation pathway proteases factor XII and factor XI protected mice from polyP-triggered lethal pulmonary embolism. Targeting polyP with phosphatases interfered with procoagulant activity of activated platelets and blocked platelet-induced thrombosis in mice. Infusion of polyP restored defective plasma clotting of Hermansky-Pudlak Syndrome patients, which lack platelet polyP. The data identify polyP as a new class of mediator having fundamental roles in platelet-driven proinflammatory and procoagulant disorders.

2 ZUSAMMENFASSUNG

Thrombozyten spielen eine zentrale Rolle bei Thrombose, Hämostase und Entzündungsprozessen. Wir zeigen, dass aktivierte Thrombozyten Polyphosphate (polyP) mit einer Kettenlänge von 60-100 Phosphatuntereinheiten sekretieren. PolyP binden und aktivieren die Serinprotease Faktor XII. PolyP-induzierte Faktor XII-Aktivierung führt über Kallikrein zur Freisetzung des Entzündungsmediators Bradykinin aus seinem Vorläufermolekül, dem hochmolekularen Kininogen. In einem Ödem-Modell zeigen wir, dass polyP die Gefäßpermeabilität in der Rückenhaut von Wildtyp-Mäusen erhöhen. Faktor XII- oder Bradykinin B2 Rezeptor-defiziente Tiere waren vor polyP-induzierter Ödembildung geschützt. PolyP aktivieren die intrinsische Blutgerinnungskaskade im Plasma. In einem polyP-vermittelten lethalen Lungenemboliemodell waren Faktor XII- und Faktor XI-defiziente Mäuse im Gegensatz zu Wildtyp Tieren geschützt. Behandlung mit Phosphatase hebt die prokoagulante Aktivität stimulierter Thrombozyten auf und blockiert die Plättchen-induzierte Thrombusbildung in Mäusen. PolyP normalisieren die verlängerte Blutgerinnungszeit von Hermansky-Pudlack Patienten. Die Daten zeigen, dass es sich bei polyP um eine neue Klasse von Mediatoren mit prokoagulanten und proinflammtorischen Eigenschaften handelt.

3 INTRODUCTION

3.1 The contact system

The contact system is an enzymatic cascade in the blood that is activated following blood contact to negatively charged surfaces. The system consists of the zymogens factor XII (FXII), plasmaprekallikrein (PPK) and the nonenzymatic cofactor high-molecular weight kininogen (HK) (Cochrane and Griffin, 1982; Colman and Schmaier, 1997). This cascade is initiated by autoactivation of FXII. Activated FXII (FXIIa) triggers four plasma cascade pathways such as the intrinsic pathway of coagulation, the fibrinolytic, the complement, and the kallikrein-kinin systems. (Fig. 1).

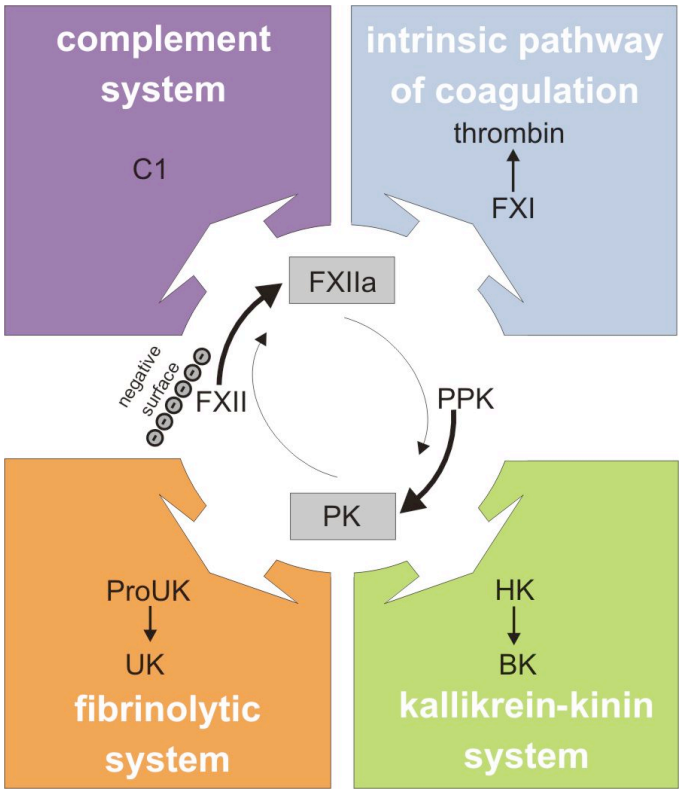


Figure 1. The contact system. The contact system consists of blood coagulation factor XII (FXII), plasmaprekallikrein (PPK) and the nonenzymatic cofactor high-molecular weight kininogen (HK). FXII becomes autoactivated following contact to negatively charged surfaces, resulting in activated factor XII (FXIIa). FXIIa cleaves PPK to form plasmakallikrein (PK). PK enhances FXIIa formation in a positive feedback loop. PK cleaves HK to liberate the proinflammatory mediator bradykinin (BK). PK also activates the fibrinolytic system through the conversion of pro-urokinase (ProUK) to the plasminogen activator urokinase (UK). FXIIa initiates plasmatic blood coagulation through conversion of factor XI (FXI) to activated FXI (FXIa), and may initiate the complement cascade through activation of complement factor C1.

Tiny amounts of FXIIa generated by autoactivation convert PPK to active plasmakallikrein (PK). In an amplification loop PK activates further FXII zymogens, thereby amplifying the initial signal (Cochrane and Revak, 1980). PK in turn liberates the vasoactive proinflammatory nonapeptide bradykinin (BK) from HK (Nishikawa et al., 1992). In addition to trigger BK formation, FXIIa also activates the fibrinolytic system through PK that activates pro-Urokinase (ProUK) to the plasminogen activator urokinase (UK) (Loza et al., 1994). PK-formed FXIIa initiates the classical pathway of the complement cascade *in vitro* via activation of the C1r and to a lesser degree the C1s subunits of the first component of complement system (Ghebrehiwet et al., 1981). Contact system-driven initiation of fibrin formation involves activation of factor XI (FXI) by FXIIa. This reaction triggers the intrinsic pathway of coagulation (Davie and Ratnoff, 1964; Macfarlane, 1964; Davie et al., 1991; Gailani and Renne, 2007).

3.2 Coagulation factor XII (Hageman factor)

Human FXII (Hageman factor) is a serine-protease primarily produced by hepatocytes that circulates in plasma as a zymogen (Revak et al., 1974; Griffin and Cochrane, 1976; Fujikawa and Davie, 1981). The FXII plasma concentration is ≈25-30 µg/ml (Revak et al., 1974; Saito et al., 1976). The zymogen comprises a polypeptide chain (M_w of 80 kDa) of 596 amino acid residues (Fujikawa and McMullen, 1983; McMullen and Fujikawa, 1985), coded by a single gene of 12 kb mapped on chromosome 5. The FXII gene consists of 13 introns and 14 exons (Cool and MacGillivray, 1987; Citarella et al., 1988). Upon activation, the zymogen is converted into a two-chain active protease, FXIIa.

Activation of FXII requires two steps: (i), binding to negatively charged surfaces that induces a conformational change (Cochrane et al., 1973; Griffin, 1978; Samuel et al., 1992) and (ii), proteolytic cleavage of FXII between the amino residues Arg^{353}-Val^{354} resulting in FXIIa (α-FXIIa). The protease consists of a heavy chain (M_w of 52 kDa) and a light chain (M_w of 28 kDa), connected by a disulfidebond, Cys^{340}-Cys^{346}.

The C-terminal light chain contains the catalytic triad of a serine-protease composed of the residues Asp[442], His[393], and Ser[554] (McRae et al., 1981). The enzymatically inactive N-terminal heavy chain is composed of five domains consisting of a type II region homologous to the collagen-binding domain in fibronectin (Fib II), an epidermal growth factor (EGF)-like domain (EGF I), followed by the type I homology or the fibrin finger in fibronectin (Fib I), and a second EGF-like domain (EGF II), preceding a kringle domain and a proline-rich region (Fig. 2) (Pixley et al., 1987a; Clarke et al., 1989; Samuel et al., 1993; Citarella et al., 2000).

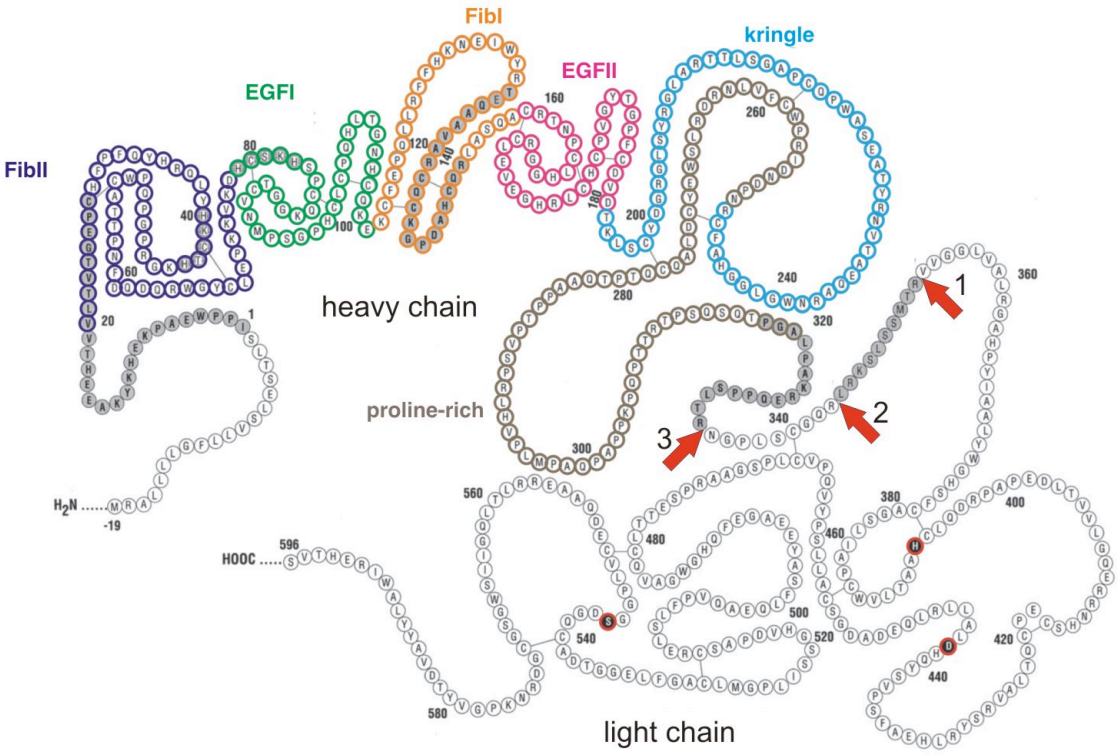


Figure 2. Structure of blood coagulation FXII. Cleavage of the peptide bond Arg[353]-Val[354] (red arrow 1) results in two-chain activated FXII (α-FXIIa). The two chains are connecetd by a disulfide-bond. Further proteolysis of the peptide bonds Arg[343]-Leu[344] and Arg[334]-Asn[335] (red arrows 2 and 3) by PK results in activated FXII-fragment (β-FXIIa). The catalytic triad of FXIIa consists of His[393], Asp[442] and Ser[544] (red). The heavy chain, which harbours the putative cell binding region of FXII, consist of five domains. Fib II (violet), EGF I (green), Fib I (orange), EGF II (purple), kringle (light blue) and the proline-rich region (brown). The light chain (white) has the enzymatic properties of a serine protease. (Structure adapted from (Cool and MacGillivray, 1987)).

The role of the individual domains of FXII is unknown. The heavy chain contains two artificial surface binding regions, one at the distal N-terminal end and another on its Fib I region (Pixley et al., 1987b; Clarke et al., 1989).

Studies using recombinant FXII deletion mutants identified a third putative binding site on the EGF II-like or kringle domain (Citarella et al., 1996).

The activation of FXII to α-FXIIa that results from binding to negatively charged surfaces, is termed autoactivation (Wiggins and Cochrane, 1979; Miller et al., 1980; Silverberg et al., 1980; Dunn et al., 1982). Some evidence suggests that binding of Zn^{2+} ions to FXII results in increased susceptibility to autoactivation and stabilizes certain conformations in the activation reaction (Shore et al., 1987; Schousboe, 1990). There are four zinc binding sites in FXII. Further cleavages of the peptide bonds Arg^{334}-Asn^{335} and Arg^{343}-Leu^{344} of α-FXIIa by PK separate the heavy from the light chain and result in activated FXII-fragment (β-FXIIa, M_w of 28 kDa), which consists of the light chain and nine amino acids of the C-terminal part of the FXII heavy chain (Dunn et al., 1982; Tankersley and Finlayson, 1984). In spite of retaining proteolytic activity towards PPK and the first component of complement C1, β-FXIIa is unable to bind to negatively charged surfaces and to efficiently induce blood clotting via FXI activation (Cochrane et al., 1973; Revak and Cochrane, 1976; Revak et al., 1978).

Mutations in the light chain of FXII result in defective enzymatic activity. There are several descriptions of inactive FXII mutants found in patients: Coagulation FXII (Washington DC) has a Cys^{571}-to-Ser^{571} substitution, that leads to complete loss of procoagulant activity *in vitro* (Miyata et al., 1989). Dysfunctional defects of coagulation FXII (Locarno and Bern) are caused by the amino acid substitution Arg^{353}-to-Pro^{353}, that abolishes the PK-cleavage site (Wuillemin et al., 1991).

The major inhibitor of α- and β-FXIIa, and PK in plasma is the C1 esterase inhibitor (C1Inh) (Forbes et al., 1970; Schreiber et al., 1973; de Agostini et al., 1984). C1Inh is a member of the serpin family, which inhibits serine proteases by irreversible binding to the enzymatic pocket (Davis, 1988). Other inhibitors of FXIIa comprise antithrombin III and plasminogen activator inhibitor-1 (PAI-1) (Pixley et al., 1985; Berrettini et al., 1989).

3.3 The kallikrein-kinin system

The kallikrein-kinin system was first considered as a proteolytic system in plasma and in tissue that liberates the vasoactive, proinflammtory kinin hormones (BK, Lys-BK (kallidin) and their biologically active des-Arg^9 metabolites) (Rocha et al., 1949). BK, a low-molecular weight nine amino acid nonapeptide, causes many characteristics of an inflammatory state, such as changes in local blood pressure, pain, vasodilatation, and increased microvessel permeability. There are two main pathways by which BK is generated: (i) the plasma, and (ii) the tissue kallikrein-kinin system. The latter involves tissue kallikrein (TK) (Margolius, 1998) and its substrate, low-molecular weight kininogen (LK) (Muller-Esterl et al., 1985). TK is secreted by various cells; tissues such as lung, kidney, intestine and brain produce large amounts of TK. The enzyme is processed intracellular from a precursor, tissueprekallikrein (TPK), to produce TK; cleavage of LK by TK yields a ten amino acid peptide, kallidin. A plasma aminopeptidase cleaves the N-terminal lysine from kallidin, forming peptide BK. The plasma kallikrein-kinin system involves PK and FXIIa. PK is immunologically and functionally distinct from TK. Although PK and TK have related functions (e.g. cleavage of kininogens), they have low sequence homology. TK prefers LK as a substrate but is capable of cleaving HK, whereas PK cleaves HK exclusively to liberate BK. The two forms of kininogen, HK and LK, results from alternative splicing of a single gene that consists of 11 exons (Kitamura et al., 1985; Takagaki et al., 1985). The kininogens, in general, can be divided into three regions: the heavy chain that is common to both HK and LK, the BK moiety, and the light chains that are unique to HK and LK, respectively. Domains 1 through 3 comprise kininogens heavy chain. Domain 4 is the BK region. Domain 5 for LK is its unique 12 amino acid long (4 kDa) light chain (Takagaki et al., 1985). The longer light chain of HK is responsible for its procoagulant activity, which is dependent upon two characteristics: its ability to form noncovalent complexes with PPK or FXI (Mandle et al., 1976; Thompson et al., 1979; Scott and Colman, 1980), and its ability to interact with negatively charged surfaces (Scott and Colman, 1980; Schmaier

et al., 1988). HK mediates binding to negatively charged surfaces with its histidine-rich region in the light chain (domain 5) and through residues of domain 3. The HK binding sites for PPK and FXI are in its extreme C-terminal portion in domain 6 (complex concentrations are ≈70-90 µg/ml) (Herwald et al., 1996; Renne et al., 2002a).

FXI and PPK are structurally related proteins sharing 58% amino acid sequence identity (Chung et al., 1986). The same structure is described for PPK and FXI, suggesting a common ancestor genic duplication event for PPK and FXI (Beaubien et al., 1991; McMullen et al., 1991a). The proteins have four tandem repeats in the N-terminal portion of the molecule due to linking of the first and sixth, second and fifth, and third and fourth half cysteine residues present in each repeat. This arrangement results in four groups of 90 or 91 amino acids that are arranged in so-called apple domains (Chung et al., 1986; McMullen et al., 1991b). The binding regions for HK have been mapped to apple domains A1, A2, and A4 of PPK (Page et al., 1994; Renne et al., 1999), and A1, A2, and A4 of FXI (Renne et al., 2002b). During contact activation, PK cleaves HK at the C-terminal portion of the BK-sequence, leaving BK attached to the C-terminal end of the heavy chain (Mori and Nagasawa, 1981), and a subsequent cleavage liberates BK from the heavy chain.

The Kinins exert their effects by specific activation of two distinct G-protein coupled receptors, BK type 1 receptor (B1R) and BK type 2 receptor (B2R) (Regoli and Barabe, 1980; Vavrek and Stewart, 1985). Non receptor bound hormones are rapidly metabolized by kininases I and II (Erdos and Sloane, 1962; Yang and Erdos, 1967; Sheikh and Kaplan, 1986). BK has a very short half-life (10-50 sec) in plasma (Decarie et al., 1996). Kininase II (angiotensin I converting enzyme, ACE) removes the C-terminal dipeptide from BK or kallidin and generates biologically inactive fragments. Kininase I enzymes (arginine carboxypeptidases) are responsible for the generation of des-Arg^9 BK and Lys-des-Arg^9 BK. Under normal conditions the B1R receptor is not expressed in normal tissue but following an inflammatory insult, receptor expression is induced and mediates action of des-Arg^9 kinins. The B2R receptor is constitutively

expressed and mediates acute vascular actions of BK and kallidin (Bhoola et al., 1992).

Activation of the kallikrein-kinin system by FXIIa leads to BK release. The peptide hormone acts in a paracrine mode and activates adjacent G-protein coupled B2R. BK increases vascular leakage and evokes pain sensations via nitric oxide (NO) and prostacyclin (PGI_2) dependent intracellular pathways in the endothelium (Busse and Fleming, 2003). NO, is derived from L-Arg by a constitutive Ca^{2+}-calmodulin-dependent endothelial nitric oxide synthase (eNOS). NO induces smooth muscle relaxation by activating soluble guanylyl cyclase. This increases intracelullar concentrations of cyclic guanosine monophosphate (cGMP), which, in turn activates the G kinase and reduces the intracellular concentration of Ca^{2+} in smooth muscle cells and results in relaxation. Because PGI_2 is also Ca^{2+} sensitive, synthesis of PGI_2 by endothelial cells is, like that of NO, a Ca^{2+}-dependent process (Chang et al., 1987).

BK formation by activation of the kallikrein-kinin system is regulated by the C1Inh (Davis, 1988). The importance of this pathway is illustrated *in vivo* by observations in patients with hereditary angioedema (HAE). These patients experience painful swelling episodes. The classical forms of HAE, types I and II, are caused by mutations in the C1Inh gene, leading to a deficiency in C1Inh or a dysfunctional C1Inh protein (Nussberger et al., 2002). In contrast to HAE types I and II, HAE type III, another form of angioedema is characterised by normal C1Inh function and plasma concentration exists (Bork et al., 2000). Clinically, HAE type III patients show the same symptoms as those with HAE types I and II and suffer from life-threatening swelling attacks. A comprehensive genome-wide linkage analysis in HAE type III affected families identified a disease-associated haplotype in the gene of FXII. The mutation leads to increased enzymatic activity of FXII in patient plasma, indicating a critical role of FXII-activity in the pathology of the disease (Cichon et al., 2006).

3.4 The intrinsic pathway of coagulation

Under physiological conditions blood circulates in a closed system. Blood coagulation is a protective mechanism of the organism in response to vascular injury. The coagulation system reacts quickly to stop blood loss from a damaged vessel wall. Defective or excessive coagulation activity leads to an increased risk of bleeding (hemorrhage) or clotting (thrombosis), respectively. In all mammals, coagulation involves both a cellular (platelet) and a protein (coagulation factor) component. Coagulation is initiated almost instantly after endothelial damage, which leads to exposure of subendothelial proteins, most notably collagen. The initial response to an injured vessel wall is vasoconstriction, which slows the flow of blood. Circulating platelets rapidly bind to collagen with collagen-specific glycoprotein Ia/IIa receptors and aggregate to form a hemostatic thrombus at the subendothelium. Platelet adhesion is strengthened by von Willebrand factor (vWF), which forms links between the platelet glycoproteins Ib/IX/V and collagen fibrils. Activated platelets release the contents of stored granules, such as ADP, serotonin, platelet-activating factor (PAF), vWF, platelet factor 4 (PF4) and thromboxane A_2 (TXA_2), which in turn activates additional platelets. This process mechanically impedes blood loss from the wound and is called primary hemostasis. The platelet plug may embolize from the vessel wall and must be stabilized by a fibrin meshwork, which is simultaneously generated by the secondary hemostatic system. Plasmatic coagulation proceeds through a series of sequential reactions involving activation of plasma coagulation proteins (Table 1). These events culminate in generation of the key coagulation enzyme thrombin, which converts fibrinogen into fibrin. Transglutaminase factor XIII (FXIII) forms intermolecular γ-glutamyl-ε-lysine cross-links between fibrin fibers, thereby stabilizing the clot and conferring resistance to proteolysis (Takahashi et al., 1986).

Table 1 : Plasma coagulation proteins.

factor	synonym	plasma level [µg/ml]	MW [kDa]	function	source
I	fibrinogen	3000	340	structural	hepatocytes
II	prothrombin*	100	72	protease zymogen	hepatocytes
III	tissue factor	-	37	cofactor/initiator	tissue
IV	calcium	-	-	-	plasma
V	proaccelerin	10	330	cofactor precursor	hepatocyte
VII	proconvertin*	0.5	55	protease zymogen	hepatocyte
VIII	antihemophilic factor	0.1	330	cofactor precursor	hepatocyte
IX	christmas factor*	5	55	protease zymogen	hepatocyte
X	stuart-prower factor*	10	55	protease zymogen	hepatocyte
XI	thromboplastin antecedent	5	160	protease zymogen	hepatocyte
XII	hageman factor	30	80	protease zymogen	hepatocyte
XIII	fibrin-stabilizing factor	30	320	protrans-glutaminase	hepatocyte
PPK	plasmaprekallikrein	40	85	protease zymogen	hepatocyte
HK	high-molecular weight kininogen	80	120	cofactor/activation	hepatocyte
vWF	von Willebrand factor	10	>220	adhesion, carrier protein	endothelium

***Vitamin K-dependent protein containing γ-carboxyglutamic acid. *Data was compiled from:* (Davie and Fujikawa, 1975; Furie and Furie, 1988).**

The classical cascade or waterfall model of coagulation, described in 1964 independently by Macfarlane (Macfarlane, 1964) and Davie and Ratnoff (Davie and Ratnoff, 1964), consists of two converging enzymatic pathways, initiated either by exposure of blood to a damaged vessel wall (extrinsic pathway) or by blood-borne components of the vascular system (intrinsic pathway) (Fig. 3). The common final steps of the coagulation cascade can be divided into three reactions: activation of factor X (FX), formation of thrombin (FIIa) from prothrombin (FII), and cleavage of fibrinogen to fibrin to form the insoluble platelet-rich clot. This classic model of coagulation provides the conceptual framework for the clotting assays used in the clinical laboratory, the prothrombin time (PT) and the activated partial thromboplastin time (aPTT), which assess extrinsic or intrinsic pathway-mediated coagulation, respectively.

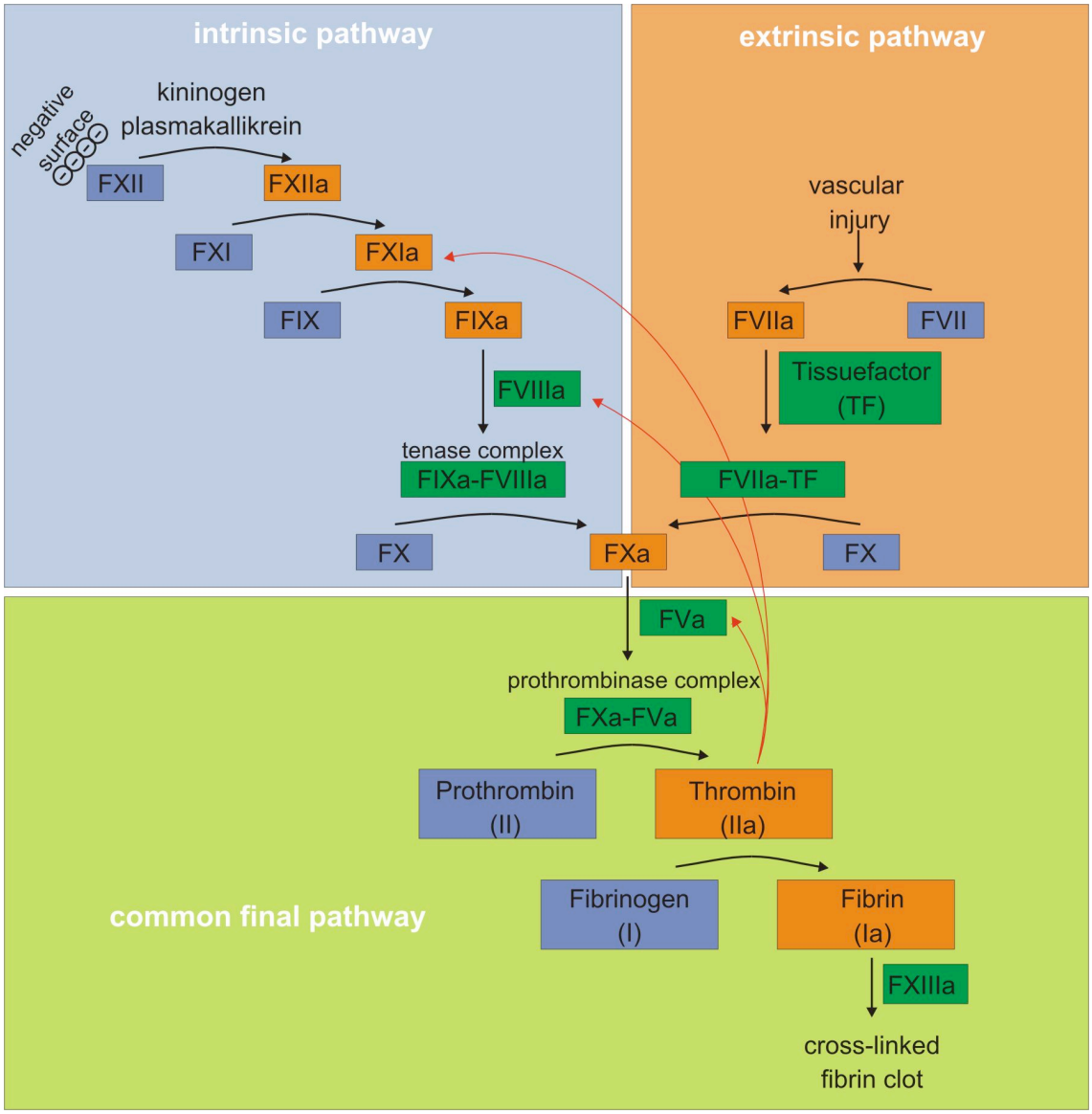


Figure 3. Coagulation cascade. Coagulation comprises the intrinsic (left, blue) and the extrinsic (right, orange) pathway. The extrinsic pathway is initiated after vascular injury by binding of cell exposed tissue factor (TF) to factor VII (FVII). Binding to negatively charged surfaces converts FXII of the intrinsic pathway to FXIIa. FXIIa then cleaves FXI to form FXIa. Factor Xa (FXa), generated by FVIIa-TF or the tenase complex, binds to factor V (FV). FXa-FVa (prothrombinase) complex converts prothrombin (FII) to thrombin (FIIa). FIIa enhances the activation of FV, factor VIII (FVIII) and FXI. During hemostasis, the tissue factor pathway is inactivated by platelet released tissue factor pathway inhibitor (TFPI).

The extrinsic pathway is initiated at the site of injury by the exposure of tissue factor (TF). TF is normally not found at high concentrations in blood, but is present on cell membranes in subendothelial layers of blood vessel. TF is exposed to activated factor VII (FVIIa) when the endothelium is injured and forms a FVIIa-TF complex (Weiss et al., 1989; Wilcox et al., 1989). The intrinsic pathway of coagulation is initiated by FXII,

in a reaction involving HK and PK. This process triggers FXIIa-mediated activation of FXI and in the presence of Ca^{2+} formation of activated factor IX (FIXa). FIXa assembles with its cofactor activated factor VIII (FVIIIa) on a phospholipid surface and forms the tenase complex (FIXa-FVIIIa). The extrinsic and intrinsic pathways converge in FX activation. Activated FX (FXa) activates FII to generate picomolar amounts of FIIa. In the presence of Ca^{2+} FXa and the co-factor Va (FVa) form the prothrombinase complex (FXa-FVa), the key enzyme complex for FIIa generation. FIIa subsequently converts fibrinogen to fibrin by cleavage of two peptide bonds, thereby releasing two small peptides, fibrinopeptide A and fibrinopeptide B (Furie and Furie, 1992; Dahlback, 2000; Davie and Kulman, 2006). FIIa may accelerate its own formation by activation of the cofactors factor V (FV) and factor VIII (FVIII). Platelet released tissue factor pathway inhibitor (TFPI) rapidly inhibits the FVIIa-TF complex, and immediate turndowns of the extrinsic pathway (Broze, 1992). As a result sustained FIIa generation is dependent on FIXa-FVIIIa and FXa-FVa complexes. FIIa activates FXI, which is an alternative way to generate FIXa (Butenas et al., 2003; Walsh, 2003).

Vitamin K is an essential cofactor for the post-translational γ-carboxylation of glutamate residues. The vitamin K-dependent proteins include the coagulation proteins FII, FVII, FIX, and FX and the anticoagulation proteins, protein S, protein C and protein Z. Thus the coagulation proteins share the requirement for the γ-carboxylation of the glutamic acid residues in order to bind Ca^{2+}. Ca^{2+} ions bind active clotting factors to membrane surfaces. The coagulation proteins contain an N-terminal module with 9-12 γ-carboxyglutamic acid (Gla) residues, followed by EGF-like regions. (Stenflo, 1991; Stenflo et al., 2000). EGF-like domains are involved in protein-protein interaction. The coagulation proteins FII, FVII, FIX, FX and protein C have two EGF-like domains, whereas protein S and protein Z have four EGF-like modules in tandem, respectively. Upon Ca^{2+} binding to the Gla residues the clotting factors are able to interact with biological membranes.

Several control mechanisms for the coagulation cascade exist because excessive clotting may be life threatening. These mechanisms include inactivation of procoagulant enzymes, fibrinolysis, and clearance of activated clotting factors (Furie and Furie, 1988). Plasma protease inhibitors (antithrombin III, tissue factor pathway inhibitor [TFPI], α_2-macroglobulin, heparin cofactor II) inactivate coagulation enzymes. Antithrombin targets FIIa, FIXa, FXa, FXIa and FXIIa. TFPI limits the action of FVIIa-TF. During wound healing, the fibrin clot in the vessel is degraded by plasmin, a serine protease generated from the plasma zymogen plasminogen. This reaction is catalyzed by several plasminogen activators, including tissue plasminogen activator (t-PA) and plasminogen urokinase activator (UK). Their activity is in turn regulated the plasma protease inhibitors, plasminogen activator inhibitor and α_2-antiplasmin (Colman and Schmaier, 1986).

Congenital deficiency in FXII (Hageman trait) is an autosomal recessive trait, first discovered in 1955 in the patient named John Hageman (Ratnoff and Margolius, 1955). Deficiency in FXII and also in the contact system proteins PPK and HK severely prolongs the aPTT. However, in sharp contrast to deficiency in the extrinsic factors TF (Bugge et al., 1996) and FVII (Rosen et al., 1997), individuals with severe deficiencies in any of the contact factors do not suffer from spontaneous or injury-related bleeding disorders (Ratnoff and Margolius, 1955; Colman et al., 1975; Sollo and Saleem, 1985). PK, HK, and FXII deficient patients can undergo surgical procedures without additional need of transfusions and clearly have a completely normal hemostatic capacity. This observation led to the current model that contact activation plays no role in hemostasis and that fibrin formation at a site of injury is exclusively initiated via the extrinsic pathway. However, FXII-deficient mice were found to have a profound defect in formation and stabilization of thrombi and suppressed BK levels, suggesting an important role for FXII in thrombus formation and inflammation (Renne et al., 2005a; Iwaki and Castellino, 2006; Kleinschnitz et al., 2006).

One third to one half of individuals with FXI deficiency have a mild bleeding diathesis, also referred to as hemophilia C (Bolton-Maggs, 2000). The FXI-related bleeding dysfunction can be explained by the ability of FIIa to activate FXI, which is considered to be an important feedback mechanism to maintain production following inactivation of the TF pathway by TFPI (Gailani and Broze, 1991). It is not clear if FXIa functions to increase the total amount of FIIa at the injured site, or act to generate FIXa at strategic locations within the clot. Furthermore, continued FIIa generation through this pathway can protect the clot from lysis by activation of thrombin activatable firbinolysis inhibitor (TAFI) (Von dem Borne et al., 1997). The antifibrinolytic effect of TAFI in FIIa-mediated FXI activation may explain the bleeding pattern in FXI-deficient patients (Asakai et al., 1991).

3.5 Activation of coagulation factor XII

FXII is activated *in vitro* by a variety of polyanionic surfaces, such as kaolin, glass, ellagic acid, certain polymers, nucleotides, sulfatides, misfolded proteins, and some types of collagen or glycosaminoglycans (Cochrane and Griffin, 1982; Maas et al., 2008; Muller and Renne, 2008; van der Meijden et al., 2009). Activation of FXII by non-physiological material like kaolin (a negatively charged silica) provides the basis for the aPTT clinical clotting assay, which is widely used to assess the integrity of the intrinsic pathway of coagulation and to monitor anticoagulation with heparin. Surface-independent mechanisms for FXII activation may also contribute. Microbial enzymes can activate FXII by direct proteolysis (Kaminishi et al., 1994), and lung damage in *Salmonella pneumonia* has been linked to activation of contact proteases and BK generation (Persson et al., 2000). Protease-driven activation of FXII may also occur on vessel walls, where FXII and HK bind to similar sites on the endothelium (Reddigari et al., 1993). Although the mechanism of activating the contact system in particular FXII is well characterized, the nature of physiological activators *in vivo* has remained unknown.

Extracellular RNA, which activates FXII (Cochrane and Griffin, 1982) and is liberated from disintegrating cells, is prothrombotic but does not contribute to hemostatic mechanisms (Kannemeier et al., 2007). These findings challenge the importance of RNA as FXII activator *in vivo*. A previous study identified activation of FXII by misfolded protein aggregates, which specifically led to activation of the kallikrein-kinin system without inducing the intrinsic pathway of coagulation (Maas et al., 2008). Given that FXIIa activates the contact system, triggering initiation of the kallikrein-kinin system as well as the intrinsic pathway of coagulation, more studies are necessary to elucidate specific activation mechanisms of FXIIa-driven intrinsic blood coagulation and BK formation. For more than 40 years platelets have been linked to the intrinsic pathway of coagulation (Castaldi et al., 1965; Nossel et al., 1969), with platelet activation promoting fibrin formation in a FXII-dependent manner (Walsh and Griffin, 1981). These findings suggest that FXII is activated on procoagulant platelets but the mechanisms involved are unclear (Furie and Furie, 2008).

3.6 Polyphosphate

Inorganic polyphosphate (polyP) (Fig. 4) is a linear, unbranched polymer of tens to hundreds of orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds similar to ATP (Kornberg, 1999; Rao et al., 2009).

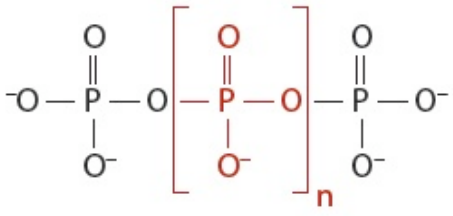


Figure 4. Inorganic polyphosphate (polyP). Number (n) of [PO_3^-] residues may vary from tens to many hundreds depending on the cellular location and the metabolic state.

PolyP is highly conserved, from archaea, bacteria, fungi, plants, insects, to mammals (Kulaev, 1975; Kulaev, 1979; Wood and Clark, 1988). PolyP was first found in yeast by Liebemann (1888). Almost 100 years ago they were observed as metachromatically stained „volutin granules“ in microbes (Robinson and Wood, 1986).

Several yeras later these volutin granules were shown to be polyP and subsequently found to be abundant in every cell in nature. Maintenance of intracellular phosphate concentrations is essential for cellular metabolism and growth. In periods of phosphate surplus, accumulation of polyP are stored as granules (Ogawa et al., 2000). Storage pools of polyP are present as osmotically inert aggregates associated with multivalent cations. Conversely, polyP reserves can be readily hydrolyzed to provide phosphate in periods of phosphate limitation by polyP degrading enzymes (Kornberg, 1999). PolyP may act as a substitute for ATP in various kinase reactions. PolyP kinase of *E.coli* catalyzes the reactions of transfer of energy-bond phosphate residues from ATP to polyP and from polyP to ADP. PolyP kinase can use polyP as a donor in place of ATP, thereby converting GDP and other nucleoside diphosphates to nucleoside triphosphates (Kulaev and Kulakovskaya, 2000). It has become apparent that polyP has other functions than acting as a phosphate storage polymer (Kornberg, 1999).

Until now polyP was studied mainly in prokaryotes. Recently it has been described to be important for diverse basic metabolisms, for some structural functions and, for stress responses (Brown and Kornberg, 2004, 2008; Rao et al., 2009). In human platelets polyP has been identified in the dense granules by staining with 4',6-diamidino-2-phenylindole (DAPI) (Ruiz et al., 2004). The granules are rich in Ca^{2+} and resemble acidocalcisomes, which are widely distributed from procaryotic to eukaryotic cells. They are responsible for the flux of Ca^{2+} into the cytosol.

A previous study demonstrated that synthetic polyP is a potent modulator of plasma clotting, affecting the intrinsic pathway, the fibrinolytic system, FV activation, and the fibrin structure (Smith et al., 2006; Smith and Morrissey, 2008b). These recent findings strongly indicate an important relevance of polyP for platelet-triggered reactions reactions *in vivo*.

3.7 Aim of the study

FXII and the contact system proteins have experienced a revival in the last years. The contact system proteins are well characterized, however, the activating physiological surface of this system *in vivo* remains unknown. Human platelets contain large amounts of polyP, accumulated in storage organelles named dense granules. Synthetic polyP has a procoagulant effect, which is exerted at several levels in the blood clotting and fibrinolytic system. PolyP accelerates blood coagulation by activating the contact system and by promoting FV activation, which in turn abrogates the anticoagulant function of TFPI (Smith et al., 2006). Furthermore, polyP modulates fibrin clot structure, resulting in thicker fibrin fibers that are more resistant to fibrinolysis (Smith and Morrissey, 2008b).

The aim of this study was to demonstrate, whether platelet derived polyP is the long sought physiological surface that activates coagulation FXII and the contact system *in vitro* and *in vivo.*

The goals of the presented work can be divided into following parts:

I) Analysis of platelet activation leading to dense granule secretion.

II) Extraction, identification and detection of polyP from platelet released material.

III) Investigation of polyP functions to trigger FXII-mediated BK formation in *vitro* and *in vivo*

IV) Analysis of polyP-driven FXII-dependent thrombin and fibrin formation *in vitro* and in genetically altered mice.

4 MATERIAL AND METHODS

4.1 Material

4.1.1 Chemicals

ADP	Sigma (Steinheim, Germany)
bovine serum albumin (BSA)	Pierce (Rockford, IL, USA)
enhanced chemoluminiscence (ECL) detection substrate	MoBiTec (Göttingen, Germany)
Dowex 50Wx8	Serva, Heidelberg, Germany
fetal bovine serum (FBS)	PAN (Aidenbach, Germany)
fibrilar penicillin/streptomycin	PAN (Aidenbach, Germany)

$PolyP_{20,}$ $polyP_{70}$ and $polyP_{1000}$ were kindly provided by BK Giulini (Ludwigshafen, Germany). All other chemicals were obtained from Sigma (Steinheim, Germany) or Roth (Karlsruhe, Germany).

4.1.2 Enzymes, Antibodies, Proteins, Substrates, Inhibitors and Markers

ENZYMES

benzonase	Roche (Penzberg, Germany)
chondroitinase ABC	Roche (Penzberg, Germany)
proteinase K	Quiagen (Hilden, Germany)
phosphatase, alkaline from calf intestinal mucosa	Fluka (Taufenkirchen, Germany)

ANTIBODIES

Antibodies were from the indicated sources and used at given concentrations in Western Blot (WB):

Antibody	Source/Class	Concentration	Supplier/Reference
α-HKL12	mAb (mouse)	1/2000	(Henderson et al., 1994)
α-FXI 199	pAb (rabbit)	1/200	(Renne et al., 2002a)
α-FXII	pAb (goat)	1/2000	Nordic Labs (Tilburg, Netherlands)
α-Fibrin	mAb (mouse)	1/500	(Hui et al., 1983)
α-Fibrinogen	pAb (goat)	1/200	Santa Cruz (Heidelberg; Germany)
α-GAPDH	mAb (mouse)	1/8000	Chemicon (Billerica, USA)
α-MBK3	pAb (rabbit)	1/250	(Haasemann et al., 1991)
α-P2	mAb (mouse)	1/1.000	(Renne et al., 2002)

Horseradish peroxidase-conjugated secondary antibodies were from Dianova (Hamburg, Germany) (WB: 1:5,000), fluorescence-conjugated antibodies (Alexa-fluor 488, 594, or 647, IF: 1:500) from Molecular Probes (Karlsruhe, Germany).

PROTEINS, SUBSTRATES AND INHIBITORS

bradykinin	Sigma (Steinheim, Germany)
collagen (Horn) type I	Nycomed (Munich, Germany)
human FXII	American Diagnostics (Greenwich, USA)
human fibrinogen	Sigma (Steinheim, Germany)
icatibant	Sigma (Steinheim, Germany)
PCK (H-D-Pro-Phe-Arg-chloromethylketone)	Bachem (Weil a. Rhein, Germany)
Trap6 (H-Ser-Phe-Leu-Leu-Arg-Asn-OH)	Bachem (Weil a. Rhein, Germany)
thrombin	Roche Diagnostics (Mannheim, Germany)
human plasmin	Sigma (Steinheim, Germany)
S-2302, S-2238	Chromogenix (Milano, Italy)

PROTEIN MARKER, DNA LADDER

Prestained Protein Ladder	Fermentas (St.Leon-Rot, Germany)
Low molecular weight DNA ladder	NEB (Frankfurt a.M., Germany)

4.1.3 Eukaryotic Cells

EA.hy926 cells, human umbilical vein endothelial cells (HUVEC, Cambrex Bioproducts) and ECV304 cells were cultivated and passaged according to ATCC recommendations.

4.1.4 Patients

Citrated human plasma was collected from healthy volunteers (University Hospital Würzburg, Germany). In some experiments pooled normal plasma and FXII deficient plasma were from George King Bio-Medical (Overland Park, Kansas, USA). Patients with Hermansky-Pudlak syndrome type 1 were enrolled in a protocol approved by the National Human Genome Research Institute's Institutional Review Board, and gave written, informed consent.

4.1.5 Animals

Animal studies were approved by the "Bezirksregierung of Unterfranken". $FXII^{-/-}$, $FXI^{-/-}$, $C1Inh^{-/-}$, and $B2R^{-/-}$ mice backcrossed for >10 generations to C57Bl/6 background were described previously (Borkowski et al., 1995; Gailani et al., 1997; Pauer et al., 2004). We used 6- to 8-week old animals and employed age- and sex-matched wild-type mice (C57Bl/6) from Charles River (Sulzfeld, Germany).

4.2 Methods

4.2.1 Cell culture

EA.hy926 cells and human umbilical vein endothelial cells (HUVEC, Cambrex Bioproducts) of passages 2-5 were cultivated as described (Profirovic et al., 2005; Renne et al., 2005). Cells were grown in a 5 % CO_2 saturated atmosphere at 37°C, and passaged according to ATCC recommendations. Cells were grown to confluence and subcultured using trypsination solution containing 0.05 % Trypsin/EDTA after removing growth medium and washing with PBS.

ECV304 cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) containing 4.5 g/l glucose, 1 mM sodium pyruvate, and 10% FBS and penicillin/strepomycin solution (100 U/0.1 mg/ml). Cells were grown to confluency in 14 cm-plastic petridishes in the recommended medium. After trypsination cells were harvested by centrifugation at 1,000x *g* for 5 min, washed several times with PBS and used for extraction of polyP.

4.2.2 Cell freezing and thawing

Cells were washed with PBS, trypsinated, pelleted (200x *g*, 5 min) and counted. The pellet was resuspended in freezing medium (20 % FBS, 100 U/0.1 mg/ml penicillin/streptomycin, 10 % DMSO in DMEM high glucose) at a final concentration of $1x10^6$/ml, aliquoted into 1.8 ml cryotubes. The aliqouts were stored over night at -80°C and then transferred in liquid N_2.

Frozen cells were thawed by warming the vials in a 37°C water-bath and immediately transferring them into 15 ml tubes with 10 ml growth medium. Cells were pelleted by centrifugation, medium was removed, and cells were resuspended in fresh growth medium and seeded in a small dish T-75.

4.2.3 Blood sampling (mouse)

Adult mice were anaesthesized, and blood from retroorbital veins was taken into tubes containing 1/10 of citrate. With this method a maximum of 1 ml blood (1/10 of body weight) can be taken per mouse. The animal survives and blood can be taken again one week later. Blood was centrifuged at 1,800 *rpm* for 5 min. Supernatant was taken and centrifuged at 800 *rpm* for 7-8 min at RT to obtain platelet rich plasma (PRP). Following centrifugation at 2,500 *rpm* for 10 min obtained platelet poor plasma (PPP).

4.2.4 Platelet isolation (mouse)

Mice were bled under anesthesia. Blood was collected into a tube containing citrate buffer and centrifuged at 1800 *rpm* for 5 min. Supernatant (plasma with platelets and a few erythrocytes) was taken and centrifuged at 800 *rpm* for 7-8 min at RT to obtain platelet rich plasma (PRP). (In addition an optional „quickrun“ could be done with remaining plasma and supernatants combined.) To wash platelets, PRP was centrifuged at 2,800 *rpm* for 5 min in the presence of prostaglandin (PGE_1) (1 µM) and the pellet was resuspended in New Tyrode's buffer (145 mM NaCl, 0.5 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, 5 mM D-Glucose, 10 mM HEPES, 2 mM $CaCl_2$, 0.35 % BSA, pH 6.5) containing PGE_1 (1 µM) incubated for 5 min in a 37°C water bath. After a second centrifugation step, platelets were resuspended in the same buffer and incubated at 37°C for additional 5 min. Platelets were finally centrifuged as above, resuspended in Tyrode's buffer (pH 7.4) and left to incubate for 30 min at 37°C before analysis.

4.2.5 Platelet and cell count

Platelet or cell suspension were diluted 1/10 in PBS (10 mM Tris pH 7.5, 150 mM NaCl) and counted with the Coulter haematological cell counter as instructed by the manufacturer.

4.2.6 Extraction of polyP

EA.hy926, ECV304 and HUVEC cells were seeded at a density of 10^8 cells/cm^2 in 14 cm plastic dishes and extraction of polyP was performed as described (Kumble and Kornberg, 1995). The washed cells were suspended in 500 µl of lysis buffer (50 mM Tris pH 7.5, 1 M urea, 0.1 %SDS, 10 mM EDTA) and sonicated in an ice-bath for three 10 sec bursts with 30 sec cooling intervals using a Polytron (Brinkmann Instruments).

For extraction of polyP from activated platelets, platelets were isolated from thrombocyte concentrate of healthy volunteers.

Platelets were harvested by centrifugation at 750x *g* for 15 min at room temperature, and the platelet pellet was resuspended in New Tyrode's buffer (pH 6.5) and PGE_1 (1 µM). The platelets were centrifuged and washed one more time. The final platelet pellet was resuspended in New Tyrode's buffer (pH 7.4) at a final concentration of $7.5x10^{11}$ platelets/ml. After one hour of rest in a 37°C water bath, washed platelets were used for experiments. Platelets were activated with thrombin (2 U/ml), Trap6 (0.2 mM), collagen (0.2 mg/ml) for 1 min, as well as with ADP (0.1 mM) for 5 min. All steps were performed at 37°C in a water bath. After activation platelets were pelleted by centrifugation at 2,700x *g* for 15 min. Supernatant containing platelet released material was collected for isolating polyP. As a control, supernatant from untreated platelets was collected.

The supernatants from activated or control platelets and cell lysates were incubated with proteinase K (750 µg/ml) at 37°C for 2 h. Proteins were removed by extraction with an equal volume of phenol/chloroform (1:1, w/v equilibrated with Tris-HCl, pH 7.5); the phases were separated by centrifugation at 14,000x *g* for 10 min. The aqueous phase was transferred to another tube and further purified. The phenol layer was backextracted with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, shaken vigorously and centrifuged at 14,000x *g* for 10 min. The pooled aqueous phases were then extracted with an equal volume of chloroform. The polyP were precipitated with barium acetate at a final concentration of 0.1 M at pH 4.5 at 4°C for 4 h, rotating overhead. The polyP precipitate was collected by centrifugation at 14,000x *g* for 30 min, resuspended in water and solubilized by the addition of few beads of ion-exchange resin Dowex 50Wx8 in NH_4^+ form (SERVA Electrophoresis, Heidelberg, Germany). The mixture was incubated by shaking for 10 min at RT, followed by centrifugation at 10,000x *g* for 30 min. The supernatant was first treated with benzonase (350 µg/ml) in 5 mM $MgCl_2$ after shaking 1 h at 37°C. Afterwards chondroitinase ABC (5 U) was added and incubated for 5 h at 37°C, pH 8. Contaminating proteins in the sample were removed

by phenol/chloroform and chloroform extraction as described above. The polyP was concentrated under vacuum and analyzed.

4.2.7 Analytical urea-PAGE of extracted polyP

Extracted polyP were determined by electrophoresis on 7 M urea/5 % polyacrylamide gels in 1xTBE (890 mM Tris, 890 mM H_3BO_4, 20 mM Na_2EDTA). The gels were pre-run at 300 V for 1 h. PolyP were mixed with 1/4 volume 5x TBE-sample buffer (50 % Sucrose, 450 mM TBE pH 8.3, 13.5 mM Na_2EDTA, 0.2 % BPB), and loaded on the gel. Electrophoresis was performed at 300 V until the dye reached half of the gel. PolyP was detected by staining with 0.05 % toluidinblue- or DAPI solution consisting of 25 % methanol, and 5 % glycerol and destaining in 25 % methanol and 5 % glycerol. PolyP of defined chain lengths ($polyP_{125}$ and $polyP_{1000}$) were run in parallel.

4.2.8 Toluidine blue staining of polyP

Electrophoresed polyphosphates were analyzed according to a method of Smith (Smith and Morrissey, 2007). Gels were stained by agitation for 15 min in fixative solution (0.05 % toluidin blue, 25 %MetOH, 5 %Glycerol) destained by three changes of destaining solution (25 %MetOH, 5 % Glycerol) over 3 h, and imaged with white light on a flatbed scanner.

4.2.9 DAPI staining of polyP

Gels were agitated for 30 min at room temperature in DAPI fixative solution (2 mg/ml DAPI, 1 mg/ml p-phenylenediamine, 10 mM EDTA pH 8.0, 25 % MetOH, 5 % Glycerol) using foil-covered containers to avoid exposure to ambient light. The gels were then destained in two 1 h changes of fixative (1 mg/ml p-phenylenediamine, 10 mM EDTA pH 8.0, 25 % MetOH, 5 % Glycerol) in foil-covered containers. Gels illuminated with 365 nm light on a UV transilluminator (ChemiImager 5500, Alpha Innotech) were documented.

4.2.10 Analytical agarose-gel and positive staining of polyP

PolyP were separated on 1.5 % agarose gel prepared using TBE (90 mM Tris, 90 mM Boric Acid, 10 mM EDTA pH 8.3) as electrophoresis buffer and stained with 0.1 % toluidine blue (Gomes et al., 2008). Alternatively, 20 µg/ml DAPI, and 1 mg/ml p-phenylenediamine in 1xTBE were used. Photographs of gels illuminated with 365 nm light on a UV transilluminator were taken (ChemiImager 5500, Alpha Innotech).

4.2.11 NMR-analysis

A sample of commercial $polyP_{125}$ (25 mg) (Sigma-Aldrich) and a sample of lyophylized lysate (10 mg) were dissolved in heavy water (D_2O) and the ^{31}P NMR spectra recorded at 202.4 MHz and 25°C using a Bruker Avance 500 NMR spectrometer. Pulse delay and repetiton time were set at 0.03 and 100 sec, respectively, to ensure correct signal integration. The content of the various species was then calculated directly from the signal intensities.

4.2.12 SDS-polyacrylamid gel electrophoresis (PAGE) and western blot

Tris/Glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 8 % or 10 % (w/v) polyacrylamide separating gels.

	Stacking gel	**Separating gel**	
	4 %	**8 %**	**10 %**
Acrylamide/Bisacrylamide 30 %/0.8 % (w/v) 29:1	0.83 ml	2.7 ml	3.3 ml
1 M Tris pH 6.8	0.63 ml	-	-
1.5 M Tris pH 8.8		2.5 ml	2.5 ml
10 %SDS	0.05 ml	0.1 ml	0.1 ml
10 % ammonium persulfate	0.05 ml	0.1 ml	0.1 ml
TEMED	0.005 ml	0.006 ml	0.004 ml
Total volume	5 ml	10 ml	10 ml

Plasma samples were diluted in 2x Laemmli sample buffer (1.25 mM Tris pH 6.8, 10 % ß-mercaptoethanol, 0.02 % BPB, 4 % SDS, 20 % Glycerin) and loaded the wells. The starting current of the gel was 80 V and increased to 150 V after samples reached the separation gel (electrophoresis buffer: 25 mM Tris pH 8.9, 192 mM Glycerin, 0.1 % (w/v) SDS). A semi-dry system was used to transfer the proteins from the gel to the nitrocellulose membrane (150 mM for 2 h at RT, transfer buffer: 25 mM Tris pH 8.9, 192 mM Glycin, 20 % (v/v) Methanol).

To verify the transfer, the nitrocellulose was stained with Ponceau S. For blocking of nonspecific binding sites the membrane was incubated in 5 % non-fat dry milk in PBS-Tween 0.05 % (PBS-T) for 1 h, followed by incubation with diluted primary antibody in 3 % non-fat dry milk in PBS-T for 2 h at RT or overnight at 4°C. Horse-radish peroxidase-labelled secondary antibody was freshly diluted in 5 % non-fat dry-milk in PBS-T and incubated with the membrane for 1 h at RT. After primary and secondary antibody incubation membranes were washed several times with PBS-T.

Protein detection was performed using an enhanced chemiluminescence detection kit, X-ray films and a Kodak developer machine.

4.2.13 Detection of contact phase proteins

Plasma samples (0.2 µl plasma/lane) were separated on SDS-PAGE gels, under reducing conditions and electrotransferred to a nitrocellulose membrane. Contact phase proteins were probed using antibodies against bradykinin (MBK3), plasma kallikrein (P2), FXII, and FXI (AS199), respectively, as well as horseradish peroxidase-conjugated secondary antibodies, followed by a chemiluminescence detection method as described above.

4.2.14 Measurement of thrombin generation

Thrombin generation was measured according to the method of Aronson with minor modifications as previously described (Aoki et al., 1997) using the chromogenic

substrate S-2238. The change in optical density was recorded spectrophotometrically at 405 nm for 40 min at 37°C by a Bio-Kinetics Reader (Biotek Instruments Inc., Friedrichshall, Germany). Thrombin generation in real time was analyzed by the calibrated automated thrombography (CAT) method as described by Hemker et al. (Hemker et al., 2006), in a Fluoroscan Ascent fluorometer (Thermo Scientific) equipped with a dispenser, according to the manufacturer`s instruction (Thrombinsocope BV, Maastricht, The Netherlands). All experiments were run in duplicate for 60 min and thrombin generation was calculated using the Thrombinoscope software package (Version 3.0.0.29).

4.2.15 Contact phase system activation assay

Human citrate plasma (PPP) was supplemented with increasing concentrations of polyphosphate (0.5-1000 µg/ml in 75 mM Tris pH 7.4, 10 mM $ZnCl_2$). Samples were incubated for 45 min in a 37°C water bath. The reaction was stopped by the addition of reducing 2x Laemmli sample buffer containing 4 % SDS. Following electrotransfer, nitrocellulose membranes were probed with HKL-12 antibody (1:2,000), anti-FXII (1:500), anti-FXI (1:250) or the anti-PK antibody PK2 (0.5 mg/ml) and horseradish peroxidase-coupled secondary antibody (1:5,000, DAKO, Hamburg, Germany).

4.2.16 Activation of the intrinsic pathway *in vitro*

Human citrate-anticoagulated plasma was incubated with polyP (0.5 – 1000 µg/ml) for 30 min at 37°C. The reaction was stopped by the addition of reducing 2x Laemmli buffer containing 4 % (w/v) SDS. 0.2 µl plasma per lane were analyzed by western blotting as described (Renne et al., 2005). BK plasma concentrations were quantified with MARKIT-M-Bradykinin ELISA (Dainippon Pharmaceutical, Osaka, Japan). Activation of purified FXII (200 nM) by kallikrein (0.5 nM) was analyzed by incubating at 37°C in the presence of either dextran sulfate (1 µg/ml), $polyP_{125}$ (5 µg/ml) or buffer alone (20 mM Hepes pH 7.4, 100 mM NaCl; HBS).

Reactions were stopped at various times (0 – 60 min) in HBS buffer containing polybrene (5.5 µg/ml) and soy trypsin inhibitor (100 µg/ml). Activity was quantified using a chromogenic substrate for FXIIa (200 nM, Bachem, USA) and read against a standard curve of purified FXIIa for 60 min at 405 nm in a VERSAmax microplate reader (Molecular Devices). Autoactivation of FXII (200 nM) was analyzed in a similar manner with addition of surfaces but in the absence of kallikrein.

4.2.17 Coagulation assays in plasma

PolyP- and kaolin-triggered clotting was assessed using a "Kugelkoagulometer" (KC10; Amelung). 100 µl of citrated human plasma was incubated with buffer or with polyP or kaolin (100 µg/ml) for 120 sec at 37°C before addition of $CaCl_2$, after which the time to clot formation was recorded. For determination of recalcification clotting times, citrated platelet-rich plasma was supplemented with Ca^{2+} ionophore A23187 (5 µM) or Trap6 (30 µM) at 37°C for 10 min in the presence or absence of alkaline phosphatase (from bovine intestinal mucosa, 10 U/ml) before addition of $CaCl_2$. Activation of FXII by polyP was analyzed as described (Renne et al., 2005a), using the chromogenic substrate S-2302 (2 mM) and a Bio-Kinetics Reader (BioTek Instruments Inc.) at 37°C. Human platelets were isolated from whole blood of consenting healthy individuals and from patients with Hermansky-Pudlak Syndrome (HPS). Isolated platelets were stimulated for 20 min at 37°C with Trap6 (50 µM) and collagen (10 µg/ml). Clotting assays were performed in a Start 4 coagulometer (Diagnostica Stago) with pooled normal plasma with addition of either normal or HPS platelets (1.5×10^{10}) in the presence and absence of additional $polyP_{125}$ (10 µg/ml). Clotting was monitored in pooled normal plasma and FXII deficient plasma over a range of polyP concentrations (0 – 650 µg/ml), using a microplate turbidity assay as previously described. Results are expressed as the mean time to reach half-maximal turbidity.

4.2.18 Determination of FXII activation by polyP

Activation of FXII by polyP was carried out in a 96-well microtiter plate. The amount of FXIIa formed in human citrate plasma was determined from the rate of hydrolysis of the chromogenic substrate S-2302 (2 mM), and the increase in absorbance at 405 nm was recorded at time intervals of 1 min for a period of 60 min by a Bio-Kinetics Reader (BioTek Instruments Inc., Friedrichshall, Germany) set at 37°C.

4.2.19 PolyP-induced pulmonary thromboembolism

Mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol and 2-methyl-2-butanol (0.15 ml/10 g of body weight from a 2.5 % solution), and polyP (300 µg/g body weight in NaCl) was slowly injected into the inferior vena cava. Alternatively Trap6 (0.7 µg/g body weight) was infused. In some experiments mice were injected i.v. with phosphatase (15 U/g body weight) before the challenge. Animals still alive after 30 min were considered survivors.

4.2.20 Histopathologic analysis

Mice were sacrificed, and lungs were rapidly removed and fixed at 4°C in buffered 4 % paraformaldehyd, pH 7.4. Tissues were dehydrated and embedded in paraffin (Histolab Products AB), cut into 8 µm sections, and stained with Mayer's hematoxylin and eosin.

4.2.21 Detection of fibrin deposition

Lung tissue was placed in buffer (0.05 M Tris, 0.15 M NaCl, 500 U/ml heparin, final pH 7.6) on ice and homogenized (Brinkmann Instruments, Inc., Westbury, NY, USA). Plasmin digestion was performed by a modification of the method of Francis (Francis et al., 1980). Human plasmin (0.32 U/ml) was added to the tissue homogenate, followed by agitation at 37°C for 6 h. More plasmin (0.32 U/ml) was then added, and samples were agitated for additional 2 h, then the mixture was centrifuged at 2,300x *g* for 15 min and the supernatant was aspirated.

Samples of plasmin-treated lung homogenates (400 mg) were boiled in reducing SDS sample buffer for 5 min and subjected to SDS-PAGE (10 %; reduced). The amount of fibrin ß-chains normalized to identical lung tissue weight was determined by western blot, using the fibrin-specific mAb59D8 antibody (Hui et al., 1983).

4.2.22 Skin vascular leakage assay

Animals were backcrossed for more than 8 generations to C57BL/6J background and their wild-type littermates were used as controls. All experiments and animal care were approved by the “Regierung of Unterfranken“. Five- to seven-week-old male mice were employed for the leakage models. Mice were anesthetized with a single i.p. injection of ketamine/xylazine (80 mg/kg ketamine and 10 mg/kg xylazine), and a total of 200 µl of sterile-filtered 0.25 % Evans blue dissolved in sterile saline (0.9 % NaCl) was injected intravenously in the retroorbital plexus. 10 min later, 50 µl of bradykinin (100 µM) or saline was injected intradermally in the dorsal region, using a tuberculin syringe. The animals were killed 10 min after the intradermal injections by decapitation.

The skin was removed, mounted, and photographed. Skin samples were removed by using a circular template, and the Evans blue dye was extracted by incubation in *N*,*N* dimethylformamide overnight at 55°C. The following day, the Evans blue fluorescence from individual skin samples was quantified spectrophotometrically at an excitation wavelenght of 620 nm and an emission wavelenght of 680 nm. The concentration of Evans blue was normalized by dividing with the value corresponding to skin samples injected with saline.

4.2.23 PolyP binding assays

Purified human FXII, FXI, PK, HK, antithrombin (AT) or albumin (10 µg) were incubated for 10 min with increasing concentrations of $polyP_{125}$ (0 – 100 µg) before being mixed with sample buffer (60 mM Tris-HCl pH 6.8, 10 % glycerol, 0.01 % bromophenol blue).

Samples were resolved on pre-cast 10 % Tris-glycine gels (Invitrogen) under native conditions and were stained for protein with Gelcode Blue (Pierce) according to the manufacturer's instructions. PolyP was immobilized onto porous zirconia beads essentially as described (Lorenz et al., 1994). Briefly, 250 mg zirconia beads were incubated with 10 mg/ml $polyP_{125}$ in water for 20 h at 37˚C. The beads were then washed thoroughly with distilled water before blocking unreacted sites with 10 % BSA for 15 h at ambient temperature. After washing in distilled water the prepared zirconia beads were dried in a vacuum oven at 80˚C for 2 h. Control beads were treated with water and BSA only. PolyP-zirconia beads (10 mg dry weight) were washed with binding buffer (200 µl; 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 % BSA) twice before addition of proteins (5 µg). After incubation at ambient temperature for 30 min the mixtures were centrifuged in mini-spin columns at 1,677x *g* for 30 sec. The flow-through was collected and the polyP-zirconia beads were washed first with binding buffer (200 µl) followed by elution buffer (200 µl; 50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.1 % BSA). Recovery of proteins from the beads at the various stages was analyzed by Western blot with specific antibodies.

4.2.24 Data analysis

All experiments were performed at least in triplicate and data shown are means ± SD. Data were analyzed using one-way analysis of variance (ANOVA) with Dunnett`s post test. P values <0.05 were considered statistical significant.

5 RESULTS

5.1 Activated platelets release polyP

Platelet dense granules contain polyP (Ruiz et al., 2004). We analyzed whether activated/procoagulant platelets release polyP. Washed human platelets were stimulated with ADP (0.1 mM), thrombin receptor-activating peptide 6 (Trap6, 0.2 mM), collagen (0.2 mg/ml), or thrombin (2 U/ml) and polyP was isolated from stimulated platelet supernatants using an ion-exchanger based method, which has been previously used to purify polyP from cell lysates (Kumble and Kornberg, 1995); it was then separated by agarose-gel electrophoresis and probed for polyP with toluidine blue (Fig. 5A) and a more sensitive DAPI-based fluorescent stain (Fig. 5B) (Smith and Morrissey, 2007). Incubation of polyP extracted from thrombin-stimulated platelets with proteinase, RNase, DNase, or glycosaminoglycan-cleaving enzymes did not alter the staining patterns (not shown). In contrast, treatment with phosphatase (Psp, 0.05 U/µg polyP), which hydrolyzes polyP, completely abolished the signals (Fig. 5A and B, “thrombin+Psp”). Platelet polyP migrated similarly to synthetic polyP with mean chain lengths of 70 ± 16 ($polyP_{70}$) phosphate units, and at higher mass compared to polymers of 20 ± 6 phosphates ($polyP_{20}$).

PolyP is not unique to platelets. In this regard we isolated the polyanions from EA.hy926, ECV304, and HUVEC endothelial cells. Examination of purified samples by urea-PAGE analysis, followed toluidine blue and DAPI staining procedures, shows the presence of polyP in all three cell lines (Fig. 5C, D). To confirm identity of the extracted material as polyP we treated cell lysates with phosphatase (Psp, 0.05 U/µg polyP for 12 h).

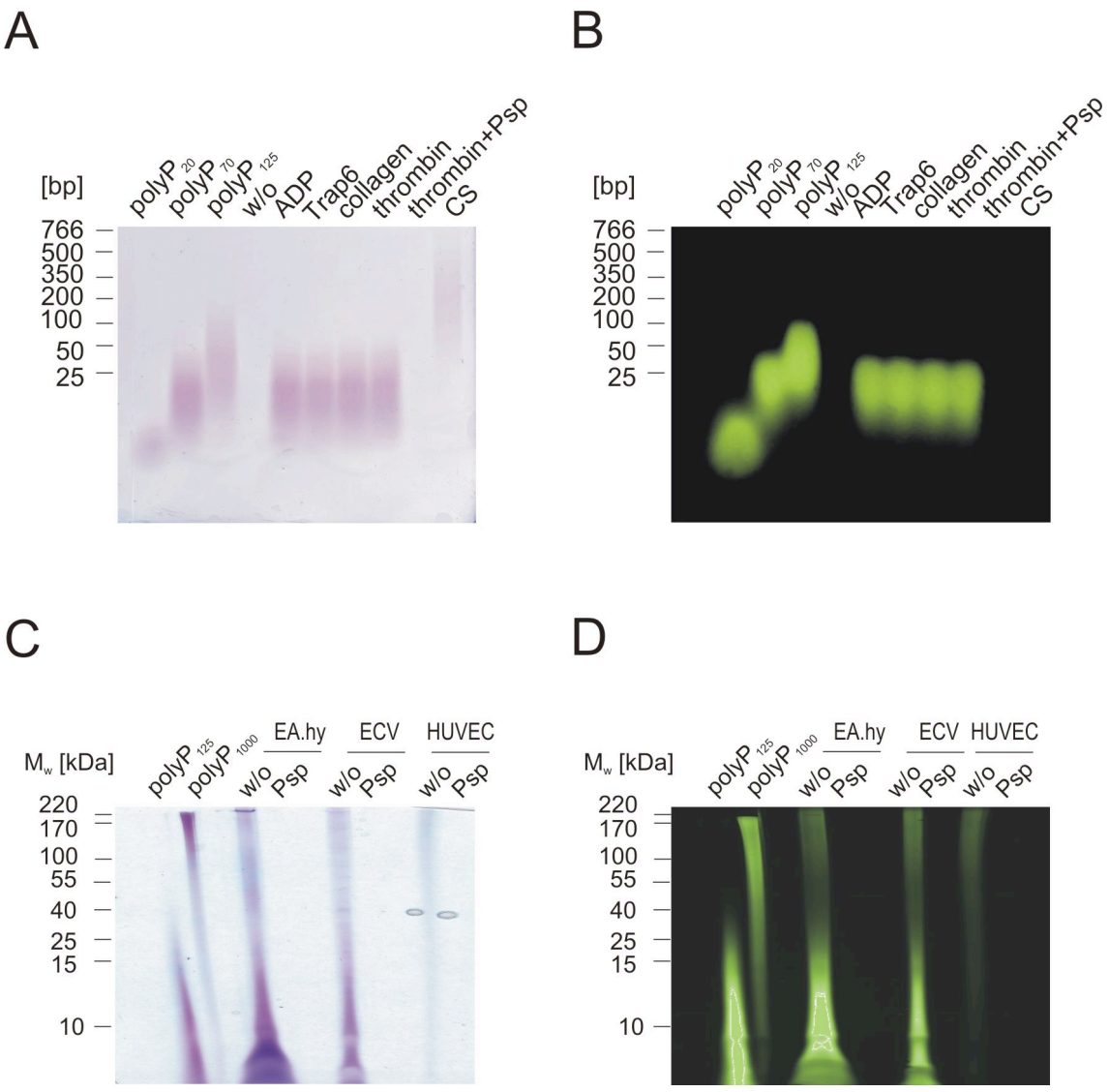


Figure 5. (**A** and **B**) **Activated platelets secrete long-chain polyP.** Washed platelets ($3.8x10^{13}$ platelets each) were stimulated with ADP (0.1 mM), Trap6 (0.2 mM), collagen (0.2 mg/ml), thrombin (2 U/ml), or buffer ("w/o") and polyP was isolated from the supernatants. PolyP were separated by agarose gel electrophoresis and stained with toluidine blue (A) or DAPI (B). Synthetic polyP with mean chain lengths of 20 ($polyP_{20}$), 70 ($polyP_{70}$) or 125 ($polyP_{125}$) phosphate units in lanes 1-3 as size standards. The purified material from thrombin-stimulated platelets was incubated with phosphatase (0.05 U/µg polyP; "thrombin + Psp") prior to electrophoresis. Negatively charged chondroitin sulfate (CS) confirmed specificity of staining. A bp DNA ladder as size standard is indicated on the left. (**C** and **D**) **Urea-PAGE analyses of endothelial cell-derived polyP.** PolyP were isolated from lysed EA.hy926, ECV304, and HUVEC endothelial cells, treated with buffer or phosphatase (0.05 U/µg polyP; lane labelled as "Psp"), and separated by urea-PAGE. Synthetic polyP of 125 ($polyP_{125}$) and 1000 ($polyP_{1000}$) mean chain lengths were loaded as size standards. PolyP was stained with toluidine blue (**C**) or DAPI (**D**). Since we used a polyacrylamid gel, we loaded a protein marker as weight standard on the left.

We determined the chain length of platelet polyP, using ^{31}P-NMR. The relative ratios of signal intensities of the phosphate end groups (∝-P, -10.2 ppm) versus central phosphates (ß-P, -22.1 ppm) yielded a mean chain length of platelet polyP of 80, consistent with its migration in the agarose matrix (Fig. 6A). Our NMR analysis indicated that the mean chain length of "polyP$_{75}$" used in previous studies (Ruiz et al., 2004; Smith et al., 2006) is 125 (Fig. 6B). Integration of all ^{31}P signals revealed that P content of total platelet released inorganic phosphates was 5 % monophosphate, 13 % diphosphate, 10 % cyclotriphosphate, 3 % ADP, and 69 % polyP.

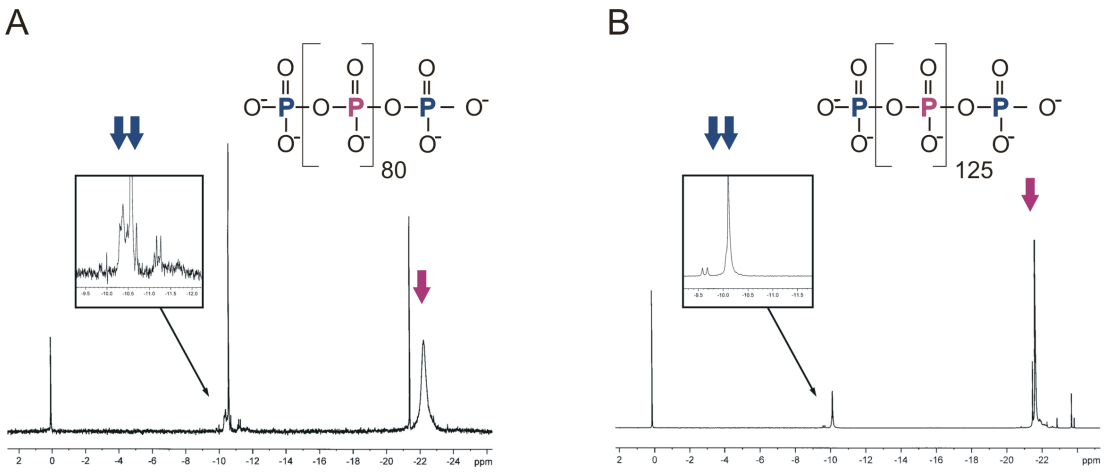
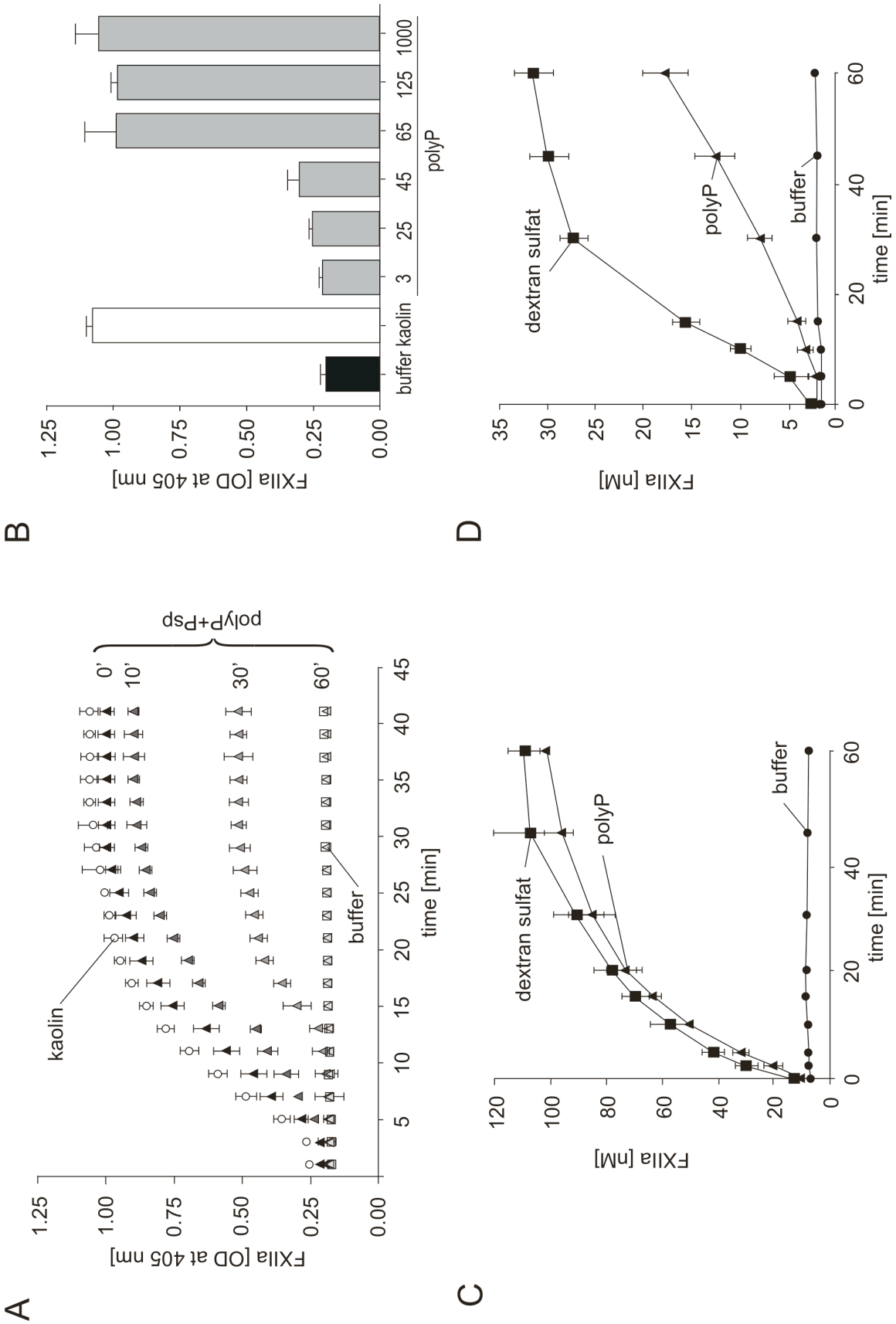


Figure 6. (**A** and **B**) **^{31}P NMR spectrum of platelet polyP and synthetic polyP$_{125}$ at 202.4 MHz.** (**A**) The terminal and internal phosphates of the polyP chain give signals at 10.2 (doublet, blue arrow) and 22.1 (red arrow) ppm, respectively; ratios of their intensities yielded a mean chain length of 80 for platelet polyP. Other phosphate species present are monophosphate (0.0 ppm, 5 %), diphosphate (-10.4 ppm, 13 %), cyclotriphosphate (-21.4 ppm, 10 %) and ADP (-11.2 ppm, 3 %). (**B**) Terminal and internal phosphates of the polyP chain give signals at 9.6 and 21.8 ppm, respectively. From the ratios of signal intensities of terminal and internal phosphates, the mean chain length of synthetic "polyP$_{75}$" was determined to be 125. The other major signals arise from the presence of monophosphate (0.0 ppm, 9.0 %), diphosphate (-10.2 ppm, 11.5 %), cyclotriphosphate (-21.6 ppm, 7.0 %) and cyclotetraphosphate (-23.8 ppm, 2.3 %).

5.2 PolyP activates the contact system *in vitro*

FXII is activated by binding to polyanions (Cochrane and Griffin, 1982). We tested platelet polyP for FXII activation, using the chromogenic substrate S-2302, which is hydrolyzed by active FXII (FXIIa) (Fig. 7A). Addition of polyP (100 µg/ml) to platelet-free plasma (PFP) initiated FXIIa generation with similar kinetics and potency as an identical concentration of kaolin. Kaolin is one of the most potent contact activators and is used in diagnostic clotting assays (Colman, 2006). Treatment of polyP with phosphatase (0.05 U/µg polyP) before addition to plasma reduced polyP-driven FXIIa generation in a time-dependent manner. Digestion for >60 min completely abolished the FXIIa-generating activity of polyP (Fig. 7A), suggesting that a minimal length of polyP is necessary for inducing FXII activation. Indeed, synthetic polyP of mean chain length ≤45 phosphate units failed to trigger FXII activation in plasma, whereas polyP, that exceeded a threshold mean chain length of 65 phosphate subunits, readily initiated FXIIa formation (Fig. 7B). To discriminate between polyP-mediated FXII autoactivation and FXII activation by kallikrein (which is generated from plasma prekallikrein (PK) by FXIIa) (Tankersley and Finlayson, 1984), we incubated purified FXII and activated PK with polyP. PolyP was as efficient a surface as dextran sulfate (a strong contact activator (Samuel et al., 1992)) in supporting kallikrein-driven activation of FXII (Fig. 7C). When autoactivation of FXII was investigated without the addition of activated PK to the reaction, polyP generated significant amounts of FXIIa compared to the no surface control, but was not as efficient as dextran sulfate (Fig. 7D).

Figure 7. PolyP triggers contact activation of factor XII. (see next page) (**A**) FXIIa formation was quantified by hydrolysis of the chromogenic substrate S-2302 at 405 nm in human plasma samples incubated with 100 µg/ml polyP (◀) or kaolin (○). Platelet polyP was either untreated (0') or pretreated with phosphatase (0.05 U/µg polyP) for 10 (◀), 30 (◀), or 60 min (◁). Data are means ± SD, *n*=5. (**B**) FXIIa activity**.** Human plasma was incubated with buffer, kaolin, or polyP of 3, 25, 45, 65, 125 or 1000 chain length (100 µg/ml each). Plasma FXIIa activity was quantified after 20 min by absorbance of the chromogenic substrate S-2302 at 405 nm. Shown are means ± SD, *n*=8. (**C** and **D**) Plasma-free activation of purified FXII (200 nM) was analyzed in the presence (**C**) or absence (**D**) of PK (0.5 nM). The mixture was stimulated with dextran sulfate (■; 1 µg/ml), polyP (◀; 5 µg/ml), or buffer (●). FXIIa concentrations were determined following S-2302 hydrolysis. Data are means ± SD, *n*=4.


A
FXIIa [OD at 405 nm]
1.25
1.00
0.75
0.50
0.25
0.00
5
10
15
20
25
30
35
40
45
time [min]
kaolin
buffer
polyP+PSP
0'
10'
30'
60'
B
FXIIa [OD at 405 nm]
1.25
1.00
0.75
0.50
0.25
0.00
buffer
kaolin
3
25
45
65
125
1000
polyP
C
FXIIa [nM]
120
100
80
60
40
20
0
0
20
40
60
time [min]
dextran sulfat
polyP
buffer
D
FXIIa [nM]
35
30
25
20
15
10
5
0
0
20
40
60
time [min]
dextran sulfat
polyP
buffer

The ability of a molecule to act as a surface for contact activation relies on interactions of contact factors with the negatively charged surface (Colman, 1984). A gel shift assay demonstrated direct binding of polyP to contact system factors (Fig. 8A). Electrophoretic mobility shifts indicated strong interaction of polyP with both FXII or HK, whereas binding to PK and FXI was weaker. PolyP did not bind to antithrombin (AT) or albumin (ALB). Zirconia beads coated with either polyP or BSA confirmed specific and direct polyP binding to FXII, PK, HK, and FXI, with FXII being the strongest polyP-interacting protein (Fig. 8B).

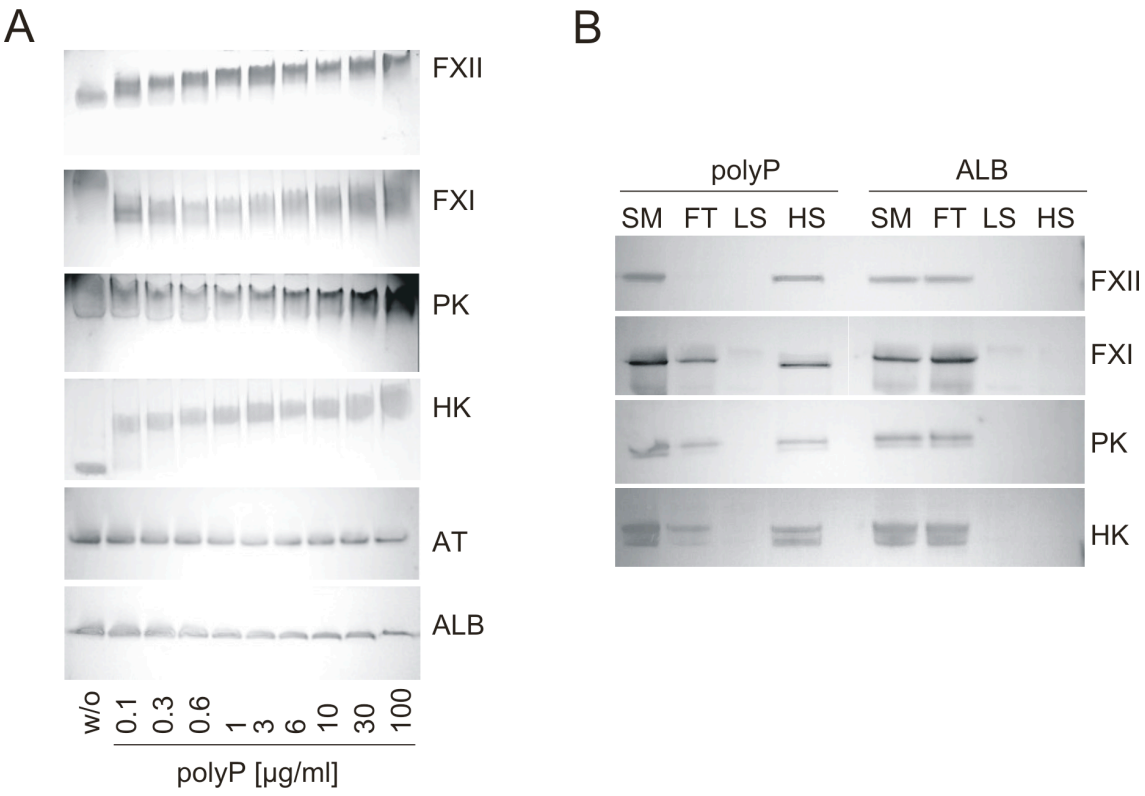


Figure 8. (**A**) **Gel-mobility shift analyses of polyP binding to proteins.** Purified FXII, FXI, PK, HK, antithrombin (AT) or albumin (ALB) were incubated with buffer ("w/o") or with increasing concentrations of $polyP_{125}$ (0 – 100 µg) for 10 min, after which mixtures were resolved on a 10 % Tris-Glycine gel under native conditions and stained for protein using Gel Code Blue. (**B**) **Protein binding to immobilized polyP.** PolyP (left) or albumin (ALB, right) were coupled to zirconia (zirconium dioxide) beads. FXII, FXI, PK, or HK (5 µg each) were incubated with the beads, after which the flow-through ("FT") fraction was collected by centrifugation using mini spin columns. The beads were washed with a low salt buffer (50 mM NaCl; "LS") followed by a high salt buffer (1 M NaCl; "HS"). Fractions were probed by Western blotting with appropriate antibodies. Starting material ("SM") refers to the initial protein load added to the beads. These data resulted from collaboration and were kindly provided by Dr. N. Mutch.

5.3 PolyP triggers bradykinin generation in plasma

FXIIa initiates BK release by PK-mediated cleavage of HK (Fig. 9A). We examined contact activation and BK release in human plasma and found that polyP at concentrations ≥1 µg/ml initiated complete HK cleavage (Fig. 9B). Consistently, BK concentrations were high (>750 ng/ml) in these samples, but low in samples treated with buffer (31 ± 4 ng/ml) or with ≤1 µg/ml polyP, which was not sufficient to initiate HK processing (Fig. 9C). To confirm that the FXII/PK cascade mediated BK generation from HK, we probed for the protease zymogens by Western blotting and found that polyP ≥1 µg/ml triggered complete PK and partial FXII activation with a slightly higher concentration (≥2 µg/ml) results in complete activation of plasma FXII (Fig. 9B).

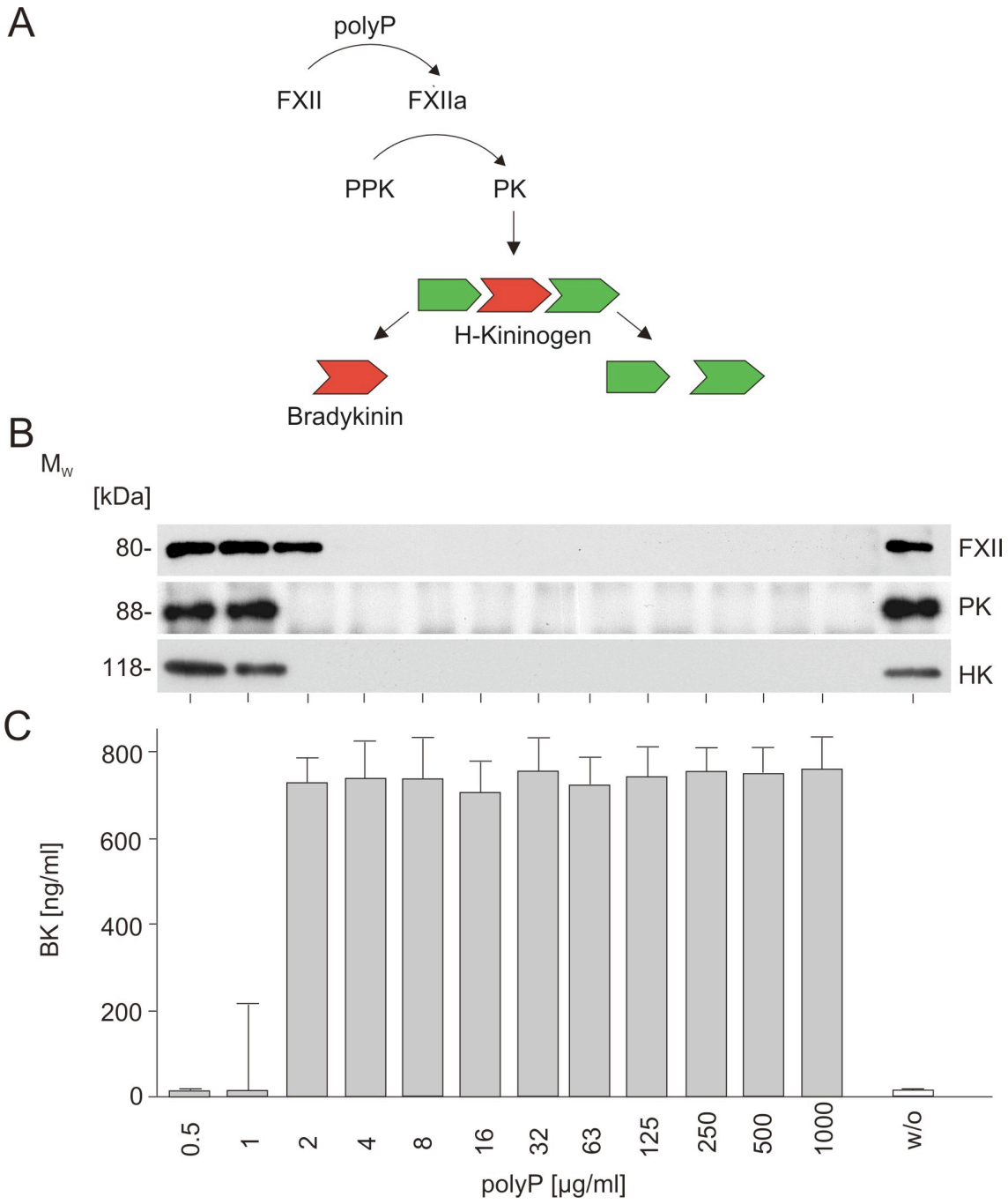


Figure 9. PolyP activates the kallikrein-kinin system. (**A**) Scheme of polyP-induced FXII-mediated HK-cleavage and release of BK. (**B**) PolyP-induced cleavage of FXII, PK and HK in plasma. Human plasma was incubated with increasing concentrations of polyP (0.5 – 1000 µg/ml) or buffer control ("w/o"), and then subjected to SDS-PAGE and western blotting. Samples were probed with antibodies against FXII, HK, and PK. (**C**) PolyP-induced bradykinin release in plasma. BK in polyP-incubated samples was quantified by ELISA. The columns give mean values ± SD, *n*=4.

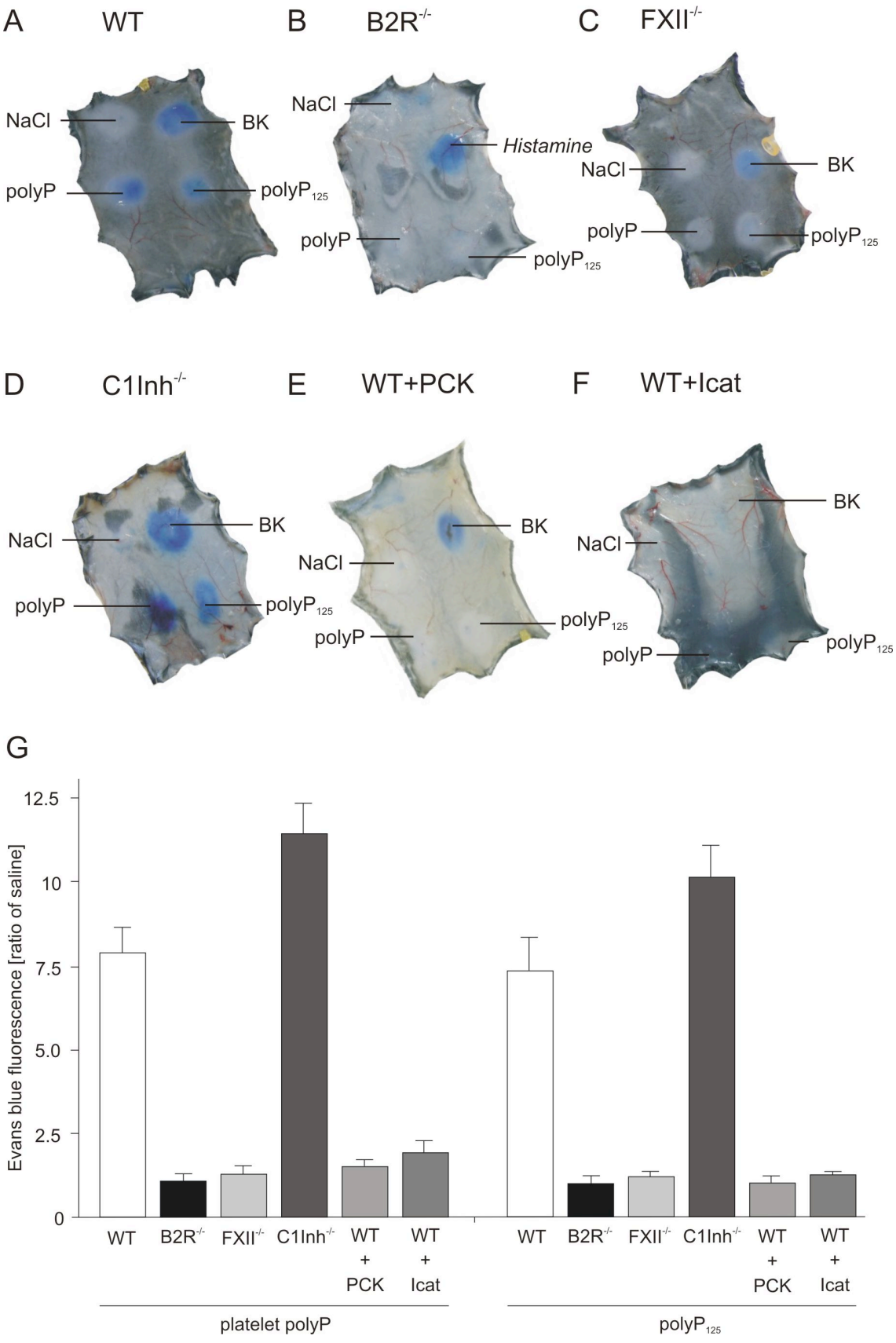
5.4 PolyP increased vascular permeability by a bradykinin-dependent mechanism

A hallmark of BK activity is increase in vascular permeability (Han et al., 2002; Nussberger et al., 2002). To analyze whether polyP initiates contact system-mediated capillary leakage via BK formation, we employed a Miles edema model (Miles and Miles, 1952) in genetically altered mice (Fig. 10A-F). Injected stimuli (50 µl each) triggered dermal vessel leakage, which was visualized by Evans Blue tracer. Extravasated tracer was extracted from the tissue, quantified by fluorescence emission, and plotted relative to the NaCl-induced signal in wild-type (WT) mice (set to 1.0) (Donelan et al., 2006). In WT mice, platelet polyP or synthetic $polyP_{125}$ (10 µg each) initiated leakage, that was increased 7.9 ± 0.8 and 7.4 ± 1.0 fold, respectively, higher than vehicle alone, respectively (*n*=10, Fig. 10A and G). BK (100 µM) stimulation in WT mice increased leakage 11.3 ± 1.5 fold of vehicle (*n*=10, Fig. 10A). To confirm that polyP induces vascular leakage by releasing BK, we employed B2R-deficient ($B2R^{-/-}$) mice, which are resistant to BK signaling (Borkowski et al., 1995) and protected from BK-driven edema formation (Han et al., 2002). $B2R^{-/-}$ mice were mostly resistant to polyP-induced increase in permeability (polyP 1.1 ± 0.2 and $polyP_{125}$ 1.1 ± 0.1, 10 µg each, *n*=5, Fig. 10B and G). Similarly, BK failed to induce a response in $B2R^{-/-}$ mice (1.1 ± 0.2, *n*=5, not depicted) whereas injection of histamine (100 µM) resulted in considerable leakage (7.7 ± 1.3, Fig. 10B), confirming that $B2R^{-/-}$ mice are susceptible for edema formation by contact system independent pathways. Because FXII-independent mechanisms for PK activation exist (Schmaier and McCrae, 2007), we analyzed polyP-triggered leakage in FXII-deficient mice ($FXII^{-/-}$), which are defective in contact system-driven BK formation (Iwaki and Castellino, 2006; Pauer et al., 2004). $FXII^{-/-}$ mice were almost completely resistant to polyP-induced leakage, and the degree of edema was not significantly different from the buffer-treated group (polyP 1.3 ± 0.2 and $polyP_{125}$ 1.2 ± 0.2, *n*=10, Fig. 10C and G). BK-stimulated edema formation in $FXII^{-/-}$ mice was similar to that observed in WT animals (10.4 ± 1.3, *n*=10, Fig. 10C).

Hereditary angioedema is characterized by recurrent attacks of swelling owing to a deficiency of a functional C1 esterase inhibitor (C1INH), which is the major plasma inhibitor for several complement proteases, FXIIa and PK (Zuraw and Herschbach, 2000). In C1INH-defective individuals, poorly defined stimuli trigger contact system-driven excess of BK, that causes attacks of edema (Davis, 2006). PolyP initiated excessive edema in C1INH null animals, that exceeded levels in WT by >40% (polyP 11.8 ± 0.9 and polyP$_{125}$ 10.9 ± 0.8, *n*=10, Fig. 10D and G), whereas injection of BK-induced (11.0 ± 0.8, Fig. 10D) tracer extravasation was similar to WT mice levels (11.3 ± 1.5, *n*=10, Fig. 10A).

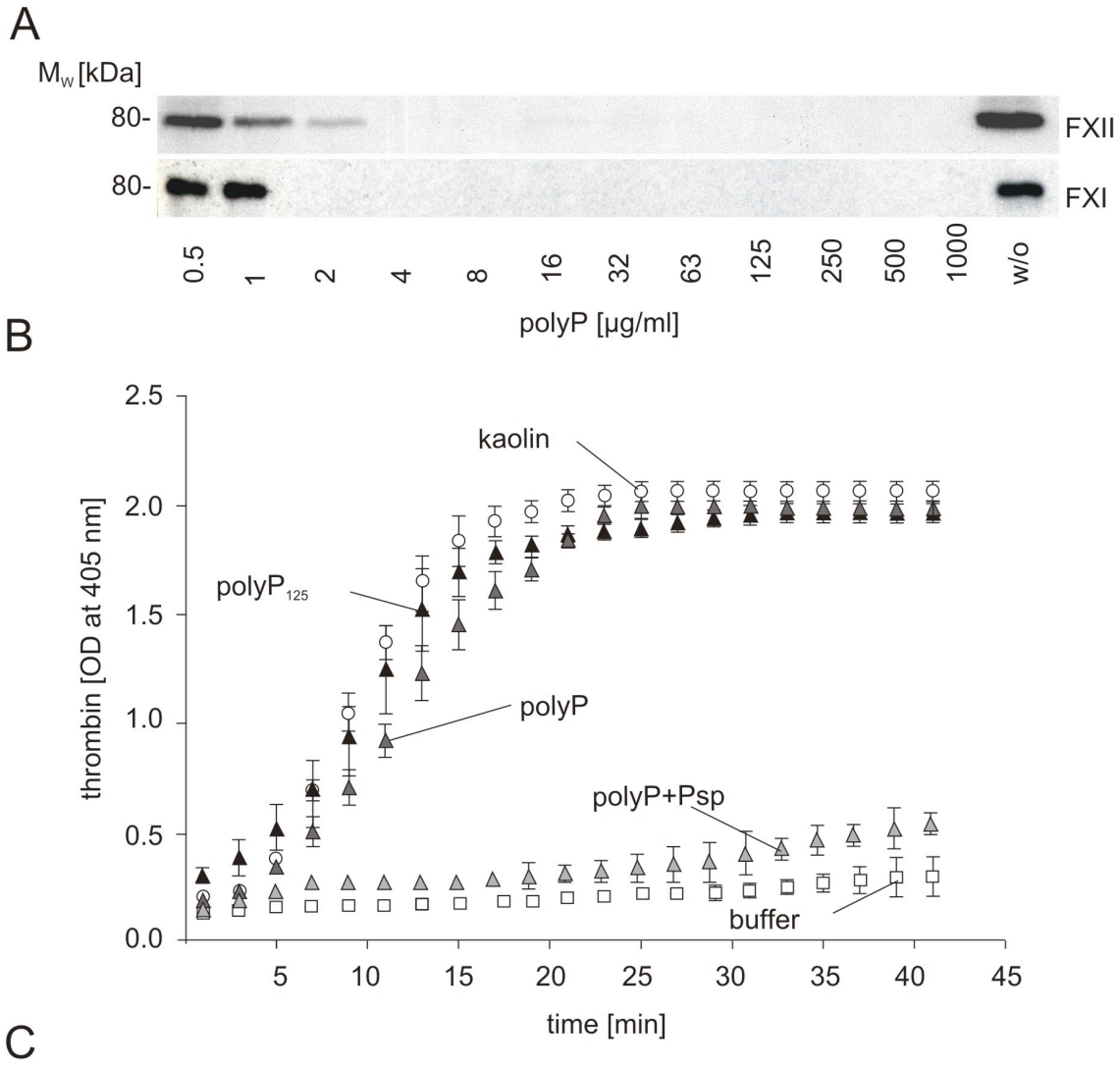
Since congenital deficiency in B2R or FXII protects mice from polyP-driven edema formation, pharmacological targeting of BK-signaling or -formation should provide similar protection. To address this hypothesis, we treated WT mice with an FXIIa inhibitor H-D-Pro-Phe-Arg-chloromethylketone (PCK, 8 mg/kg body weight) (Kleinschnitz et al., 2006), or with the selective B2R antagonist icatibant (Icat, 175 µg/kg body weight) 5 min prior to polyP application. PolyP was unable to induce leakage and edema formation in WT mice pretreated with PCK (polyP 1.9 ± 0.4 and polyP$_{125}$ 1.2 ± 0.2, *n*=10, Fig. 10E and G) or Icat (polyP 1.6 ± 0.2, polyP$_{125}$ 1.1 ± 0.2, *n*=10, Fig. 10F and G). BK function was blocked by Icat (Fig. 10F), but was independent of FXII activity (1.2 ± 0.1, *n*=10, Fig. 10E). Inhibitor application after polyP injection did not significantly reduce edema formation (not shown). Together, these findings in loss- and gain-of-function models are consistent with the *in vitro* findings and indicate that polyP activates FXII-driven BK formation *in vivo*.

Figure 10. PolyP triggers contact system-mediated bradykinin-induced edema in mice. (see next page) (**A-F**) Evans blue was intravenously infused as a tracer into the following: (**A**) WT mice, (**B**) B2R$^{-/-}$ mice, (**C**) FXII$^{-/-}$mice, (**D**) C1INH$^{-/-}$ mice, (**E**) PCK-infused WT mice (8 mg/kg body weight), and (**F**) icatibant-administered WT mice (175 µg/kg body weight). Dorsal skin edema formation was induced by intradermal injection of 50 µl saline (NaCl), BK (100 µM), platelet polyP (10 µg), or synthetic polyP$_{125}$ (10 µg), respectively, and visualized by tracer extravasation after 30 min. (B2R$^{-/-}$ animals were treated with 100 µM histamine instead of BK). (**G**) Evans blue tracer from skin welts was extracted and quantified. Extravasated tracer is plotted as fold-increase of saline-induced signal in WT mice to control for inter-animal variability. Data are means ± SD of 10 independent experiments.


A WT
NaCl
BK
polyP
polyP125
B B2R-/-
NaCl
Histamine
polyP
polyP125
C FXII-/-
NaCl
BK
polyP
polyP125
D C1Inh-/-
NaCl
BK
polyP
polyP125
E WT+PCK
NaCl
BK
polyP
polyP125
F WT+Icat
NaCl
BK
polyP
polyP125
G
Evans blue fluorescence [ratio of saline]
12.5
10
7.5
5.0
2.5
0
WT
B2R-/-
FXII-/-
C1Inh-/-
WT + PCK
WT + Icat
platelet polyP
polyP125

5.5 PolyP initiates the intrinsic pathway of coagulation in plasma

FXIIa initiates fibrin formation by the intrinsic pathway of coagulation (Gailani and Renne, 2007). To analyze the procoagulant activity of polyP we incubated human plasma with increasing concentrations of polyP (0.5 – 1000 µg/ml) and followed generation of FXIIa and activation of its principal substrate in the intrinsic pathway of coagulation, FXI, by Western blotting (Fig. 11A); we also quantified thrombin generation (Fig. 11B). PolyP ≥1 µg/ml triggered the conversion of FXII and FXI to the active proteases as indicated by the disappearance of the zymogen forms at 80 kDa (Renne et al., 2002a) (Fig. 11A). FXIIa/FXIa formation correlated with polyP-triggered thrombin generation (Fig. 11B). Digestion of polyP with phosphatase (0.05 U/µg polyP) impeded the procoagulant activity of the polymer. Furthermore, we compared polyP with kaolin for initiating thrombin formation in plasma in real time, using a fluorogenic thrombin substrate (Hemker et al., 2006). PolyP and kaolin (100 µg/ml each) driven thrombin generation had almost identical kinetics (lag time = time to achieve 10 nM thrombin of 6.6 ± 0.1 for polyP *vs.* 6.3 ± 0.2 min for kaolin and time to maximal thrombin/peak 13.1 ± 0.3 *vs.* 13.8 ± 0.2 min), potency (peak = maximal thrombin of 142.9 ± 5.2 *vs.* 144.8 ± 1.1 nM), and effects on total thrombin generation (endogenous thrombin potential (ETP) = integrated thrombin generation over 60 min of 2700 ± 97 and 2682 ± 34 nM*min, *n*=5 each) (Fig. 11C). Pre-incubation of polyP with phosphatase (0.05 U/µg polyP) largely abolished thrombin formation stimulated by polyP (ETP <100 nM*min). We assessed the procoagulant activity of polyP by *in vitro* clotting tests.



C

	lagtime [min]	peak [nM]	time to peak [min]	ETP [nM*min]
polyP	6.6 ± 0.1	142.9 ± 5.2	13.1 ± 0.3	2700 ± 97
kaolin	6.3 ± 0.17	144.8 ± 1.1	13.8 ± 0.2	2682 ± 34

Figure 11. PolyP activates the intrinsic pathway of coagulation in plasma. (**A**) PolyP-induced cleavage of FXII and FXI in plasma. Plasma was incubated for 30 min at 37°C with increasing concentrations of polyP (0.5 – 1000 µg/ml) and analyzed for zymogen forms of FXII and FXI by Western blotting. Untreated plasma ("w/o") shows initial FXII and FXI levels. (**B**) Thrombin generation in plasma incubated with buffer alone (□), 100 µg/ml kaolin (○), or platelet polyP (▲; "polyP"), synthetic $polyP_{75}$ (▲), platelet polyP treated with phosphatase prior to addition of plasma (△; "polyP+Psp"). Data are means ± SD, *n*=5. (**C**) Comparison of thrombin-forming activities of polyP and kaolin. Clotting was initiated by addition of 100 µg/ml polyP or kaolin to plasma. Thrombin generation was monitored in real time by using a fluorogenic substrate for thrombin and analyzed using Thrombinoscope software. Lag time is the time to achieve 10 nM thrombin; peak indicates the maximal thrombin concentration; time to peak is the time to achieve maximal thrombin starting from addition of the stimulus, and ETP is endogenous thrombin potential (integrated thrombin generation over 60 min). Data are means ± SD, *n*=5. PolyP and kaolin-stimulated coagulation parameters do not differ significantly using Student's *t* test.

We assessed the procoagulant activity of polyP by *in vitro* clotting tests. Addition of polyP (0 – 5 µg/ml) to normal plasma, but not to FXII-depleted plasma shortened the clotting time in a dose-dependent manner (Fig. 12A). Clotting times in recalcified plasma triggered by synthetic $polyP_{125}$, and $polyP_{1000}$ were similar to kaolin (139 ± 16 and 77 ± 2 vs. 69 ± 14 sec in PFP, and 52 ± 16, and 87 ± 156 *vs.* 51 ± 14 sec in PRP), whereas $polyP_3$ stimulated clotting did not significantly differ from buffer control (833 ± 43 *vs.* 957 ± 58 sec in PFP, and 743 ± 31 sec *vs*. 813 ± 57 in PRP, $P>0.05$, $n=5$) (Fig. 12B). These findings demonstrate a similarity in the mechanism of polyP and kaolin-driven clotting and strongly suggest that polyP contributes to fibrin formation predominantly via activation of the intrinsic pathway of coagulation.

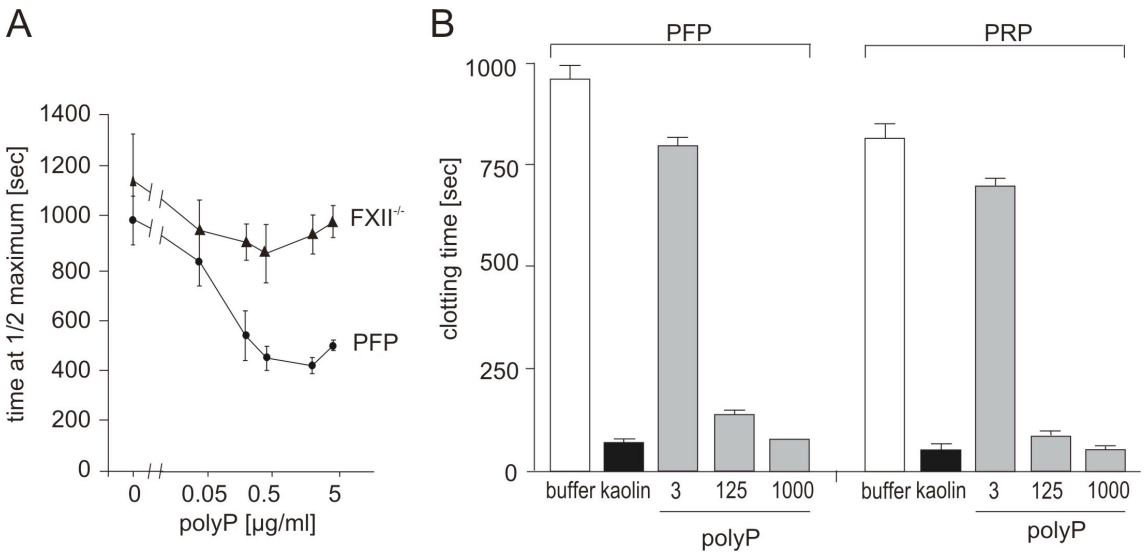


Figure 12. Plasma clotting times. (**A**) PolyP was added to normal plasma (●) or FXII-immunodepleted plasma (▲) at a range of concentrations (0 - 5 µg/ml) with clot formation quantified as the half-maximal change in turbidity at 405 nm. Data are means ± SD, $n=3$. These data resulted from a collaboration and were kindly provided by Dr. N. Mutch. (**B**) Recalcification clotting times were determined in human platelet-free plasma (PFP) and platelet-rich plasma (PRP) incubated with 100 µg/ml $polyP_3$, $polyP_{125}$, or $polyP_{1000}$, respectively, for 1 min prior to $CaCl_2$ addition. Data are means ± SD, $n=5$.

5.6 Deficiencies in intrinsic pathway proteases protect mice from polyP-induced pulmonary embolism

To analyze polyP for fibrin formation *in vivo* we challenged WT and FXII$^{-/-}$ mice in a model of lethal pulmonary thromboembolism (PE) by intravenous infusion of polyP (300 µg/g body weight). All WT mice with the exception of a single animal (14/15) died within 5 min after polyP application (Fig. 13A). In contrast, FXII$^{-/-}$ mice were significantly protected from polyP-induced PE, with 12 out of 15 FXII$^{-/-}$ mice surviving the challenge for >30 min (***P*<0.01 FXII$^{-/-}$ *vs.* WT). To show that the high level of survivors observed in FXII$^{-/-}$ mice was a direct result of defective polyP-driven FXII activation rather than a secondary effect associated with hereditary FXII deficiency, we reconstituted FXII null mice with human FXII (hFXII) (2 µg/g body weight). Intravenous infusion of human protein corrected the prolonged aPTT clotting time of FXII-deficient murine plasma to normal values (28 ± 4 sec) and restored susceptibility for lethal pulmonary embolism after polyP infusion, with 12 out of 15 reconstituted animals dying (Fig. 13A). This supports the critical role of polyP-driven FXII activation in this model. Pretreatment of polyP with phosphatase (0.05 U/µg polyP) abolished its procoagulant activity, with 14/15 mice surviving infusion of degraded polyP (Fig. 13B). The infestin4-based recombinant FXIIa-inhibitor CSL829 (Schmidbauer, 2009) and PCK irreversibly inhibit the amidolytic activity of FXIIa and PK-mediated activation of FXII. CSL829 (150 µg/ml) blocked polyP-driven FXII and consecutive FXI, PK and HK activation steps in plasma (Fig. 13C). To test the protective potential of FXIIa-inhibitors for polyP-driven thrombosis, WT mice were intravenously injected with CSL829 (15 µg/g body weight) 10 min prior to polyP infusion, which prolonged the aPTT (48 ± 9 sec) and significantly protected mice from lethal PE (12 out of 15 survived; ***P*<0.01 *vs.* WT, Fig. 13A). PCK (8 µg/g body weight) provided similar protection from polyP-triggered thromboembolism (12/15 survived, not shown). Histologic sections of lung tissue from polyP treated mice and counting of formed thrombi are shown in Figure 13D and E.

While the majority of vessels are obstructed in WT and hFXII-reconstituted FXII$^{-/-}$ mice (dead and survivors), virtually no thrombi were found in FXII$^{-/-}$ mice and in WT animals treated with FXIIa-inhibitors. Some interstitial edema were observed in WT and reconstituted FXII$^{-/-}$, but not in FXII$^{-/-}$ or inhibitor treated mice, indicating that thrombosis was associated with increased permeability in these animals. If the prothrombotic effect of polyP is mediated by the intrinsic pathway *in vivo*, FXI$^{-/-}$ mice (deficient in the primary FXIIa substrate in the intrinsic pathway of coagulation) should be protected from pulmonary embolism similarly to FXII$^{-/-}$ animals (Fig. 13A). Indeed, FXI deficiency conferred resistance to polyP-induced PE and FXI null mice were largely protected from polyP challenge (11/15 survived; $^{**}P<0.01$ FXI$^{-/-}$ *vs.* WT). Targeting B2R was shown to confer protection from arterial occlusion induced by vascular injury (Shariat-Madar et al., 2006). We analyzed B2R$^{-/-}$ mice in our PE model and found that almost all (13/15) animals died (Fig. 13A), suggesting that BK did not contribute to polyP initiated PE. To confirm that polyP-initiated FXII activity contributes to pathological thrombosis by the intrinsic pathway of coagulation, we measured fibrin formation in lung tissues by quantitative immunoblot analysis of urea-insoluble tissue extracts. We used the antibody 59D8 (Hui et al., 1983), which recognizes a neo-epitope exposed on fibrin, following thrombin-dependent cleavage of fibrinogen and does not cross-react with the precursor protein. Fibrin accumulation was significantly reduced in the lungs of FXII$^{-/-}$ and inhibitor treated mice, compared to WT and reconstituted animals (Fig. 13F).

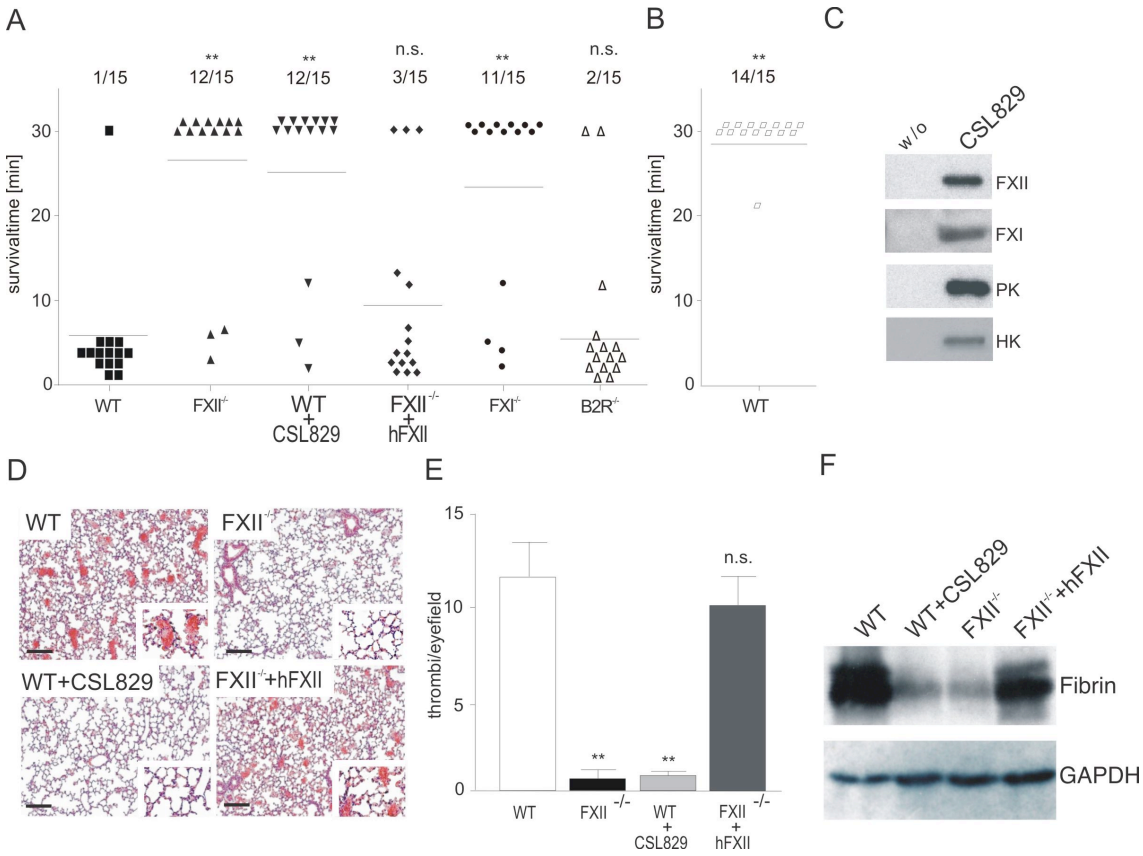


Figure 13. PolyP triggers thrombosis *in vivo*. (**A** and **B**) Survival times following i.v. polyP challenge. (**A**) Pulmonary thromboembolism was induced by i.v. infusion of platelet polyP (300 µg/g body weight) in WT mice, FXII$^{-/-}$ mice, FXII$^{-/-}$ mice reconstituted with human FXII ("hFXII", 2 µg/g body weight), WT mice infused with FXII inhibitor CSL829 (150 µg/g body weight), FXI$^{-/-}$ mice, or B2R$^{-/-}$ mice. Mortality was assessed in each group (*n*=15); animals still alive 30 min after challenge were considered survivors. Asterisks indicate significant reduced mortality in FXII$^{-/-}$, CSL829-treated WT, and FXI$^{-/-}$ animals as compared to WT (*P*<0.05). (**B**) PolyP was phosphatase digested (0.05 U/µg polyP) prior to infusion into WT animals and survival was analyzed as in panel A. (**C**) Efficacy of CSL829 inhibitor. Plasma supplemented with CSL829 (150 µg/µl) or buffer ("w/o") was incubated with polyP (500 µg/ml) for 30 min, and analyzed for zymogen FXII, FXI, PK and HK by Western blotting. (**D**) Hematoxylin and eosin stained sections of lungs of WT, FXII$^{-/-}$, CSL829 infused WT, and hFXII reconstituted FXII$^{-/-}$ mice 30 min after polyP administration (bar = 100 µm). (**E**) Thrombi per visual field were counted at 10x magnification from sections such as those in panel D. Data are mean ± SD for 100 fields. (**F**) Accumulation of fibrin in lung tissue of polyP treated WT, FXII$^{-/-}$, CSL829 pretreated WT, and reconstituted FXII$^{-/-}$ mice. Fibrin formation 30 min after polyP challenge was analyzed by western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control.

5.7 PolyP initiates fibrin formation on activated platelets

The intrinsic pathway critically contributes to activated platelet-promoted clotting in plasma (Walsh and Griffin, 1981) and arterial thrombus formation in mouse models (Renne et al., 2005a), suggesting that FXIIa is generated by platelets and that FXIIa-driven fibrin formation proceeds on activated platelet surfaces (Colman, 2006; Furie and Furie, 2008). To assess whether polyP may present the "foreign surface", that initiates FXII-driven fibrin formation on platelets, we determined whether phosphatase, which efficiently degrades polyP (Fig. 7A), inhibits procoagulant platelet activity. If polyP contributes to fibrin formation initiated by activated/procoagulant platelets, targeting polyP should interfere with clotting. Recalcification times in platelet-rich plasma (PRP) were determined in the absence or presence of phosphatase (10 U/ml), and platelets were either stimulated with calcium ionophore A23187 (5 µM, Fig. 14A) or Trap6 (30 µM, Fig. 14B) 10 min prior to $CaCl_2$ addition. In the absence of phosphatase, platelet activation with A23187 and Trap6 reduced clotting times up to 3.2- and 2.9-fold, respectively, as compared to untreated plasma (1st *vs.* 3rd column, Fig. 14A and B). Addition of phosphatase prior to recalcification almost completely abrogated the procoagulant activity conferred by stimulated platelets; clotting times in Ca^{2+}-ionophore or Trap6 treated PRP were not significantly different from unstimulated PRP (A23187: 365 ± 8 sec and Trap6: 411 ± 32 sec *vs.* 450 ± 2 sec, $P>0.05$, $n=6$ each, 4th column, Fig. 14A and B). Phosphatase did not alter the recalcification time in PRP without platelet activation (399 ± 13 sec, 2nd column in Fig. 14A and B). Consistent with our initial observation that clotting in A23187–stimulated murine PRP depends on FXII (Renne et al., 2005a), *in vitro* clotting in human FXII deficient PRP in response to either platelet agonist was severely impaired in the presence or absence of phosphatase (Fig. 14C and D). These findings demonstrate that activated/procoagulant platelets promote clot formation by polyP-stimulated intrinsic pathway of coagulation *in vitro*.

PolyP is procoagulant in plasma and in mouse models (Fig. 12 and 13). Platelets secrete polyP upon activation (Fig. 5) and targeting polyP interferes with clotting *in vitro* (Fig. 14A and B). To analyze polyP functions for pathological clotting driven by stimulated platelets *in vivo,* we established a model of lethal PE initiated by Trap6 infusion (Fig. 14E). The thrombin receptor-activating peptide Trap6 activates platelets (Hamad et al., 2008) and initiates polyP secretion (Fig. 5), but has no direct effect on the plasma coagulation cascade and does not trigger fibrin formation in platelet-free plasma by another mechanism (not shown). Almost all (13/15) WT mice died within 5 min after intravenous infusion of Trap6 (0.7 µg/g body weight) (Fig. 14E). In contrast, $FXII^{-/-}$ mice were largely protected from Trap6 induced lethal PE (13/15 survived; $^{**}P<0.01$ *vs.* WT). Infusion of phosphatase (15 U/g body weight) prior to Trap6 application protected WT mice from lethal PE and 13 out of 15 animals survived the challenge for >30 min. Lung histology from Trap6 treated mice confirmed PE (Fig. 14F). While the vast majority of vessels were obstructed in WT animals, virtually no thrombi were found in $FXII^{-/-}$ mice and in WT mice treated with phosphatase. Together these results show that polyP initiates plasma coagulation on procoagulant platelets *in vivo*.

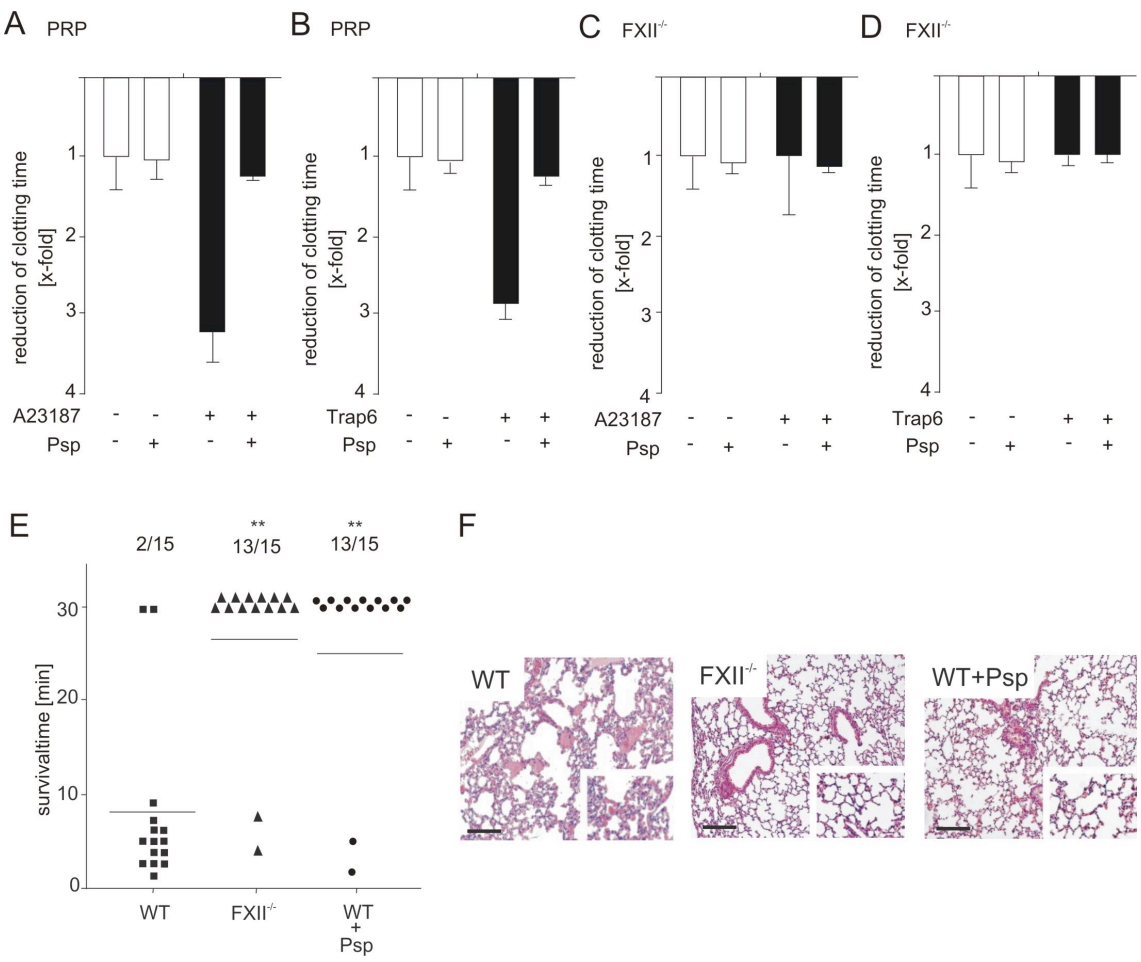


Figure 14. Targeting polyP blocks platelet procoagulant activity *in vitro* and thrombosis *in vivo.* (A and **B)** Recalcification clotting times were determined in platelet-rich plasma stimulated with (A) A23187 (5 µM) or (B) Trap6 (30 µM) in the presence (+) or absence (-) of phosphatase (10 U/ml; "Psp"). Reductions in clotting times are given relative to untreated plasma. Data are means ± SD, *n*=6. (**C** and **D**) The polyP-driven procoagulant activity of platelets depends on FXII. Recalcification clotting times were determined in platelet-rich plasma from a factor XII deficient individual (FXII levels <1 % as determined with the Siemens BSC-XP system) stimulated with A23187 (5 µM, C) or Trap6 (30 µM, D) in the presence (+) or absence (-) of phosphatase (10 U/ml, "Psp"). Reduction in clotting time is plotted relative to untreated plasma. Representative data are given from one of four tested FXII-deficient humans (means ± SD, *n*=5). (**E**) Mortality associated with i.v. injection of 0.7 µg/g body weight Trap6 in WT mice; FXII$^{-/-}$ mice; and WT mice injected i.v. with phosphatase (15 U/g body weight; "WT+Psp") before Trap6 challenge. 13 of 15 WT mice died within 10 min of challenge, whereas FXII$^{-/-}$ and phosphatase-treated WT mice were significantly protected from lethal pulmonary embolism induced by Trap6; 13/15 survived for >30 min each (FXII$^{-/-}$ and WT+Psp *vs.* WT, $P<0.05$, *n*=15 per genotype). (**F**) Targeting polyP interferes with pulmonary embolism in mice. Hematoxylin and eosin-stained lung sections of WT, FXII$^{-/-}$ mice, and phosphatase-treated WT animals ("WT+Psp") 30 min after Trap6 injection (0.7 µg/g body weight) (bar = 100 µm).

We further tested the importance of this concept for human disease states. Hermansky-Pudlak Syndrome (HPS) is a rare, complex hereditary disease (Gahl et al., 1998), in which a bleeding diathesis results from platelet storage pool deficiency. PolyP is accumulated principally in dense granules secretory organelles, which are absent in HPS patients (Ruiz et al., 2004). We compared the ability of platelets isolated from these individuals to initiate coagulation to platelets from normal individuals (Fig. 15A). Platelets were Trap6-stimulated before addition to recalcified normal (platelet-free) plasma. Supplementing plasma with normal platelets resulted in a recalcification time of 280 sec with no additional shortening of the clotting time upon addition of exogenous $polyP_{125}$ (10 µg/ml). Time to clot formation triggered by stimulated HPS platelets was longer (425 sec) and could be shortened to a 'normal' time of approximately 300 sec by addition of polyP. These results indicate that the concentration of polyP found in platelets is sufficient to trigger plasma coagulation, but that a reduction in the normal range of these concentrations, such as in HPS platelets, impairs the procoagulant potential of activated platelets.

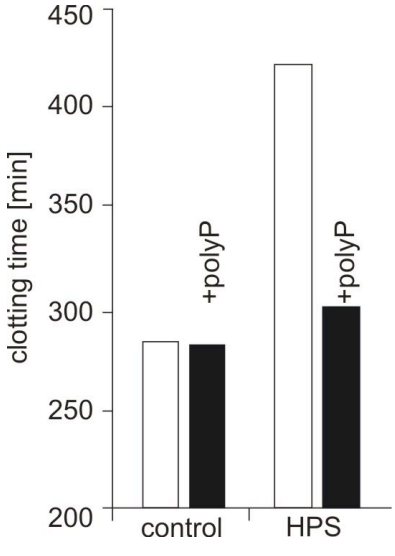


Figure 15. (**A**) **PolyP "rescues" defective fibrin formation in Hermansky-Pudlak Syndrome (HPS) patients.** Human platelets were isolated from healthy blood donors (control) and from patients with HPS and stimulated for 10 min at 37°C with Trap6 (50 µM) and collagen (10 µg/ml). Recalcification clotting times in normal plasma on addition of either normal (control) or HPS platelets (HPS, 1.5 $x10^7$) in the absence (open bars) or presence (closed bars) of synthetic $polyP_{125}$ (10 µg/ml). The graph shown is representative of 3 different experiments performed on different HPS and normal individuals. This data resulted from a collaboration and were kindly provided by Dr. N. Mutch.

Cumulatively these findings support the concept that inorganic polyP is a new class of platelet-derived proinflammatory and procoagulant mediator, that exerts its effect by activation of the FXII-driven contact activation system. PolyP initiates fibrin formation on procoagulant platelets, linking primary to secondary hemostasis.

6 DISCUSSION

Thrombosis may occur in the venous or arterial circulation, causing pulmonary embolism or myocardial infarction and stroke, the most common causes of death in the developed world (Mackman, 2008). Platelets play a pivotal role in vascular occlusive disease (Davi and Patrono, 2007; Furie and Furie, 2008; Ruggeri, 2002). These anucleate cells contribute to fibrin formation and inflammation leading to the concept of "procoagulant platelet activity". This study demonstrates that the inorganic polymer, polyP, which is secreted upon platelet activation, is responsible for platelet-driven fibrin formation and vascular leakage. PolyP mediates its effects by activating the FXII-driven contact activation system. Targeting polyP attenuates procoagulant and proinflammatory platelet activity *in vivo* and may serve as a new strategy to interfere with platelet-driven thromboembolic and inflammatory diseases. PolyP links platelet plug formation (primary hemostasis) and fibrin generation (secondary hemostasis). PolyP functions are not limited to procoagulant disorders, but also contribute to platelet-driven capillary leakage, which is a hallmark of inflammatory reactions.

In clinical aPTT clotting assays the anionic silicate kaolin is employed to activate FXII, which potently triggers FXII activation and induces fibrin formation by the intrinsic pathway *in vitro* (Cochrane and Griffin, 1982). Despite the importance of polyanion-driven FXII activation for *in vitro* clotting assays, FXII activation is dispensable for hemostasis *in vivo* as FXII deficient individuals do not suffer from increased hemorrhage. However, using FXII null mice we demonstrated that FXII activity has an essential function for thrombus formation (Kleinschnitz et al., 2006; Renne et al., 2005a). Severe deficiency in the clotting factor (<10 % plasma level) impaired the stability of the growing thrombus from the vessel wall without interfering with fibrin formation at wound sites. During pathological clotting FXII is likely to operate through the intrinsic pathway as factor XI-deficient mice are similarly protected from thrombus formation in $FeCl_3$-induced arterial injury models (Renne et al., 2005a; Wang et al., 2006).

Deficiency in FXII or FXI also impairs fibrin formation induced by ischemic brain injury (Kleinschnitz et al., 2006). The importance of the intrinsic pathway for thrombosis in humans remains less clear compared to animal models since individuals with severe deficiency of contact system proteases are rare (Gailani and Renne, 2007). However, factor XI may contribute to thrombosis in humans, since a congenital deficiency of this protein (<1 %) is associated with a reduced risk of ischemic stroke (Salomon et al., 2008). The decisive role of the contact system for thrombosis is not restricted to intrinsic pathway proteases. HK-deficient mice have a normal hemostatic capacity, but impaired thrombus formation in the carotid artery (Merkulov et al., 2008). These *in vivo* studies are consistent with the critical role of the intrinsic pathway for fibrin formation by activated platelets *in vitro* (Johne et al., 2006; Walsh et al., 1976) and suggest that FXII activation proceeds on procoagulant platelet surfaces within the thrombus. In this study we have shown that polyP is released by activated platelets (Fig. 5), and that a phosphatase, which degrades polyP blocks fibrin formation promoted by procoagulant platelets *in vitro* and *in vivo* (Fig. 14). Circulating exopolyphosphatases in plasma may limit the activity of platelet-derived polyP (Schroder et al., 1999) and provide thromboprotection similar to infused exogenous phosphatase.

PolyP is a “foreign” surface, that is not present under “normal” non-activated conditions. We failed to stimulate endothelial cells (EA.hy926, ECV304, HUVEC) to release polyP. Although these cells contain polyP, that could be liberated following cell injury (Fig. 5C and D), the polymer apparently does not significantly contribute to fibrin formation under these conditions, since FXII-deficient individuals do not bleed excessively. Either the endothelial polyP concentration is not sufficient to initiate the contact pathway, or yet unknown regulatory mechanisms interfere with polyanion-driven FXII activation at the vessel wall. RNA, which activates FXII (Cochrane and Griffin, 1982) and is liberated from disintegrating cells, is prothrombotic, but does not contribute to hemostatic mechanisms (Kannemeier et al., 2007).

The mechanism by which polyP-initiated fibrin formation contributes to thrombosis is not entirely clear, but polyP appears to stabilize the growing thrombus distant from the vessel wall. In plasma polyP promotes fibrin formation (Smith et al., 2006) and improves the stability of the fibrin clot structure (Smith and Morrissey, 2008b). Thromboelastography assays (which measure mechanical stability of a clot in whole blood) indicate that FXII-driven coagulation contributes to clot stability and intravital microscopy showed rapid embolisations of the platelet aggregate in the vessels of FXII null mice (Renne et al., 2005a).

FXII is activated by a variety of natural polyanions such as ellagic acid, nucleotides, sulfatides, misfolded proteins, and some types of collagen, or glycosaminoglycans (Cochrane and Griffin, 1982; Kannemeier et al., 2007; Maas et al., 2008; May et al., 2008; Muller and Renne, 2008; van der Meijden et al., 2009). Some contact activators specifically trigger BK formation (Johne et al., 2006; Maas et al., 2008; Schwartz, 2008), but do not initiate fibrin formation. Remarkably, patients with hereditary angioedema, who suffer from increased BK formation due to kallikrein-kinin system activity, do not have an increased thrombotic risk (Cugno et al., 1997; Zuraw, 2008). The selective activation of the FXIIa-driven cascade suggests that delicate regulatory mechanisms of the contact system may exist, involving cell type-specific FXIIa effects (Renne et al., 2002a; Renne et al., 2005a) and FXIIa proteolysis products (Schmaier, 2008).

Besides its importance for endogenous thrombus formation, synthetic polyP may be used as a hemostatic agent to reduce blood loss at sites of injury or during surgical interventions. Application of polyP has been shown to promote clot formation in plasma from hemophilia A and B patients (who are deficient in coagulation factor VIII or IX, respectively) (Smith and Morrissey, 2008a). In such plasmas procoagulant activities of polyP were additive to those of a recombinant form of active factor VII (rFVIIa), indicating that polyP-mediated fibrin formation is independent of the rFVIIa-driven extrinsic pathway.

In the present study we showed that polyP could “rescue” the prolonged clotting time of plasma containing platelets from HPS patients (Fig. 15). HPS is a disorder affecting endosome sorting and multiple platelet defects e.g. reduced ATP or serotonin storage and secretion result in bleeding syndromes of varying severity in effected patients (Nurden and Nurden, 2008), and whose platelets should therefore be unable to secrete polyP upon activation. As polyP-triggers FXIIa production (Fig. 7), which has the ability to efficiently activate FVII to FVIIa (Osterud and Rapaport, 1977), the polymer acts as a hemostatic adjuvant (Ong et al., 2008; Smith and Morrissey, 2008a). Indeed, FXIIa may bypass deficiency in factors VIII and IX by generating FVIIa in monkeys (Ton-That et al., 2000).

Fibrin formation is initiated at a site of vessel injury when plasma factor VIIa binds to TF, a membrane protein found on cells underlying blood vessel endothelium. The importance of FVIIa/TF (the extrinsic pathway of coagulation) initiate thrombin and fibrin formation is highlighted by the fact that mice lacking factor VII or TF die perinatally in utero from severe hemorrhage. However the activity of the FVIIa/TF complexes at the vessel wall is likely limited by inhibitors such as tissue factor pathway inhibitor (TFPI) (Lu et al., 2004), and by accumulation of fibrin and platelets over the area of injury (Hathcock and Nemerson, 2004). TF has been recognized to be circulating blood and the blood borne TF may drive fibrin production apart from the vessel wall (Giesen et al., 1999). The concept that there may be functional significant amounts of tissue factor on platelets remains controversial (Panes et al., 2007). TF is expressed on procoagulant leucocyte-, monocyte- or vessel wall cell-derived microparticles, which become incorporated into a growing thrombus in a P-selectin–dependent manner (Falati et al., 2003) and arterial thrombus formation is defective in mice expressing low levels of TF, although the relative contribution of vessel wall or blood cell–derived TF is not entirely clear (Chou et al., 2004; Day et al., 2005). As tiny amounts of active TF induce rapid clotting in plasma (Butenas and Mann, 2004), TF on microparticles may exist in a latent (encrypted) form that lacks coagulant activity.

Mechanism of TF encryption may involve lipid reorganization, secretion of TF containing granules, protein disulfide isomerase (PDI)-mediated dimerization (Reinhardt et al., 2008), and splicing of TF pre-mRNA (Schwertz et al., 2006). The relative contribution of TF- and FXII-driven fibrin formation for thrombosis is not know and may differ depending on the vascular bed, type of injury, and flow conditions. At least in human plasma, and in mouse PE models, blood borne TF is not sufficient to mediate fibrin formation on activated platelets in the absence of polyP (Fig. 14) indicating that polyP-driven FXII activation is an essential component for thrombus formation.

FXIIa initiates several protease cascades in plasma, such as the kallikrein-kinin system, the intrinsic pathway of coagulation, and the complement and fibrinolytic systems (Gailani and Renne, 2007). In this study we have shown that polyP increases vascular leakage in mice by FXII-initiated BK formation (Fig. 9 and 10). Pre-formed FXIIa has been shown to induce leakage in skin microvessels in a BK-dependent manner in mice (Renne et al., 2005). Contact system-mediated kinin release is a critical component of *Escherichia coli* (*E.coli*) and *Salmonella* induced sepsis and septic shock (Herwald et al., 1998). It is not precisely known how these bacteria initiate BK-formation, but *Salmonella* (Kim et al., 2002) and *E. coli* (Rao et al., 1998) are known to contain high amounts of polyP. One can speculate that disintegrating bacteria may release the polymer, which could activate FXII and initiate BK-mediated leukocyte chemotaxis, pain sensations, and leakage (Leeb-Lundberg et al., 2005). Pharmacological inhibition of PK/FXIIa interferes with *Salmonella* induced pulmonary plasma leakage and blood cell infiltration in rats (Persson et al., 2000), again leading us to speculate that polyP may contribute to infectious disease. FXIIa can initiate the classical complement system, that generates C3a and C5a in host-defense reactions (Ghebrehiwet et al., 1981), and platelet activation triggers initiation and propagation of the complement system (Del Conde et al., 2005). PolyP released from activated platelets might therefore contribute to this response by generating FXIIa.

In addition to being anti-thrombotic phosphatase may serve as a potent anti-inflammatory agent in reducing FXIIa-driven complement and BK effects with possible implications for acute and chronic inflammatory responses, such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and also in the progression of malignancies, or in immune responses to bacteria.

7 CONCLUDING REMARKS

Beyond an eminent role in hemostasis and thrombosis at sites of injury, platelets have also prominent functions in inflammatory processes. Upon stimulation platelets release various kinds of proinflammatory and procoagulant mediators from their storage granules. In the present study, we identified polyP from human platelet dense granules as the long sought physiological surface for contact activation, culminating in inflammation as well as in blood coagulation. Platelets released this inorganic polymer after stimulation; polyP directly bound and activated coagulation factor XII. PolyP triggers in a FXII-dependent manner the activation of kininogen-processing kallikreins, and capillary leakage via release of bradykinin. Furthermore, polyP appeared to contribute to intrinsic blood coagulation. These findings establish inorganic polyP as a new class of proinflammatory and procoagulant substances that exert their activity by the activation of the FXII-driven contact phase system activation (Fig. 16).

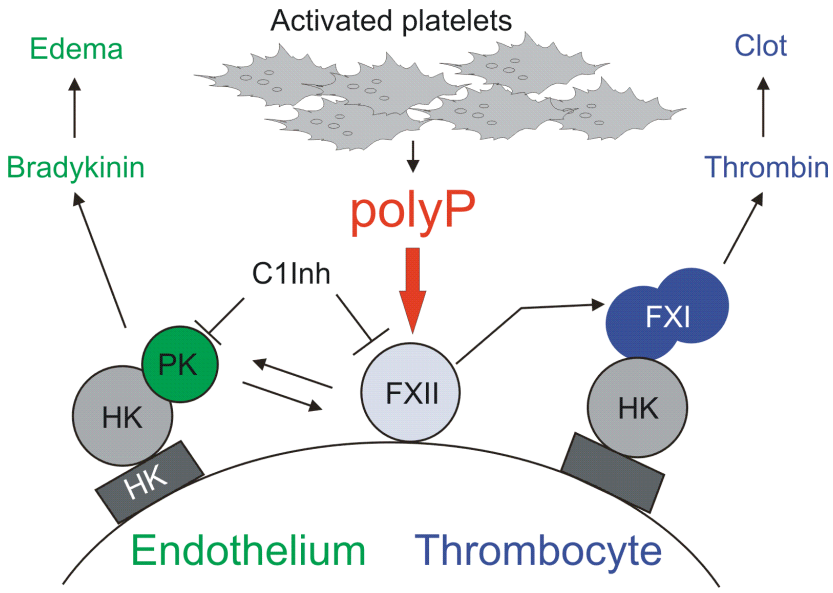


Figure 16. Function of platelet polyP in FXII-driven pathways. Activated platelets release polyP from their dense granules. This polyanion triggers on the one hand FXII-mediated thrombin formation via activation of the intrinsic pathway of coagulation and on the other hand edema formation via activation of the kallikrein-kinin system.

Interrupting polyP-driven fibrin- and BK-generation interferes with thrombosis and edema formation *in vivo*. Platelet derived polyP may present a heretofore unrecognized target for intervention in FXIIa driven inflammatory and thromboembolic diseases.

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LIST OF ABBREVIATIONS

°C	degrees Celcius
% (w/v)	mass-volume percentage
% (v/v)	volume-volume percentage
µ	micro
µg	microgram
µl	microliter
µM	micromolar
ADP	adenosinediphosphate
aPTT	activated partial thromboplastin time
ATP	adenosine-5'- triphosphate
BK	bradykinin
bp	base-pair
BPB	bromphenol blue
BSA	bovine serum albumin
Da	dalton
DMEM	Dulbecco´s modified eagle´s medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescene
EDTA	ethylene diamine tetraaceticacid
ELISA	enzyme linked immunosorbentassay
EtOH	ethanol
ECV	human umbilical vein endothelial cells
FBS	fetal bovine serum
Fig.	figure
FXI	plasma coagulation factor XI
FXII	plasma coagulation factor XII
g	gram
GPDH	glycerol 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
HK	high-molecular weight kininogen
HRP	horseradish peroxidase
IgG	immunoglobulin G
kb	kilo base
kDa	kiloDalton
M	Molarity (mol/L)
mM	millimolar
mRNA	messenger RNA
M_W	molecular weight
nm	nanometer
nM	nanomolar
N-terminal	amino terminal
PAGE	polyacrylamide gel
PBS	phosphate buffered saline
PCK	H-D-Pro-Phe-Arg-chloromethylketone
pH	negative base-10 proton concentration
PK	plasmakallikrein
polyP	polyphosphate
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodiumdodecylsulfate
TF	tissue factor
TFPI	tissue factor pathway inhibitor
Trap6	H-Ser-Phe-Leu-Leu-Arg-Asn-OH
Tris	trishydroxyl-methylaminomethane
U	unit
V	Volt
w/o	without

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Presentations

F. Mueller, N. J. Mutch Mueller, W. A. Schenk, W. A. Gahl, J. H. Morrissey, T. Renné. Platelet polyphosphate are procoagulant and proinflammtory *in vivo*. XXII Congress of the International Society on Thrombosis and Haemostasis, Boston, USA, July 11-16, 2009. Abstract in *J Thromb Haemost*. 2009; 7 (1): AS-WE-034. [Oral presentation]

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F. Mueller, S. Wilhelm, S. Cichon, M. Nöthen, T. Renné. Pathophysiology and therapy of hereditary angioedema type III. 52. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung, Wiesbaden, Germany, February 20-23, 2008. Abstract in *Hämostaseologie*. 2008; 28 (1-2): A38. [Oral presentation]

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F. Mueller, B. Schinke, U. Walter, W. Schenk, T. Renné. Polyphosphates initiate thrombosis and inflammation by factor XII activation *in vivo.* Gemeinsamer Kongress der Österreichischen Gesellschaft für Laboratoriumsmedizin und klinische Chemie und der Deutschen Vereinten Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, Wien, Austria, September 19-22, 2007. V44. [Oral presentation]

F. Mueller, S.Cichon, M. Nothen, U. Walter, T. Renne. Hereditary angioedema type III are caused by a gain of function mutation in coagulation factor XII, which increases vascular permeability mediated by bradykinin generation. XXIst Congress of the International Society on Thrombosis and Haemostasis, Geneva, Switzerland, July 6-12, 2007. Abstract in *J Thromb Haemost.* 2007; 5 (2): O-S-090. [Oral presentation]

F. Mueller, S. Cichon, A. Karpushova, K. Bork, M. Nöthen, U. Walter, T. Renné. Increased activity of coagulation factor XII (Hageman factor) causes hereditary angioedema type III. 51. Jahrestagung der Gesellschaft für Thrombose und Hämostaseforschung, Dresden, Germany, February 21-24, 2007. FV73. [Oral presentation]

F. Mueller, S. Cichon, A. Karpushova, K. Bork, M. Nöthen, U. Walter, T. Renné. Pathophysiologie des Hereditären Angioödems Typ III. IV. Minisymposium „Interaktion von Endothelzellen und Leukozyten im SFB 688“, Würzburg, Germany, October 26, 2006. [Oral presentation]

F. Mueller, S. Cichon, A. Karpushova, K. Bork, M. Nöthen, U. Walter, T. Renné. Hereditary angioedema type III is caused by an activating missense mutation in the gene for coagulation factor XII (Hageman factor). Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, Mannheim, Germany, October 1-4, 2006. [Poster and oral presentation]

PUBLICATIONS

1. **Mueller F**., Mutch N.J., Schenk W.A., Smith S.A., Esterl L., Spronk H.M., Schmidbauer S., Gahl W.A., Morrissey J.H., Renné T. (2009) Platelet polyphosphate are proinflammatory and procoagulant mediators *in vivo. Cell,* in press

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3. **Mueller F**., Renné T. (2008) Novel roles for FXII-driven plasma contact activation system. *Curr Opin Hematol.* 15 (5): 516-21.

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5. Grimmler M., Otter S., Peter C., **Mueller F**., Chari A., Fischer U. Unrip, a factor implicated in cap-independent translation, associates with the cytosolic SMN-complex and influences its intracellular localization (2005). *Hum Mol Genet*. 14 (20): 3099-111.

Publications 1-4 are results of the present PhD-thesis.

AFFIDAVIT

(Eidesstattliche Erklärung)

I hereby declare that my thesis entitled:

„Analysis of the factor XII-driven contact phase system activation *in vivo“*

is the result of my own work.

I did not receive any help or support from commercial consultants.

All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor similar form.

Würzburg, ...