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Caveats in studies of the physiological role of polyphosphates in coagulation.

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Key words

Blood, coagulation, contact activation, platelets, polyphosphate, phosphatase

Abbreviations

ADP, adenosine diphosphate; ATP, adenosine triphosphate; F, coagulation factor; CTI, corn trypsin inhibitor; polyP, polyphosphate; PS, phosphatidylserine.

Abstract

Platelet-derived polyphosphates (polyP), stored in dense granule and released upon platelet activation, have been claimed to enhance thrombin activation of coagulation factor XI (FXI) and to activate FXII directly. The latter claim is controversial and principal results leading to these conclusions are likely influenced by methodological problems.

It is important to consider that low-grade contact activation is initiated by all surfaces and is greatly amplified by the presence of phospholipids simulating the procoagulant membranes of activated platelets. Thus, proper use of inhibitors of the contact pathway and a careful choice of materials for plates and tubes is important to avoid artefacts.

The use of phosphatases used to degrade polyP has an important draw-back as it also degrades the secondary activators ADP and ATP, which are released from activated platelets. In addition, the use of positively charged inhibitors such as polymyxin B to inhibit polyP in platelet-rich plasma and blood is problematic, as polymyxin B also slows coagulation in the absence of polyP.

In conclusion we hope awareness of the above caveats may improve research on the physiological roles of polyphosphates in coagulation.

Introduction

Coagulation is initiated through two pathways, the contact system and the extrinsic system. An essential part of the initiation step in the former is activation of coagulation factor XII (FXII) by surfaces ("contact activation"), in the latter binding of factor VII to tissue factor, exposed to the blood upon vessel wall injury. Clotting of blood ex vivo in the test tube is driven by the contact pathway. *In vivo*, the extrinsic pathway is considered to be the physiological relevant pathway since humans completely lacking FXII have no bleeding problems but low levels of FVII are associated with a bleeding disorder. In recent years physiological substances released from cells *in vivo,* such as denaturated DNA and RNA, histones and polyphosphates (polyP) have been suggested to induce contact activation. Polyphosphates of varying chain-lengths are found in acidocalcisomes in bacteria and also in human cells [1]. For coagulation analysis the blood sample is commonly anticoagulated with 11 mmol/L citrate to complex ionised calcium in order to stop activation of FX and prothrombin

and thus arrest coagulation. Citrate does not block contact activation of factors XI and XII. Upon analysis, the sample is often re-calcified to allow coagulation to take place.

However, there are many caveats in the methods used to study the roles of polyP in haemostasis, which if not properly considered, may lead to erroneous conclusions.

Caveat 1: PolyP of from different sources and of different chain length have different properties

Platelet-derived polyP have a size of 60-100 phosphate monomers in length, are stored in dense granule and released upon platelet activation [2]. Platelet-derived polyP has been claimed to enhance thrombin activation of coagulation factor XI (FXI) and to activate FXII directly [3, 4]. The latter claim is contested since polyPs of short chain lengths are inefficient as contact activators, in contrast to the long chains often found in bacteria [5, 6]. Synthetic polyP may have different chain lengths. Furthermore, we showed in a recent article in Blood that activated platelets do not generate physiologically relevant amounts of FXIIa by the release of polyP [6]. Purified platelet-derived polyphosphates have been shown to enhance activation of FXI, at least *in vitro* [4]. If this mechanism is of any physiological relevance is uncertain. Patients with inherited deficiency of FXI have a bleeding diathesis, and attenuation of production of FXI in the liver has recently been shown to reduce post-operative thrombosis [7].

Caveat 2: Instruments used for clotting time measurements induce contact activation of coagulation

It is well known that negatively charged surfaces induce activation of FXII, but in fact, most material surfaces will induce some degree of FXII activation. The effects of material-induced contact activation on coagulation is much larger in the presence of phospholipid membranes such as those presented by activated platelets, since the contact activation pathway is greatly amplified by phosphatidylserine (PS) present on the membrane of activated platelets. Therefore, also minor contact activation from materials will have a great impact on coagulation in platelet-rich plasma and whole blood when PS containing membranes are present. This can be demonstrated by thrombin generation in plasma with increasing concentration of PS containing phospholipids, simulating the procoagulant membranes of activated platelets [8], as shown in Figure 1. In this experiment it is evident that thrombin generation in the “vehicle” wells (without externally added contact activator) is also greatly amplified by the presence of PS membranes.

Choosing an appropriate detection method is important when designing experiments to measure contact activation-dependent coagulation. For experiments in whole blood, optical clot detection is problematic, and instruments employing mechanical detection methods are commonly employed, which entail various degrees of artificial contact activation. As an example, we have shown that the Amelung KC4 instrument induces much more contact activation than the ReoRox 4 instrument [6, 9]. The clot detection method in the Amelung instrument is based on the detection of deceleration of a moving metal ball in the plastic cuvette as the clot is forming, whereas the ReoRox measures the attenuation of frequency of oscillation for a plastic cup containing the blood sample [10]. The main disadvantage of the Amelung instrument is that the blood contacting surfaces of the plastic cuvette and metal ball by itself induce a rather large degree of contact activation. Another disadvantage is that it is difficult to measure longer clotting times, as the metal ball may destroy the forming fibrin fibers instead of being stopped Thus, the previously reported shortening of clotting times upon platelet activation, which was interpreted as platelet induced contact activation [3], was most likely caused by platelet-dependent amplification of the already existent material-induced contact activation.

The fact that low level FXII generation by material surfaces greatly amplifies coagulation in the presence of phospholipid membranes also has to be taken into account when trying to measure

tissue factor activity on microparticles using thrombin generation, as the commercial reagents contain synthetic phospholipids [8].

Caveat 3: Unspecific effects of polyP antagonists may lead to misleading results

Furthermore, activation of platelets causing release of dense granule will not only cause release of polyP but also the well-known secondary platelet activators ADP and ATP. The pathophysiological role of ADP has been convincingly shown by the benefit of inhibitors of the ADP-receptor P2Y12 on outcome in cardiovascular diseases in large clinical studies the last decades [11-13]. Phosphatases have been used to elucidate the role of polyP in haemostasis. These enzymes cleave phosphate bonds, but such bonds are also present in ATP and ADP. Indeed, initial experiments showed that the phosphatase (same source as in [3]) not only degraded polyP, but also exhibited a strong negative effect on coagulation-independent platelet activation mechanisms (Fig. 2)[6].Platelet aggregation induced by a PAR1-activating peptide (Trap6) was decreased in phosphatase-treated samples (Figs. 2A, C). Furthermore, in contrast to control samples, the aggregation with added phosphatase was found to be reversible, which is well known to occur when using ADP receptor blockade. This is also shown in our experiments (Figs. 2B, C). We therefore investigated this further and found that incubation of ADP with phosphatase resulted in rapid degradation of ADP (Fig. 2D), and that within four minutes, the ADP concentration fell below the threshold required to provoke an aggregation response in platelets. These results were expected as the two molecules, polyP and ADP, share the phosphate-phosphate bond structure, which is the target for phosphatase. Thus, phosphatase can degrade both polyP and ADP and thereby not only affect the procoagulant effect of platelet polyP, but also the important platelet ADP signaling and activation pathway. The degradation of ADP may cause a critical attenuation of the final procoagulant state of the platelets, which if not taken into account may lead to incorrect conclusions regarding the origin of the platelet procoagulant contribution. Experiments employing phosphatases to elucidate polyP-dependent effects of platelets on coagulation will thus need to include control experiments where the ADP-receptors P2Y1 and P2Y12 and the ATP-receptor are blocked in order to account for these unspecific effects of phosphatases.

In addition, the use of positively charged inhibitors of polyphosphates, such as polymyxin B, in platelet-rich plasma and blood is problematic. We show that polymyxin B does attenuate coagulation also when platelets are substituted with PS containing phospholipid vesicles in platelet-free plasma, i.e. in absence of any source of polyP, see Figure 3. From this it is impossible to tell whether polymyxin B interacts with negatively charged residues on the material surfaces in the instrument and/or blocks the phospholipid membranes, but it is obvious the effect is present in absence of polyP.

Caveat 4: Platelet-derived Ca^{2+}-saturated polyP is poorly soluble

We have previously shown that calcium-saturated compositions of purified platelet-derived polyphosphates are prone to form precipitates [6]. The use of calcium-saturated or non-saturated polyP have varied between different studies and may be one of the reasons for the different results obtained [3, 5]. The question regarding calcium precipitation of polyP becomes interesting since native blood and platelet dense granule contains calcium, and using standard procedures for coagulation experiments with citrate anticoagulation (Ca^{2+} -chelating agent) and re-calcification (adding more calcium) to start the coagulation experiment, will increase the calcium concentration even further. We therefore believe it is important in such polyP experiments to investigate if polyP-calcium precipitate forms and if so, what size distribution the precipitate has, to better enable discussions regarding the physiological relevance of such experiments. For example, using platelet polyP-precipitate particles in the micrometre scale, as we have shown before can occur [6]could be questionable since platelets themselves have a diameter of only a few micrometres. It is plausible that the polyP-calcium precipitate interact differently with the coagulation cascade than soluble

polyP, since the former more resembles a material surface, and could therefore be more prone to induce the contact activation pathway of coagulation.

Conclusions

All kind of surfaces may induce contact activation of the coagulation system, not only the better known negatively charged surfaces such as glass and kaolin, but also plastics and metals to various degrees. The PS containing membrane of activated platelets enhance the coagulation steps after FXIIa has been formed and can thereby accelerate coagulation initiated by contact activation on materials in the experimental system. Phospholipid vesicles may substitute for platelets in this regard, and can be used in control experiments to verify a possible platelet-specific mechanism. However, platelets or substances released from platelets have previously been shown incapable to initiate coagulation by the contact activation pathway (FXII) [6, 9]. Positively charged substances like polymyxin B inhibit effects of polyP on coagulation, but are not specific to polyP and are demonstrated to inhibit coagulation also in the absence of polyP. Phosphatase inhibits the action of polyP by degradation, but also efficiently degrade the important platelet activators ADP and ATP. Phosphatase inhibition of platelet polyP should therefore be used with caution. PolyP of different chain lengths and from different sources have different properties. We hope awareness of these caveats may improve research on the physiological roles of polyphosphates in coagulation.

Figure legends

Figure 1. Minor activation of FXII boosts thrombin generation in the presence of phospholipid membranes. Thrombin generation measurements were performed in platelet-free plasma using different concentrations of kaolin in the presence and absence of phospholipids. Kaolin was used at 0, 1 and 100 μg/mL and phospholipids at 0, 0.5 and 2 μM. The thrombin generation curves are average values from five donors. This figure was part of Fig. 1 in Boknäs et al. J Thromb Haemost 2014;12:515-518.

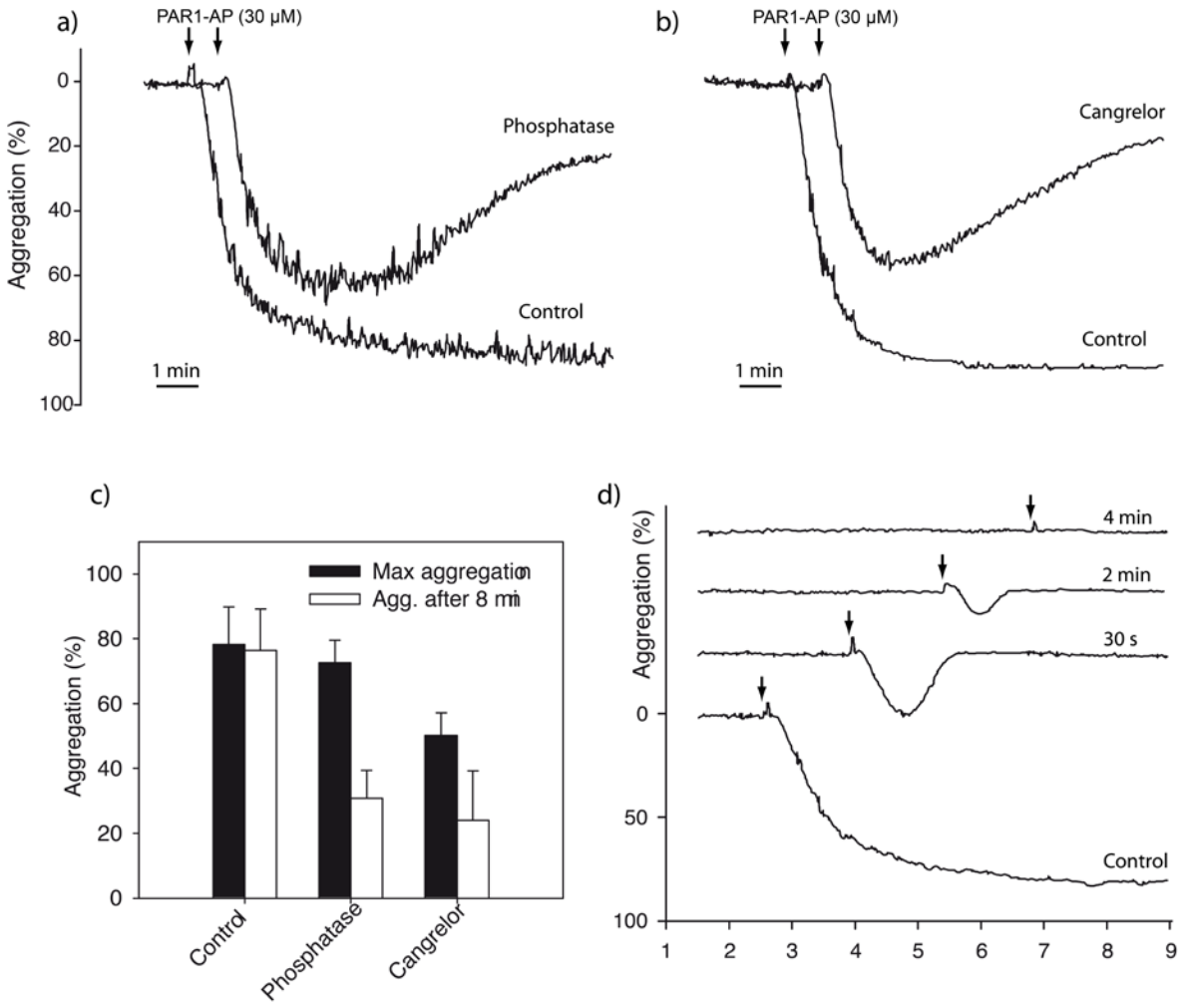

Figure 2. Phosphatase degrades ADP and thereby attenuates platelet activation. Platelet aggregation in anticoagulated platelet-rich plasma samples with the addition of A) phosphatase (10 U/mL) and B) ADP P2Y12 receptor inhibitor (cangrelor, 1 µM). Platelet activation was induced by addition of 30 µM Trap6. C) Platelet aggregation in platelet-rich plasma with added phosphatase (10 U/mL) or cangrelor (0.1 µM), compared with control samples (n=4). Platelets were activated with 30 µM Trap6. Values are presented as mean+SD. D) ADP (10 µM final conc.) was mixed with phosphatase (10 U/mL final conc.) and incubated 30 s, 2 min and 4 min before addition to platelet-rich plasma in the aggregometer. Control trace shows platelet aggregation induced by 10 µM ADP in the absence of phosphatase.

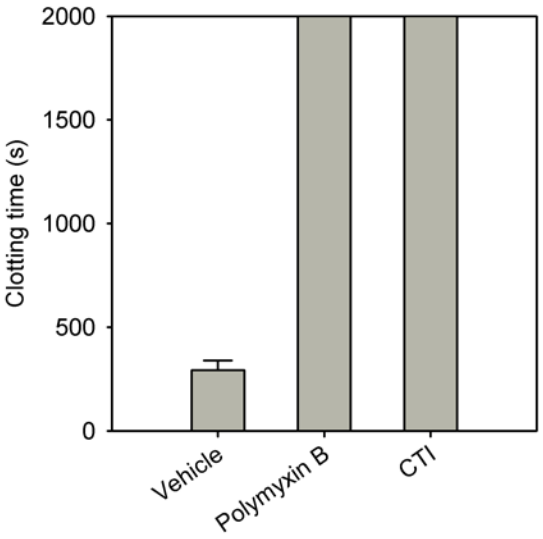

Figure 3. Corn trypsin inhibitor and polymyxin B prolongs clotting times in platelet-free plasma substituted with phospholipids. Clotting times measured using Amelung KC4 in platelet-free plasma with 2.5 µM phospholipids (PS/PC/PE, 20:60:20) as a substitute for activated platelet membranes were measured with or without FXIIa inhibitor (CTI, 50 µg/mL) or polymyxin B (10 µg/ml), n=3. Data presented as mean+SD. Extraction of data from Figure 3 in Faxälv et al. Blood 2013;122:3818-3824 with addition of polymyxin B data.

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Disclosures

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