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THROMBIN RECEPTOR ACTIVATING PEPTIDE DOES NOT STIMULATE PLATELET PROCOAGULANT ACTIVITY

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SUMMARY: Platelets after challenge with α-thrombin alone, collagen alone or thrombin/collagen mixture were observed to increase the rate of activation of prothrombin by factor Xa in the presence of factor Va and calcium ion (platelet procoagulant activity) by a maximum of 25, 45 and 110 fold, respectively. The increase in platelet procoagulant activity due to these agonists has been described previously and arises from increased expression of phosphatidylserine on the platelet surface. When platelets were treated with the thrombin receptor activating peptide (TRAP) (SFLLRNPNDKYEPK), alone or in the presence of collagen or thrombin, no change in platelet procoagulant activity was observed at concentrations of TRAP sufficient to cause increased intracellular calcium levels and protein phosphorylation in a manner similar to that of thrombin. In addition, no increase in platelet procoagulant activity was seen upon treatment with TRAP in the presence of inactivated thrombin (PPACK-thrombin). These results suggest that the thrombin-mediated increase in procoagulant activity may be due to activation of a thrombin receptor distinct from the recently cloned G-protein-coupled receptor, or to other proteolytic events on the platelet surface.

Membrane lipids play an important role in the function of blood platelets, especially in their haemostatic activities. In human platelets, phospholipids are heterogeneously distributed between the two halves of the membrane bilayer. Anionic phospholipids which enhance thrombin formation from prothrombin (by factor Xa, factor Va and Ca^{2+}) are enriched on the inner leaflet of resting platelets [1-4]. Following platelet activation by physiological agonists such as thrombin and/or collagen, or by non-physiological stimuli such as Ca^{2+} ionophore, anionic phospholipids are exposed on the outer membrane surface where they serve as binding sites for prothrombin, factors Xa and Va [5-7]. Thrombin activation of platelets therefore acts as a positive feedback enhancing further formation of the molecule.

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Recently, a thrombin receptor with a unique mechanism of action has been cloned from a megakaryocyte cell line [8]. Thrombin cleaves the receptor within the large N-terminal extracellular domain creating a new amino-terminus which, by insertion into an as yet unidentified location, activates the receptor leading to cellular activation. A thrombin receptor activating peptide (TRAP) corresponding to the initial 14 residues of the newly created N-terminus can mimic this insertion and has been shown to activate the thrombin receptor in platelets [8,9], endothelial cells [10,11], smooth muscle cells [12] and fibroblasts [13]. Evidence derived from studies in human platelets indicates that the actions of α-thrombin in these cells can be entirely attributed to activation of the cloned receptor [8,9,10,14,15]. In the present study, we have examined the effects of TRAP on exposure of anionic phospholipid on the outer platelet membrane. Our results show that although both TRAP and thrombin activate platelets to a similar degree, TRAP does not promote platelet procoagulant activity.

Materials and Methods

Human α thrombin and γ thrombin kindly provided by Dr Freysinnet (Strasbourg) and Dr J. Fenton (Albany), respectively. PPACK thrombin was prepared from human α thrombin as described [15] previously. Human prothrombin and factor Xa was obtained from Enzyme Research (Swansea) and bovine factor V from Dr T. Lindhout, University of Limburg. It was activated to factor Va as described previously [16]. The calcium ionophores A23187 and ionomycin were obtained from Sigma Chemical Co, and from Calbiochem, UK, respectively. Stock solutions of ionophores prepared in either acetonitrile (A23187) or DMSO (ionomycin) were stored at -20°C until use. Soluble collagen was obtained as a solution in 0.1M acetic acid which was diluted in physiological buffer just before use. TRAP peptide (SFLLRNPNDKYEPF) corresponding to the sequence of 42-55 of the human platelet thrombin receptor, was synthesized on a Milligen 9050 automated peptide synthesiser using standard solid phase Fmoc t-BOC chemistry.

Preparation of washed human platelets

Human blood was obtained by forearm venepuncture of healthy volunteers who had denied taking medication for at least 10 days. Blood was drawn into acid:citrate:dextrose and platelet-rich plasma (PRP) was obtained by centrifugation at 200g for 15 min as previously described [17,18]. The PRP was centrifuged at 1500g for 10 min and the platelet pellet resuspended in a citrate washing buffer containing prostacyclin as described previously [18]. Washed platelets were finally resuspended at the appropriate cell count in a Hepes-Tyrode buffer (pH 7.4) composed of 10 mM-Hepes, 145 mM-NaCl, 5 mM-KCl, 1mM-$MgSO_4$, 1 mM $CaCl_2$, 5 mM-D-glucose and 10 μM indomethacin.

Measurement of platelet procoagulant activity

The ability of the platelet surface to enhance the activation of prothrombin by factor Xa in the presence of factor Va and calcium (platelet procoagulant activity) was measured as described previously [19].

Protein phosphorylation studies

For the measurement of protein phosphorylation platelets (1 x 10^9/ml) in washing buffer were incubated with 0.25 mCi of carrier-free [^{32}P]P_i/ml for 90 min at 37°C in the presence of

20 nM prostacyclin. Following incubation, platelets were washed twice by centrifugation (10 min; 1500g) and were subsequently resuspended in Hepes-Tyrode buffer to a final count of 4 x 10^8/ml. Incubations were carried out using 100 μl aliquots of cell suspension and were terminated with 50 μl of Laemmli sample buffer (3x). Phosphoproteins were separated on gradient polyacrylamide gels (7-20%) and ^{32}P incorporation was assessed by autoradiography (-70°C; Fuji RX X-ray film).

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} concentration was assessed in fura-2/AM-loaded platelets as previously described [18]. Free Ca^{2+} was calculated by the method of Grynkiewicz *et al.* [20].

Results

After preincubation with activator for 5 minutes at 37°C, an enhancement of the rate of activation of prothrombin was observed (Fig.1) and calculated as fold acceleration.

We initially examined the ability of several agonists, previously shown to be potent activators of procoagulant activity [5-7], to enhance the rate of activation of prothrombin by factor Xa/factor Va. Fig.1 includes the results obtained following treatment of platelets with α and γ thrombin, collagen, collagen plus thrombin mixture, or calcium ionophore. In agreement with previous findings [5,6], the greatest acceleration was observed following challenge with calcium ionophore, ionomycin or A23187 and α-thrombin/collagen. Of the remaining agonists the rank order of potency (according to maximal acceleration observed) was collagen/α-thrombin > α-thrombin > collagen, >γ-thrombin. In marked contrast, addition of TRAP caused no apparent increase in the rate of prothrombin activation when studied at concentrations ranging between 1 and 100 μM (Fig.1A).

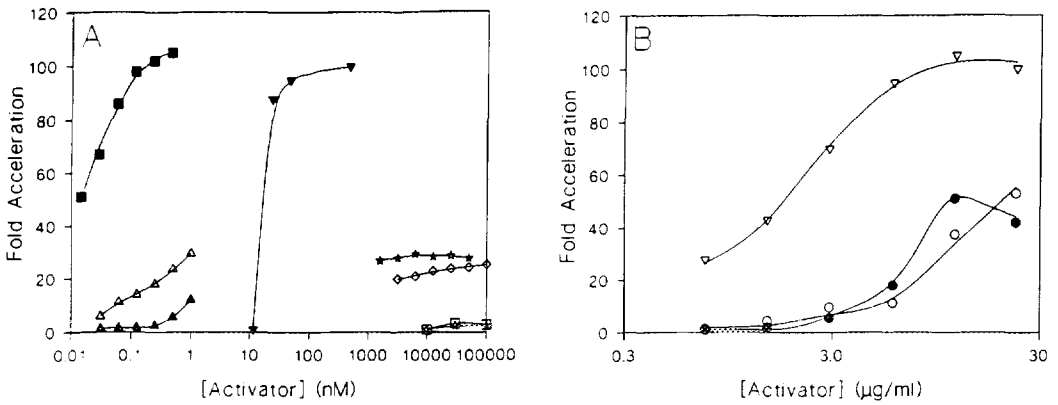
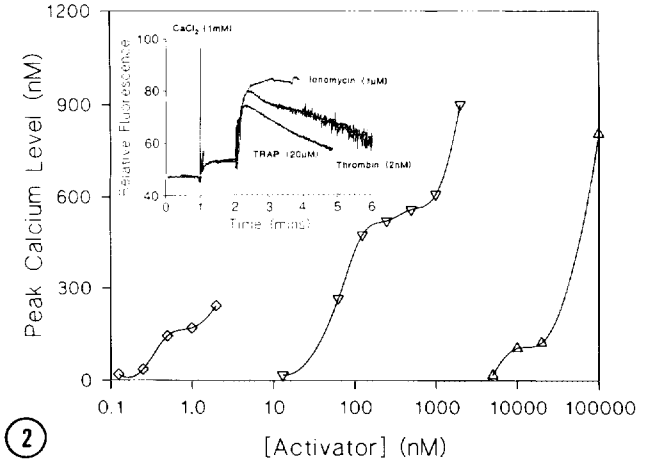


Figure 1. Increase in platelet procoagulant activity measured as fold increase in the rate of activation of prothrombin by factor Xa/factor Va upon treatment of cells with different concentrations of various agonists A) △-△, α-thrombin; ▲-▲, γ-thrombin; □-□, TRAP; ☆-☆ , TRAP in the presence of amstatin (1 μM); ■-■, α-thrombin/collagen (5 μg/ml); ▼-▼, ionomycin; ★-★, TRAP in the presence of α-thrombin (1 nM); ◇-◇, TRAP in the presence of collagen (5 μg/ml); B) ●-●, collagen; ▽-▽,collagen in the presence of thrombin (1nM); ○-○, collagen in the presence of TRAP (50 μM).

Recent studies have shown that TRAP can be cleaved and therefore inactivated by aminopeptidase M [21]. In order to determine whether the lack of effect of TRAP was due to peptide cleavage during the pre-incubation period, experiments were performed in the presence of the aminopeptidase inhibitor amastatin. Incubation with 1 μM amastatin did not increase procoagulant activity following challenge with TRAP (Fig.1A). Similarly, various concentrations of TRAP had no potentiatory effect on procoagulant activity measured in the presence of either collagen or thrombin (Fig.1A,B). A proteolytically inactive form of thrombin, PPACK-thrombin (27 nM), did not promote procoagulant activity either alone or in the presence of TRAP (1 to 50μM) (data not shown).

Although TRAP had no influence upon platelet procoagulant activity, the peptide potently induced platelet activation. Fig.2 illustrates the effects of thrombin, TRAP, and ionomycin on Ca^{2+} mobilisation in fura-2/AM-loaded platelets. These experiments were performed on platelets prepared on the same day and from the same donors as those depicted in Fig.1. A dose-dependent increase in the peak $[Ca^{2+}]_i$ attained in the presence of each agonist was observed. TRAP at a concentration of 20 μM elevated $[Ca^{2+}]_i$ to a comparable level, and with the same time-course, as that achieved with 2 nM thrombin (inset to Fig.2). Platelet



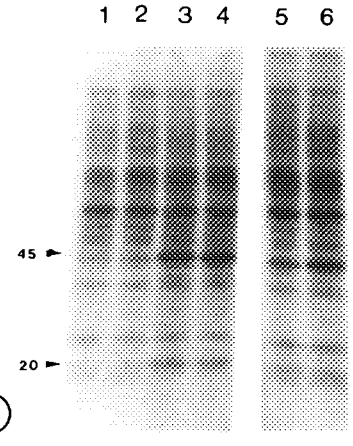


Figure 2. Platelets were preloaded with fura-2/AM and changes in intracellular Ca^{2+} levels were monitored as described in Materials and Methods. Data show the peak $[Ca^{2+}]_i$ attained following challenge with various concentrations of either α-thrombin, (□-□); TRAP, (△-△); ionomycin, (▽-▽) for 2 min. Experiments were performed in the presence of 1 mM $CaCl_2$. Data are derived from 4 experiments performed on platelets from different donors. The resting $[Ca^{2+}]_i$ level was 75 ± 11 nM (mean ± SEM from 4 experiments). The inset shows representative fluorescence traces from a single experiment in response to thrombin, TRAP, or ionomycin at the indicated concentrations.

Figure 3. Phosphorylation of proteins in platelets by thrombin and TRAP. Lanes 1 + 2 resting, 3 + 4 thrombin-treated (0.91nM) and 5 + 6 TRAP-treated (10μM). All incubations were for 3 min. 45 and 20 reflect relative migration points for 45 and 20 kDa proteins.

activation by thrombin is associated with the phosphorylation of a number of endogenous proteins including pleckstrin (45 kDa) and myosin light chain (20 kDa). In [^{32}P]-labelled platelets TRAP enhanced [^{32}P] incorporation into the 45 and 20 kDa proteins to the same extent as that induced by a maximal dose of thrombin (Fig.3).

Discussion

Thrombin cleaves a seven-transmembrane domain receptor and binding of the resulting N-terminal tethered ligand promotes receptor-mediated events within the cell [8]. This hypothesis is supported by the observation that a synthetic peptide that duplicates a portion of the tethered ligand has effects which parallel those induced by α-thrombin in a number of cell types [8-14]. Since insertion of the tethered ligand will be kinetically favoured with respect to free peptide [22] it has been noted that approximately 1000 fold greater concentrations of TRAP are required to produce an equivalent thrombin-mediated response [8-14]. Thus, in the present study, TRAP elevated $[Ca^{2+}]_i$ and enhanced phosphorylation of the 20 and 45 kDa proteins to a maximal level similar to that of thrombin but at much greater concentrations. These results confirm previous findings [8,9,23] and suggest that the effects of thrombin, at least on Ca^{2+} mobilisation and protein phosphorylation, relate to the generation of tethered ligand. In marked contrast, treatment of platelets with TRAP did not cause expression of procoagulant activity on the cell surface. A recent study has shown that TRAP-induced platelet aggregation is enhanced in the presence of inhibitors of aminopeptidase M [24], suggesting that TRAP can be rapidly degraded *in vitro*. However, the lack of effect of TRAP on procoagulant activity in the present study could not be accounted for by peptide degradation since procoagulant activity was not observed in response to TRAP in the presence of the aminopeptidase inhibitor, amastatin. A substantial amount of evidence indicates that the proteolytic activity of thrombin is essential for its effectiveness as a platelet agonist [23,25]. We attempted to investigate whether binding of thrombin to membrane component(s) other than the cloned thrombin receptor was necessary for the generation of procoagulant activity in these cells. However, addition of an active site-inhibited thrombin, PPACK thrombin, which retains its ability to bind to the thrombin receptor, did not cause PS exposure either alone or in the presence of TRAP. In addition, high concentrations of TRAP had no effect upon the activation induced by thrombin itself.

The significance of these findings should be considered in relation to the mechanism by which prothrombinase activity is increased, namely by expression of phosphatidylserine (PS) on the outer plasma membrane surface. The relative enrichment of PS to the inner leaflet is known to be due to a specific aminophospholipid translocase which actively transports PS to the inner membrane [26]. Inhibition of this enzyme by thiol reagents [27] and/or by elevated

concentrations of intracellular calcium [28] leads to rapid phospholipid rearrangement and subsequent PS exposure. In certain situations this rearrangement may be accompanied by ruffling of the membrane surface with release of microvesicles [29]. The results from the present study indicate that Ca^{2+} mobilisation by TRAP peptide alone is not a sufficient stimulus for the expression of procoagulant activity since TRAP potently elevated intracellular Ca^{2+} but clearly had no effect upon PS exposure.

Taken together, our results indicate that the proteolytic activity of thrombin is required for the generation of prothrombinase activity but that the cloned thrombin receptor is unlikely to be the site of proteolysis. Our data therefore suggest that thrombin may additionally bind to other site(s) distinct from the receptor identified by Vu *et al.* [8] and/or that it may cleave other sites on the platelet surface in order to cause PS exposure. In this respect, evidence is accumulating to suggest that activation of the cloned thrombin receptor may not be responsible for generating all of the cellular effects of thrombin, pointing to the existence of further thrombin receptors in some cell types. For example, in human endothelium TRAP, like thrombin, elevates intracellular $[Ca^{2+}]$ and promotes prostacyclin release [30] but does not induce either expression of intracellular adhesion molecule 1 or enhanced endothelial permeability [31,32]. Similarly, the p44 mitogen-activated protein kinase is differentially regulated by TRAP versus thrombin in lung fibroblasts [33]. Previous studies have shown that α-thrombin binds to high, moderate and low-affinity sites on the human platelet membrane [34] and it has been proposed that the high affinity site is contained within the glycocalicin portion of glycoprotein Ib [35]. It remains to be established whether cleavage of glycoprotein Ib is a determinant of platelet procoagulant activity.

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