Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators

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Synthetic thrombin receptor peptides (TRPs), comprising the first 6–14 amino acids of the new N-terminus tethered ligand of the thrombin receptor that is generated by thrombin's proteolytic activity, were reported to activate platelets equally with thrombin itself and are considered to be full agonists [Vu et al. (1991) *Cell* **64**, 1057–1068]. Using aspirin plus ADP-scavengers or the ADP-receptor antagonist adenosine 5'-[α -thio]triphosphate to prevent the secondary effects of the potent agonists that are normally released from stimulated platelets (i.e. ADP and thromboxane A_2), we assessed the direct actions of thrombin and TRPs (i.e. TRP^{42–47} and TRP^{42–55}). Compared with thrombin, under these conditions, TRPs: (1) failed to aggregate platelets completely; (2) produced less activation of glycoprotein (GP)IIb–IIIa; (3) did not cause association of GPIIb and pp60^{c-src} with the cytoskeleton; and (4) caused less α -granule secretion, phosphorylation of cytoplasmic phospholipase A_2 , arachidonic acid release and phosphatidyl inositol (PtdOH) production. Furthermore, TRPs induced transient increases in protein phosphorylation mediated by protein kinase C and protein tyrosine phosphorylation, whereas these same responses to thrombin

were greater and more sustained. Hirudin added after thrombin accelerated protein dephosphorylation, thereby mimicking the rate of spontaneous dephosphorylation seen after stimulation by TRPs. Platelets totally desensitized to very high concentrations of TRPs, by prior exposure to maximally effective concentrations of the peptides, remained responsive to α - and γ -thrombins. Thrombin-stimulated PtdOH production in permeabilized platelets desensitized to TRPs was abolished by guanosine 5'-[β -thio]diphosphate (GDP[β S]), as in normal platelets. These results are discussed in terms of the allosteric Ternary Complex Model for G-protein linked receptors [Samama et al. (1993) *J. Biol. Chem.* **268**, 4625–4636]. We conclude that: (1) TRPs are partial agonists for the thrombin receptor and produce incomplete receptor desensitization in keeping with their lower intrinsic activity; (2) thrombin's effects in platelets, even in TRP-desensitized platelets, are entirely mediated through the recently cloned G-protein linked receptor, and (3) thrombin's ability to produce sustained signals, compared with TRPs, may require the continued progressive proteolytic activation of naive thrombin receptors.

INTRODUCTION

The cloning of the thrombin receptor (Vu et al., 1991a), a seven transmembrane domain protein of the G-protein linked family, has brought about an understanding of thrombin's molecular mechanism of action. The serine protease attacks the Arg⁴¹–Ser⁴² peptide bond of the extracellular N-terminal region of the receptor protein, thereby generating a new N-terminus. This new N-terminus appears to behave as a tethered ligand (TL) which self-activates the receptor (TLR). This conclusion was deduced from the fact that thrombin receptor peptides (hereafter referred to as TRPs), such as SFLLRN (TRP^{42–47}) and SFLLRNPNDKYEPF (TRP^{42–55}), comprising the first 6 and 14 amino acids, respectively, of this new N-terminus, mimic thrombin in inducing maximal aggregation of human platelets and the secretion of ATP (Vu et al., 1991a). Similar thrombin receptors were cloned from CCL39 hamster lung fibroblasts (Rasmussen et al., 1991) and rat smooth muscle cells (Zhong et al., 1992). Both contain a thrombin cleavage site followed by a homologous TL sequence in the extracellular domain. Thrombin mutants that

bind to the receptor, but lack protease activity, are antagonists of thrombin (Hung et al., 1992a), and receptor mutants that are uncleavable by thrombin are insensitive to thrombin (Vu et al., 1991a), whereas when an enterokinase cleavage sequence (DDDD) is substituted at the normal thrombin cleavage site the receptor responds to enterokinase rather than thrombin (Vu et al., 1991b). Thus, the specificity for thrombin's actions lies in its protease activity and in the nature of the susceptible peptide bond that is hydrolysed to generate the TL.

Several studies have compared the effects of thrombin and various TRPs on responses in platelets and other cells: e.g. the TRPs, like thrombin, have mitogenic activity (Reilly et al., 1993; McNamara et al., 1993; Joseph and MacDermot, 1993) and stimulate wound healing (Warren et al., 1992; Carney et al., 1992), and they have similar actions on vascular endothelium (Ngaiza and Jaffe, 1991; Sugama and Malik, 1992; Tiruppathi et al., 1992), vascular smooth muscle (Muramatsu et al., 1992) and human monocytes (Joseph and MacDermot, 1993). In platelets, TRP^{42–55}, like thrombin, induces aggregation, ATP secretion (Vu et al., 1991a), tyrosine phosphorylation, inositol phosphates

Abbreviations used: TRPs, thrombin receptor peptides; TL, tethered ligand; TLR, tethered ligand receptor; PKC, protein kinase C; cPLA₂, cytoplasmic phospholipase A_2 ; CP, creatine phosphate; CK, creatine kinase; PtdOH, phosphatidic acid; TXA₂, thromboxane A_2 ; AA, arachidonic acid; PLC, phospholipase C; CSK, platelet cytoskeleton; ECL, enhanced chemiluminescence; ICAM, intercellular adhesion molecule.

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production (Vassallo et al., 1992), formation of phosphatidyl inositol (PtdOH) and 3-phosphorylated phosphoinositides (Huang et al., 1991) and inhibition of adenylyl cyclase (Seiler et al., 1992; Vassallo et al., 1992). In this paper, we additionally show that TRPs stimulate phosphorylation of cytosolic phospholipase A₂ (cPLA₂), the release of arachidonic acid (AA) and α -granule proteins, and the translocation of glycoprotein (GP)IIb-IIIa (integrin $\alpha_{IIb}\beta_3$) and pp60^{c-src} to the cytoskeleton. It was previously concluded that TRPs were full agonists for the thrombin receptor in platelets (Vu et al., 1991a), meaning that maximal activation of the receptor by TRPs produced responses equal to those caused by thrombin and that this agonist activity was contained entirely within the potential sites of interaction present in the N-terminal six amino-acid sequence of SFLLRN (Chao et al., 1992; Scarborough et al., 1992; Coller et al., 1992; Vassallo et al., 1992; Sabo et al., 1992).

Before the cloning of the TLR, it was postulated by some workers that platelets contained two thrombin receptors with K_D values of about 0.3 nM and 11 nM, respectively, and these two sites were proposed to initiate different signal transduction effects (Greco and Jamieson, 1991). From several indirect lines of evidence the high-affinity receptor was postulated to be the membrane glycoprotein GPIb, which selectively mediated such responses as AA release, inhibition of adenylyl cyclase and Na⁺/H⁺ exchange, whereas the moderate-affinity receptor mediated activation of phospholipase C (PLC) (Greco and Jamieson, 1991). GPIIb contains a highly negatively charged domain that probably interacts with the anion-binding exosite of thrombin (DeMarco et al., 1994), similar to the receptor-thrombin and thrombin-hirudin interactions. However, with the discovery of the agonist TRPs, it became apparent that virtually all the known effects of thrombin on platelets could be reproduced by the peptide analogues of the N-terminal region of the TL. Furthermore, Huang et al. (1991) found that the formation of PtdOH and 3-phosphorylated phosphoinositides by TRP⁴²⁻⁵⁵ and thrombin was non-additive, and concluded that these effects are mediated solely through the TLR. Antibodies developed against two different regions of the TL inhibited platelet activation by thrombin (Hunt et al., 1992b; Brass et al., 1992). Yet, in both cases, the inhibition was completely overcome by increasing concentrations of thrombin. Although this was attributed to a competitive antagonism between thrombin and the antibodies, the possible existence of a second receptor for high concentrations of thrombin could not be excluded. Other studies have proposed the existence of more than one receptor in platelets, and other cells, based on observed differences in the responses to TRPs compared with thrombin itself (Seiler et al., 1991; Sugama and Malik, 1992; Muramatsu et al., 1992; Kinlough-Rathbone et al., 1993).

Interpretation of the previous studies on platelets is further complicated by the fact that ADP and thromboxane A₂ (TXA₂) are normally released or generated by activated platelets, and these potent platelet agonists act on their own specific receptors. Under these conditions a true assessment of the direct actions of thrombin and the TRPs is compromised. Therefore, in this study we evaluated the direct effects of the TRPs and thrombin on platelet aggregation, secretion of α -granules and several biochemical responses by abolishing these secondary mediators with aspirin plus ADP scavengers [apyrase or creatine phosphate/creatine kinase (CP/CK) or the ADP-receptor antagonist adenosine-5'-[α -thio]triphosphate (ATP[α S])] (Greco et al., 1991)]. These studies revealed heretofore unknown significant differences between thrombin and the TRPs with respect to agonist efficacy and the homologous desensitization of thrombin receptors. Despite these differences, our data support the conclusion that

activation of human platelets by thrombin is probably fully accounted for by the cloned G-protein-linked TLR. However, our data also suggest that the agonist (A) TRPs are less effective than thrombin: (1) in driving the normal equilibrium between the unactivated state (R) and the active state (R*) of the receptors towards the activated state; and (2) in promoting the formation of the ternary complexes with G-protein, i.e. AR*G.

MATERIALS AND METHODS

Materials

Receptor peptides SFLLRNPNDKYEPF (TRP⁴²⁻⁵⁵) and SFLLRN (TRP⁴²⁻⁴⁷) were synthesized by Dr. R. J. Freer (Department of Pharmacology, Virginia Commonwealth University, VA, U.S.A.) and were also obtained from Bachem Bioscience Inc. Human α - and γ -thrombins were gifts from Dr. J. W. Fenton II (New York State Department of Health, Albany, NY, U.S.A.). [³²P]Phosphate (5 mCi/ml) and ¹²⁵I-protein A (142 mCi/ml) were from Du Pont-New England Nuclear. α -Thrombin (T3010, 4000 units/mg), apyrase [two preparations: (a) ATPase 491 units/mg, ADPase 57 units/mg; (b) ATPase 260 units/mg, ADPase 150 units/mg], CP, CPK (150–300 units/mg), aspirin, amastatin, peptides RGDS and GPRP were obtained from Sigma Chemical Co. ATP[α S] was a product of Calbiochem. Monoclonal antibody Mo327 was kindly supplied by Dr. J. Brugge (ARIAD, Cambridge, MA, U.S.A.) and anti-GPIIb (SZ22) was purchased from Amac (Westbrook, ME, U.S.A.). Anti-phospholipase A₂ was kindly provided by the Genetics Institute (Cambridge, MA, U.S.A.), anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Inc., horseradish peroxidase-conjugated goat anti-mouse IgG from Bio-Rad, and rabbit anti-(mouse IgG) from Sigma. Poly(vinylidene difluoride) (PVDF) membranes were obtained from Millipore, and enhanced chemiluminescence (ECL) reagent was from Amersham or Kirkegaard and Perry Labs.

Platelet preparation and measurement of aggregation and secretion of α -granule proteins

Washed platelets were prepared from human platelet-rich plasma purchased from the American Red Cross Blood Center, Farmington, CT, U.S.A. after incubation with 0.2 mM aspirin for 20 min to inactivate prostaglandin endoperoxide synthetase, as we described previously (Halenda et al., 1989). The platelets were washed and resuspended at the indicated concentrations in modified Tyrode's solution (145 mM NaCl/5 mM KCl/5.5 mM glucose/0.04 mM CaCl₂/1 mM MgCl₂/10 mM Hepes). Platelet aggregation was measured in a Chronolog lumi-aggregometer using 0.5 ml aliquots of platelets at 3×10^8 /ml at 37 °C. Secretion of α -granule proteins from platelets (1×10^9 /ml) was initiated by stimulation with α -thrombin or the TRPs. At various times the platelets were rapidly sedimented in a microcentrifuge (15 s, 12000 g) and aliquots of the supernatant were subjected to SDS/PAGE and staining with Coomassie Blue. The most prominent secreted protein was thrombospondin ($M_r = 180$) which was quantified by a Molecular Dynamics laser densitometer.

Measurement of intracellular free calcium [Ca²⁺]_i and expression of activated GPIIb-IIIa on platelet surface

[Ca²⁺]_i was measured spectrofluorometrically in a PTI Deltascan using fura-2 as the Ca²⁺ detector as previously described (Zavoico et al., 1986). Expression of activated GPIIb-IIIa (the fibrinogen

receptor) on the platelet surface was measured as previously described (Pumiglia et al., 1992) by fluorescence flow cytometry using PAC-1 antibody that was supplied by Dr. Sanford Shattil (University of Pennsylvania Medical School).

Immunoblotting of pp60^{c-src} and GPIIb in cytoskeleton fraction of platelets

Platelet cytoskeletons (CSK) were isolated as Triton X-100 insoluble proteins as described in detail by Pumiglia and Feinstein (1993). After SDS/PAGE of CSK proteins immunoblotting was performed using monoclonal antibodies Mo327 against pp60^{c-src} and SZ22 against GPIIb, with detection by an ECL kit (Pumiglia and Feinstein, 1993).

Protein phosphorylation and tyrosine phosphorylation

Protein phosphorylation was measured in washed platelets (1×10^9 /ml) preincubated with [³²P]phosphate (0.7 mCi/ml; 800 Ci/mmol) to label the metabolic ATP pool to equilibrium, as previously described (Halenda et al., 1989). After exposure to thrombin or TRPs, the platelets were added to an equal volume of $2 \times$ concentrated electrophoresis sample buffer [3% (w/v) SDS/5% (v/v) β -mercaptoethanol/10% (v/v) glycerol/60 mM Tris, pH 6.8] plus 0.2 mM sodium orthovanadate/20 μ M Na₃MoO₄/10 mM EDTA, in a boiling water-bath. After SDS/PAGE, the gels were stained, dried and then exposed to phosphorimager screens. Our analyses centred on p47, known as pleckstrin, the major substrate for protein kinase C (PKC) in platelets. For the determination of tyrosine phosphorylation, the platelet proteins were electrotransferred to PVDF membranes after SDS/PAGE and immunoblotted with the monoclonal anti-phosphotyrosine antibody (4G10), using rabbit anti-mouse IgG (1:5000) as the second antibody and ¹²⁵I-protein A for detection (Pumiglia et al., 1992). Alternatively, detection was by an ECL method using a second antibody of peroxidase-labelled rabbit anti-mouse IgG as described by the manufacturer (Amersham). Emphasis was placed on the analysis of p128, p92 and p79 as major bands whose tyrosine phosphorylation was substantially stimulation-dependent. Phosphorylation of cPLA₂ was measured by the migration shift of the phosphorylated form of the enzyme when subjected to SDS/PAGE (Lin et al., 1992; Kramer et al., 1993) using a monoclonal antibody to cPLA₂ and detection by ECL. In all cases, radioactive protein bands were quantified by a Molecular Dynamics phosphorimager and the chemiluminescent bands by laser densitometry of X-ray films with a Molecular Dynamics personal densitometer using chemiluminescent standards of an appropriate range of peroxidase-labelled IgG.

Phosphatidic acid production

Washed platelets (0.5 ml aliquots, 1×10^9 /ml) were incubated with 0.8 mCi/ml [³²P]orthophosphate, to label the metabolic ATP pool, and stimulated with thrombin or TRPs. Reactions were stopped with 1.88 ml of ice-cold CHCl₃/CH₃OH/HCl (10:20:1). The lipids were extracted as described previously (Pumiglia et al., 1992) and chromatographed on Whatman K5 silica gel 150 Å t.l.c. plates with CHCl₃/CH₃OH/20% NH₂CH₃ (180:108:30). Radioactivity of the PtdOH bands, identified by co-migration with authentic standards stained with iodine vapour, was quantified by the phosphorimager.

RESULTS

TRPs, unlike thrombin, do not cause formation of large platelet aggregates in the absence of released extracellular ADP or when ADP receptors are blocked

Aggregation of washed platelets requires the secretion of platelet fibrinogen, activation of GPIIb-IIIa (the fibrinogen receptor) and divalent cations in the medium. In aspirinized platelets, α -thrombin and both TRPs induced shape change (decreased light transmission) followed by maximal aggregation, characterized by the formation of very large aggregates that were readily detectable by eye and by the large increase in light transmission measured by the aggregometer (Figure 1). The EC₅₀ values for aggregation by TRP⁴²⁻⁴⁷ and TRP⁴²⁻⁵⁵ were approximately 5 μ M and 8 μ M respectively. The additional enzymic elimination of secreted ADP by ADP-scavengers further reduced maximal TRP-induced aggregation and often caused some spontaneous disaggregation and shifted the dose-response relationship to higher concentrations of TRPs. The ADP-scavengers, apyrase (ATPase + ADPase) or CP/CK totally prevented the formation of large aggregates induced by even supramaximal concentrations of TRPs (e.g. 400 μ M) (Figure 1b). The same result was obtained when the ADP-receptor was blocked by 50–200 μ M ATP[α S] (not shown). Although apyrase or CP/CK, and ATP[α S], also shifted the α -thrombin dose-response about 5-fold, higher concentrations of α -thrombin could still induce maximal aggregation characterized by very large aggregate size (Figure 1a, right). These inhibitory effects of the ADP-scavengers can be ascribed largely to the destruction of ADP as they were not duplicated by adding the respective products of the scavenger reactions, namely AMP or ATP, at concentrations equal to, or in excess of, the maximum amounts of ADP + ATP normally released from the platelets. In addition, the inhibitory potency of different apyrase preparations was directly proportional to their ADPase activity (not shown). Amastatin (25–100 μ M), an inhibitor of aminopeptidase M which can inactivate TRPs, did not potentiate the responses of washed platelets to TRPs and did not prevent their spontaneous disaggregation in the presence of ADP-scavengers or ATP[α S].

TRP-induced secretion of the α -granule protein, thrombospondin, was also diminished by an average of 30% in aspirinized platelets plus ADP-scavengers (not shown). The reduced secretion of α -granule proteins, including fibrinogen, might account for the sub-maximal aggregation by TRPs. However, normal maximal aggregation was not restored by adding additional fibrinogen and Ca²⁺ to the medium (Figure 2a), suggesting that the availability of fibrinogen receptors was limiting (see below). The larger aggregates produced by α -thrombin in the presence of ADP-scavengers were not the result of entrapment of platelets in clumps of polymerized thrombin-generated fibrin because large aggregates were formed in the presence of the peptide GPRP (Figure 2b), which totally blocks fibrin polymerization (Figure 2c), and by γ -thrombin (Figure 2b), which lacks the fibrinogen clotting activity of α -thrombin (Wilner et al., 1979). These results demonstrate that the TRPs, in contrast to either α - or γ -thrombin, are incapable of producing maximal aggregation by their direct actions in the absence of secreted ADP. Therefore, when compared with thrombin, the TRPs behave as partial agonists with respect to their ability to produce platelet aggregation.

TRPs cause less association of pp60^{c-src} and GPIIb with the platelet CSK

Maximal platelet aggregation by thrombin is associated with

Figure 1 Effect of aspirin plus ADP-scavengers on α -thrombin and TRP⁴²⁻⁵⁵ induced aggregation

Aspirinized washed platelets in modified Tyrode's solution (3×10^8 /ml) were stimulated with 1.4–20 nM α -thrombin (a), or 20–400 μ M TRP⁴²⁻⁵⁵ (b), at time zero. The initial decrease in light transmittance is due to the shape change of activated platelets. The first series of responses (left) in each case were in the absence of apyrase and the second series (right) were in the presence of 2 units/ml of apyrase. Identical results were obtained using TRP⁴²⁻⁴⁷. Similar inhibition of TRP-induced aggregation was observed with 5 mM CP plus 8 units/ml of CK as the ADP scavenger, or with 50–200 μ M ATP[α S] as an ADP-receptor antagonist. These results are representative of more than 30 experiments.

Figure 2 Aggregation of platelets in response to TRP⁴²⁻⁵⁵, α -thrombin and γ -thrombin

(a) Aspirinized platelets (3×10^8 /ml) were stimulated with 40 μ M TRP⁴²⁻⁵⁵ in a medium containing 1 mM Ca^{2+} and 1 mg/ml fibrinogen: A, without apyrase; B, plus apyrase, 2 units/ml. (b) Aspirinized platelets plus apyrase were stimulated in the presence of 120 μ M GPRP with: A, 20 nM α -thrombin; or B, 1 μ M γ -thrombin. (c) A, Aggregation of glass beads by 20 nM α -thrombin plus 56 μ g/ml fibrinogen ($2 \times$ amount secreted by 3×10^8 platelets/ml); B, total inhibition of glass bead aggregation by 120 μ M GPRP.

substantial translocation of GPIIb–IIIa and the tyrosine kinase pp60^{c-src} from the Triton X-100-soluble fraction to the Triton X-100-insoluble fraction which represents the platelet CSK. Translocation of both proteins to the CSK occurred in aspirinized platelets aggregated maximally with TRP⁴²⁻⁴⁷, but the response to thrombin was 1.7–2-fold greater (Figure 3). The addition of apyrase virtually totally prevented the increase of pp60^{c-src} and GPIIb in the CSK of TRP-stimulated platelets (Figure 3) but not in thrombin-stimulated platelets, which still aggregated maximally.

Thrombin produces larger and longer-lasting intracellular signals than TRPs

We also compared the ability of thrombin and both TRPs to induce signal transduction events in the absence of ADP and TXA₂: i.e. increase of $[\text{Ca}^{2+}]_i$, the surface expression of activated

GPIIb–IIIa, phosphorylation of cPLA₂, AA release (a measure of cPLA₂ activation), PtdOH formation (a measure of PLC activation), p47 (pleckstrin) phosphorylation (a measure of PKC activation), and tyrosine phosphorylation of proteins. Even in the absence of apyrase, the transient rise in $[\text{Ca}^{2+}]_i$ by the peptides was of similar magnitude to thrombin, but the return to basal levels was somewhat faster in the case of the TRPs, so that $[\text{Ca}^{2+}]_i$ remained elevated for a slightly longer time after thrombin (Figure 4). TRPs also caused substantially less surface expression of activated GPIIb–IIIa, the fibrinogen receptor (Figure 4). TRPs also released less AA from phospholipids, compared with both α - and γ -thrombins, even in the absence of apyrase, as shown in the dose–response curves (Figure 5). The release of AA is catalysed by cPLA₂ and thrombin increases phosphorylation of that enzyme (Kramer et al., 1993) and thereby enhances its catalytic activity (Lin et al., 1992; Kramer et al., 1993). We therefore measured the dose–responses for phosphorylation of

Figure 3 Translocation of GPIIb-IIIa and pp60^{c-src} to the platelet CSK

Aspirinized washed platelets ($3 \times 10^9/\text{ml}$) were stimulated with 6.8 nM α -thrombin or 40 μM TRP⁴²⁻⁴⁷ with stirring at 37 °C, ± 2 units/ml apyrase. At the times indicated, Triton-insoluble CSK proteins were isolated (see Pumiglia and Feinstein, 1993) and subjected to SDS/PAGE and immunoblotting using Mo337 and SZ22 with detection by ECL. Platelet aggregation was measured in parallel with results like that in Figure 2a. The mean values for CSK content are expressed as a percentage of whole platelet content (two experiments in duplicate). Unstimulated control: GPIIb 2.5%, pp60^{c-src} 0.8%; thrombin (6.7 nM) minus apyrase: GPIIb 44%, pp60^{c-src} 69%; TRP minus apyrase: GPIIb 26%, pp60^{c-src} 33%; TRP + apyrase (2 units/ml): GPIIb 4%, pp60^{c-src} 1.3%.

Figure 4 Mobilization of internal Ca^{2+} and surface expression of activated GPIIb-IIIa by α -thrombin and TRP⁴²⁻⁵⁵

(a) Aspirinized platelets ($1 \times 10^9/\text{ml}$) pre-loaded with fura-2 AM were stimulated with: A, 6.8 nM α -thrombin; or B, 40 μM TRP⁴²⁻⁵⁵ to produce maximal rise of $[\text{Ca}^{2+}]$. Note that $[\text{Ca}^{2+}]$ rose somewhat more slowly and fell slightly more rapidly in TRP-treated platelets. (b) Measurement of surface GPIIb-IIIa activation in aspirinized platelets (+ apyrase 2 units/ml) with PAC-1 antibody by flow cytometry: A, unstimulated; B, stimulated by 40 μM TRP⁴²⁻⁵⁵; C, stimulated by 6.8 nM α -thrombin.

cPLA₂ caused by TRP⁴²⁻⁴⁷ and α -thrombin in aspirinized platelets (minus apyrase) at 1, 5, 10 and 15 min time points. In four experiments, platelets were then each stimulated by maximally

Figure 5 Release of AA by α -thrombin, γ -thrombin and TRP⁴²⁻⁴⁷

Aspirinized washed platelets ($1 \times 10^9/\text{ml}$) were stimulated with various concentrations of α - and γ -thrombins and TRP⁴²⁻⁴⁷ for 5 min. No apyrase was present. The data are from an experiment done in duplicate and are typical of three other experiments, in two of which 2 units/ml of apyrase was present. Mean maximal AA release by TRP⁴²⁻⁴⁷ in 4 experiments was 53% of that by α -thrombin (range 35–60%).

effective concentrations of α -thrombin and TRP⁴²⁻⁴⁷. The maximum phosphorylation of cPLA₂ was 70.05% at 35 nM thrombin and 48.25% at 100 μM TRP⁴²⁻⁴⁷ (paired *t*-test $P < 0.05$).

For other biochemical responses (i.e. PtdOH production, phosphorylation of p47 and tyrosine phosphorylation of p128 (p92 and p79 not shown), the very early phases of the responses were quite similar for both TRPs and α -thrombin, but those caused by the peptides did not attain the same maximum and were consistently more transient, returning to resting levels much sooner (Figure 6, left column). For example, α - and γ -thrombins caused longer-lasting PtdOH production than either of the TRPs and the maximal amount of PtdOH generated by the thrombins was 2.34-fold greater ($n = 5$; paired *t*-test $P < 0.01$). The persistent biochemical responses to α -thrombin were not the result of fibrin polymerization as they were not affected by GPRP and were also observed with γ -thrombin (not shown), which does not clot fibrinogen. Furthermore, amastatin (25 μM) did not increase or prolong PtdOH production by TRPs, indicating that the lower response was not due to destruction of peptides by aminopeptidase M.

Thrombin can activate platelets that are totally desensitized to TRPs

Homologous desensitization of thrombin receptors in human erythroleukaemia (HEL) cells by α -thrombin and TRPs has been described by Brass (1992). Although α -thrombin and TRPs can cause internalization of receptors in the HEL cells, no internalization of receptors was observed in platelets (Norton et al., 1993). To investigate this process further, we initially measured aggregation. TRP⁴²⁻⁵⁵ or TRP⁴²⁻⁴⁷ (40 μM) was added to unstirred platelets (to prevent aggregation) and then stirring was begun after 1–60 min to measure the ability of those platelets to aggregate. Total loss of responsiveness to both TRPs, even up to a concentration of 400 μM peptide, was evident within 10 min (Fig. 7a, C). This was accompanied by a virtual total loss of PtdOH production over the same concentration range (Fig. 7b). Aggregation caused by low concentrations of α -thrombin was inhibited in TRP-desensitized platelets, but several-fold higher concentrations of the protease (or γ -thrombin) still caused

Figure 6 Phosphatidic acid production and protein phosphorylation caused by α -thrombin and TRP⁴²⁻⁵⁵ in normal and TRP-desensitized platelets

Left column: aspirinized washed platelets (1×10^9 /ml) labelled with [32 P]phosphate were stimulated in the presence of 2 units/ml of apyrase with 20 nM α -thrombin (○), or 40 μ M TRP⁴²⁻⁵⁵ (●) at 37 °C. Right column: aspirinized platelets plus 2 units/ml of apyrase were pretreated with 40 μ M TRP⁴²⁻⁵⁵ at 37 °C for 20 min without stirring, and then stimulated with 20 nM α -thrombin (○), or an additional 40 μ M TRP⁴²⁻⁵⁵ (●) at time zero on the abscissa; at time zero the basal values were the same as in unstimulated platelets. Reactions were stopped at various time points indicated on the abscissa and reaction products were measured: (a) PtdOH; (b) p47 protein phosphorylation; (c) tyrosine phosphorylation of p128. The data are typical of 3–5 experiments in duplicate.

maximal aggregation (Fig. 7a A and B), even after 60 min of exposure to a TRP (not shown). Even when platelets were totally desensitized by pretreatment with 400 μ M TRPs, they were still able to aggregate strongly in response to α -thrombin (Fig. 7a, D). It is unlikely that these responses of TRP-desensitized platelets to α -thrombin can be attributed to the putative receptor GPIb (Greco and Jamieson, 1991; Yamamoto et al., 1991), because identical responses were elicited by γ -thrombin (Fig. 7a, E) which does not interact with GPIb (Jandrot-Perrus et al., 1988, 1990).

TRP⁴²⁻⁵⁵ can be inactivated by plasma aminopeptidase M (Coller et al., 1992), so that selective loss of response to the peptides might be due to their metabolism by a secreted or membrane-bound enzyme of washed platelets. To test this possibility, platelets were incubated with 200–400 μ M TRP⁴²⁻⁵⁵ for 60 min at 37 °C. Aliquots of the supernatant, after centrifuga-

Figure 7 Platelet aggregation by α -thrombin and γ -thrombin persist after desensitization by TRP⁴²⁻⁴⁷

(a) Aspirinized platelets (3×10^8 /ml; plus 2 units/ml of apyrase and 120 μ M GPRP) were preincubated with 40 μ M TRP⁴²⁻⁴⁷ without stirring in an aggregometer. After 10 min, stirring was commenced and the following were added: A, 6.8 nM α -thrombin; B, 1 μ M γ -thrombin; or C, 400 μ M TRP⁴²⁻⁴⁷. Platelets (as above) were also pretreated with 400 μ M TRP⁴²⁻⁴⁷ for 10 min. Upon stirring, the platelets did not aggregate (as in C). The records show the subsequent response to the addition of: D, 20 nM α -thrombin and E, 3 μ M γ -thrombin. (b) Phosphatidic acid production. Platelets (as above, at 1×10^9 /ml) were stimulated with 1–400 μ M TRP⁴²⁻⁴⁷: Δ , control; $\square$, undesensitized platelets; $\blacktriangle$, platelets pre-desensitized with 40 μ M TRP⁴²⁻⁴⁷ for 10 min.

tion to remove platelets, were added to unstimulated platelets from the same donor to attain final TRP⁴²⁻⁵⁵ concentrations of 2–20 μ M. The aggregation responses to incubated TRP⁴²⁻⁵⁵ were exactly equal to that of the native peptide (not shown). Thus, there was no loss of peptide activity during the incubation with washed platelets, demonstrating that proteolysis of TRP⁴²⁻⁵⁵ does not occur under these conditions and cannot account for either the short-lived signals elicited by TRPs in naive platelets or the lack of response following desensitization. By contrast, a similar incubation of TRP⁴²⁻⁵⁵ with plasma resulted in total loss of the peptide's activity. In addition, although the aminopeptidase inhibitor amastatin potentiates the aggregation by TRPs of platelets in plasma (Coller et al., 1992), it had no effect on aggregation by TRPs in washed platelets (not shown).

Several intracellular signal transduction events were also studied in desensitized platelets, i.e. PtdOH formation, p47 phosphorylation and tyrosine phosphorylation of p128 (p79, p92 not shown). Platelets were treated with 40 μ M TRP⁴²⁻⁵⁵ at 37 °C for 20 min to allow the initial responses to dissipate totally. The platelets were then stimulated with 20 nM α -thrombin or an additional 40–400 μ M TRP⁴²⁻⁵⁵. The TRP-pretreated platelets were totally or substantially desensitized to the subsequent addition of TRP⁴²⁻⁵⁵ (Fig. 6, right column), so that PtdOH formation, p47 phosphorylation and tyrosine phosphorylation were virtually absent. For example, the EC₅₀ for these responses was about 10 μ M TRP⁴²⁻⁵⁵ and maximum responses were attained at 20–40 μ M, but after desensitization the platelets were virtually totally unresponsive to as much as 400 μ M TRP⁴²⁻⁵⁵ or TRP⁴²⁻⁴⁷ (e.g. see Fig. 7b). However, the TRP-desensitized platelets still responded significantly to α -thrombin, although the responses were slowed and the dose-response was shifted about 2–10-fold to the right. To test the possibility that a different receptor mechanism was responsible for these persistent actions of α -thrombin we measured the production of [32 P]PtdOH in normal and TRP-desensitized platelets that were permeabilized by saponin in the presence of [32 P]ATP (Pumiglia et al., 1992). In both cases, [32 P]PtdOH production by thrombin in permeabilized platelets was totally blocked by GDP[β S] (not shown), indicating

Figure 8 Inactivation of α -thrombin with hirudin: effects on platelet aggregation and p47 protein phosphorylation

(a) Aspirinized platelets plus 2 units/ml apyrase were stimulated with 6.8 nM α -thrombin to measure aggregation. Hirudin (15 units/ml) was added (arrows) at 1 or 10 min (second panel), 30 s or 15 s (3rd and 4th panels respectively) after thrombin. (b) [³²P]Phosphate-labelled aspirinized platelets incubated with $\circ$, 6.8 nM α -thrombin or $\bullet$, 40 μ M TRP⁴²⁻⁵⁵. ∇ , Hirudin (15 units/ml) added 1 min after thrombin (arrow).

that the response to thrombin even after desensitization by TRPs was a G-protein-mediated event.

Inactivation of α -thrombin with hirudin terminates and/or reverses signal transduction and mimics the responses to TRPs

Hirudin was added at various times after initial stimulation by α -thrombin to inactivate the enzyme rapidly and strip it from its receptors (Tam et al., 1979). At early time-points (15–30 s), hirudin arrested aggregation before a maximum could be attained, which resulted in a small degree of partial disaggregation (Fig. 8a). However, if hirudin was added at 1 min when the rate of aggregation was maximal, or at 10 min when aggregation was fully attained, it did not cause disaggregation, as was described earlier by Holmsen et al. (1981). However, adding hirudin at 1 min (Fig. 8b), or at the height of aggregation (5–10 min, not shown), initiated more rapid dephosphorylation of p47 and several tyrosine phosphorylated proteins (e.g. p128, p92, p79 not shown). The rate of protein dephosphorylation after addition of hirudin (Fig. 8b) was similar to the spontaneous decline seen after TRPs.

DISCUSSION

Thrombin activates its receptor by proteolysis and is presumably not itself actually an agonist in the classical sense. The agonist activity resides within the receptor itself, in the form of the new N-terminal TL sequence generated by the hydrolysis of the Arg⁴¹–Ser⁴² peptide bond by thrombin (Vu et al., 1991a). The TRPs, which are based on the N-terminal TL sequence are believed to mimic the action of the TL by reversibly binding to the same docking site as the TL to activate the receptor. If TRPs were the full agonist equivalent to the TL in platelets then the maximal responses (i.e. intrinsic activity) to each should be the same, as was originally reported by several labs (Vu et al., 1991a; Vassallo et al., 1992). However, these studies did not take into account the contribution of the potent secondary mediators that are released by activated platelets, and the possible synergism between them and TRPs. When these secondary mediators, which have their own specific receptors, are eliminated by aspirin and ADP-scavengers, or ADP-receptor antagonists, the actions of TRPs should be restricted entirely to their direct actions,

mediated solely by the thrombin receptor. When TXA₂ and ADP were both eliminated there were three major differences between TRPs and thrombin: (i) unlike thrombin, TRPs could not produce large and irreversible platelet aggregates; (ii) they caused much less surface expression of the activated fibrinogen receptor GPIIb–IIIa than is necessary for aggregation and they failed to cause the association of GPIIb and pp60^{c-src} with the CSK which normally accompanies the formation of very large aggregates (Pumiglia and Feinstein, 1993); and (iii) thrombin produced longer lasting and often greater maximal intracellular biochemical signals than TRPs (i.e. PtdOH production, phosphorylation of cPLA₂ and protein Ser/Thr and Tyr residues, and AA release).

Differences between responses to thrombin and various TRPs have been noted in other systems: e.g. (i) TRP⁴²⁻⁵⁵ caused expression of P-selectin and neutrophil adhesion to endothelial cells, but unlike α -thrombin it did not increase expression of intercellular cell adhesion molecule-1 (ICAM-1) or ICAM-1 dependent neutrophil adhesion (Sugama et al., 1992); (ii) TRP⁴²⁻⁵⁵ unlike α -thrombin did not cause translocation of PKC from cytosol to membranes in endothelial cells (Lum et al., 1993); (iii) α -thrombin, but not SFFLRNP, caused cytosol to nuclear translocation of p42^{mapk} and p44^{mapk} in chinese hamster fibroblasts (CCL39) (Lenormand et al., 1993); and (iv) rabbit and rat platelets did not aggregate or secrete in response to TRPs and TRPs did not desensitize rabbit platelets to thrombin (Kinlough-Rathbone et al., 1993). In some cases the presence of additional agonists may have contributed to the responses to TRPs. For example, TRPs were mitogenic in CCL39 hamster fibroblasts only in the presence of tyrosine kinase-dependent growth factors such as nerve growth factor (NGF) (Vouret-Craviari et al., 1993). In contrast, TRP⁴²⁻⁵⁵ stimulated the same maximal proliferation of cultured rat aortic smooth muscle cells as thrombin in the absence of added growth factors other than insulin, but the authors concede that growth factors known to be produced by the smooth muscle cells [e.g. platelet derived growth factor (PDGF-AA) and transforming growth factor (TGF- β)] may have contributed to the mitogenic response elicited by the TRP (McNamara et al., 1993). Two other studies described actions of TRPs that were short-lived compared with thrombin: i.e. the activation of p44^{map} kinase in CCL39 hamster cells (Vouret-Craviari et al., 1993), and P-selectin mediated adhesion of neutrophils to human umbilical endothelial cells (Sugama and

Malik, 1992). However, it is now evident that the transient nature of the responses to TRPs are due in some cases to the metabolism of the peptides by cellular aminopeptidase M, an enzyme present in plasma, leucocytes, smooth muscle, vascular endothelium and other cell types, which removes the terminal Ser to inactivate the TRPs, but which apparently does not attack the N-terminal Ser of the TL in the large native receptor molecule (Coller et al., 1992). Amastatin, a very potent inhibitor of the peptidase, potentiated aggregation of platelets in plasma by TRPs (Coller et al., 1992) and enabled a long-term mitogenic activity of TRPs in kidney epithelial cells (Zacharias et al., 1993). However, the differences between TRPs and thrombin in our experiments on washed human platelets were clearly not attributable to the metabolic destruction of the TRPs, because the incubation of TRPs with stimulated platelets and their secretory products did not diminish their agonist activity, and responses to TRPs were not potentiated or prolonged by amastatin. This is consistent with an analysis of platelet exopeptidases that did not reveal any aminopeptidase M activity (Scharpe et al., 1991).

The lower intrinsic activity (maximal responses) of TRPs compared with thrombin in human platelets may be ascribed to the lower pharmacological efficacy (Stephenson, 1956; Furchgott, 1966) of the agonist peptides, as described by the classical Ternary Complex Model of G-protein-linked receptors (DeLean et al., 1980). This theory was recently modified to encompass evidence obtained from constitutively active receptor mutants. Based in part on studies with wild-type and mutant adrenergic receptors, it was postulated that inactive receptors (R) can isomerize to an active state R^* governed by an equilibrium constant J . In this allosteric model, agonists (A) stabilize the active receptor conformation because of a preferentially higher affinity for R^* and, furthermore, enhance its subsequent interaction with G-protein to form a ternary complex AR^*G leading to biological responses (Samama et al., 1993). In this model, the intrinsic activity of an agonist depends on its ability to affect this equilibrium by discriminating between R and R^* . The extent to which an agonist shifts this equilibrium to R^* and then stabilizes the ternary complex AR^*G is described by two dimensionless constants β and α respectively, so that the pharmacological efficacy of an agonist is given by the product $\alpha\beta$ (Samama et al., 1993). Partial agonists would therefore have lower affinity for R^* defined by low values for $\alpha\beta$. Indeed, the intrinsic activity of various partial agonists on β_2 -adrenergic receptors correlates well with their relative affinities for binding to R^* (Samama et al., 1993). Thus, we account for the lower intrinsic activity of TRPs compared with thrombin in human platelets as owing to a lesser specificity for binding to R^* and lesser ability to stabilize AR^*G , which is quantitatively expressed by the value of $\alpha\beta$ for the peptides. For the unique case of the thrombin-activated receptor, it is the proteolytic action of thrombin that generates R^* . This state of the thrombin receptor is comparable to that of mutant adrenergic receptors that are constitutively active in the absence of an agonist ligand (Kjelsberg et al., 1992). The spontaneous activity of mutant adrenergic receptors can be inhibited by negative antagonists which reverse the isomerization from R^* to R (Samama et al., 1994). This suggests that it may be possible to design negative antagonists that are able to suppress thrombin-activated receptors.

We found thrombin was able to evoke various responses in platelets that are totally desensitized to TRPs. Wilhelm and Siess (1993) also noted that platelets pretreated with $2.5 \mu\text{M}$ TRP⁴²⁻⁵⁵ were more effectively desensitized to a subsequent addition of the peptide than to thrombin when their effects on pertussis-toxin-catalysed ADP-ribosylation of G-proteins was studied. These differences in desensitization can also be explained by the partial

agonist nature of the peptides. However, it is also necessary to first consider alternative theories: i.e. (i) responses to thrombin in TRP-desensitized platelets may be mediated by a second receptor such as GPIb, which was proposed to be the high-affinity receptor for α -thrombin (Yamamoto et al., 1991; Greco and Jamieson, 1991); (ii) the TL generated by thrombin may activate TRP-desensitized receptors; and (iii) thrombin may have an additional effect on its receptors beyond generation of the TL. The first proposal is unlikely because γ -thrombin, which does not interact with GPIb (Jandrot-Perrus et al., 1988, 1990), also stimulated TRP-desensitized platelets, and the responses to γ -thrombin can be attributed to its ability to activate the TL receptor (Brass et al., 1992). Furthermore, the generation of PtdOH by thrombin was totally blocked by GDP[β S] in TRP-desensitized permeabilized platelets, as it is in undesensitized platelets, indicating that the same G-protein-dependent mechanism is involved in both cases. The second hypothesis is also unlikely because thrombin and TRPs cause homologous desensitization in megakaryoblastic HEL and CHRF-288 cells (Brass, 1992; Hoxie et al., 1993), suggesting that individual receptors desensitized by a TRP are likely to be desensitized to the TL as well. Thirdly, it remains to be determined whether the binding of thrombin plays any additional role in the R to R^* transition or whether the intramolecular binding of TL alone is sufficient to generate R^* . Apart from its hydrolytic action at the Arg⁴¹-Ser⁴² bond, the evidence for any further effect of thrombin on its receptor remains controversial. Huang et al. (1991) showed that occupancy of receptors by thrombin had no further effect on phosphoinositide metabolism beyond that required for catalytic generation of the TL. In contrast, Brass et al. (1994) reported an additional action on receptor internalization that was exerted by thrombin even after hydrolysis of receptors had occurred. On the other hand, the case for attributing the differential desensitization by TRPs and thrombin to the partial agonist activity of TRPs is more clear cut. It is well demonstrated in the case of G-protein-linked β -adrenergic receptors that: (i) partial agonists cause less maximal desensitization than full agonists (Su et al., 1980); and (ii) the ability of an agonist to cause receptor desensitization correlates well with its intrinsic activity. This is because increased intrinsic activity translates into greater formation of R^* , which is the substrate for phosphorylation by the receptor-specific kinase β -ARK (Benovic et al., 1988). Receptor phosphorylation appears to be a mechanism for desensitization of thrombin receptors also (Brass, 1992). Partial agonists, by producing less R^* , would cause incomplete desensitization of the total receptor population. Since TRPs behave as partial agonists with lower intrinsic activity than thrombin we can expect that even maximally effective concentrations of TRPs should leave some fraction of receptors in an undesensitized state, but the number of these receptors would be inadequate to generate significant signals in response to the low efficacy TRPs. However, proteolysis of the remaining undesensitized receptors by α - or γ -thrombin could convert them all to R^* , which would be sufficient to initiate significant cellular responses.

Because thrombin-activated receptors appear also to undergo rapid desensitization (Brass, 1992), the ability of thrombin to cause sustained signals may be due to its ongoing enzymic action on the total population of receptors to generate R^* . This would require the continued presence of proteolytically competent thrombin. It is known that hirudin, which rapidly inactivates thrombin and can strip it from its receptors, also quickly terminates PtdOH production (Holmsen et al., 1981; Huang and Detwiler, 1987), AA release and phosphoinositide breakdown (Huang and Detwiler, 1987), and causes accelerated protein dephosphorylation (this paper). This hypothesis is also supported

by Huang et al. (1991) who showed that an active-site inhibitor, which does not inhibit thrombin's binding to the receptor, also arrested PtdOH production. The most direct evidence comes from Ishii et al. (1993) who devised a thrombin receptor construct with an epitope-tagged activation peptide transfected into Rat1 fibroblasts to directly monitor the hydrolysis of receptors by thrombin. They found that response (i.e. the cumulative hydrolysis of phosphoinositides measured as total inositol phosphates) correlated exactly with the total extent of receptor hydrolysis over time, and it was concluded that cells probably sense varying concentrations of thrombin as different rates of receptor hydrolysis (activation). Downstream biochemical effects, which depend on the steady-state levels of second messengers, would thereby be a function of the rate of receptor hydrolysis and the rates of metabolic inactivation of the second messengers. In contrast, Brass et al. (1994) concluded that a thrombin molecule activates a very restricted population of receptors and that molar ratios of thrombin:receptor greater than 1 are necessary to continuously hydrolyse additional receptors. In our experiments, based on an assumed 1800 receptors per platelet (Brass et al., 1992) and the concentration of platelets used, we calculate the thrombin/receptor stoichiometry to be very much greater than 1, so that the situation observed by Ishii et al. (1993) would be applicable.

We conclude that in human platelets, TRPs are partial agonists for the thrombin receptor and that their behaviour is explained by the Ternary Complex Model for receptors that are linked to G-proteins. The basis for the lower efficacy of TRPs may be their differential affinity for the R and R* states of the receptor, whereas thrombin produces the R* state irreversibly by its proteolytic action. It remains to be determined if thrombin exerts additional effects on the TL and if ligands can be developed to reverse the isomerization: i.e. from R* to R. Finally, differences between TRPs and thrombin that have often been interpreted as evidence for the existence of other types of thrombin receptors can in many cases be adequately explained by the partial agonist nature of the TRPs and/or the metabolism of the peptides by cellular aminopeptidase activity.

Since the submission of this paper for publication, Ishii et al. (1994) have directly demonstrated phosphorylation of the thrombin receptor expressed in *Xenopus* oocytes by the β -adrenergic receptor kinase BARK2, and the concomitant inhibition of receptor signalling.

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