Thrombin Receptor-Activating Peptide Releases Arachidonic Acid from Human Platelets: A Comparison with Thrombin and Trypsin

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ABSTRACT
The serine proteases thrombin and trypsin are both powerful platelet agonists that act by cleaving the terminal portion of the thrombin receptor and allowing the new C-terminal to auto-stimulate the receptor. Synthetic peptides, termed thrombin receptor-activating peptides (TRAPs), have been shown to mimic many of the effects of thrombin. Here we have compared the effects of inhibitors on platelet aggregation and [14C]-arachidonic acid release from [14C]-arachidonic acid-prelabeled platelets to control levels. In contrast, leupeptin did not affect either aggregation or [14C]-arachidonic acid release in platelets stimulated by TRAP. Thrombin-induced aggregation and [14C]-arachidonic acid release were only partially inhibited by leupeptin. These data are consistent with the activation of platelets by both trypsin and TRAP occurring via the proteolytic receptor, whereas thrombin-induced platelet activation appears to occur by a dual mechanism of action. One component of thrombin-induced platelet activation is by a proteolytic action on the moderate-affinity receptor. This effect is sensitive to inhibition by leupeptin and is mimicked by trypsin and TRAP. The other component of thrombin is nonproteolytic and may occur by an action at a high-affinity receptor such as glycoprotein Ib.

The serine protease thrombin is the most powerful and the most commonly used platelet agonist in vitro. Thrombin initiates a wide range of platelet responses, such as shape change, pseudopod extension, eicosanoid production, granule release, adhesive receptor expression and aggregation (McNicol and Gerrard, 1993). These effects of thrombin are mediated by a series of biochemical events, including phospholipase C activity, intracellular calcium changes and protein phosphorylation (Seiss, 1989; Nozawa et al., 1991; McNicol and Gerrard, 1993; McNicol et al., 1993b). Similarly, trypsin has been shown to stimulate platelet activation, including aggregation and phosphoinositide metabolism (Davey and Luscher, 1967; Martin et al., 1975; Ruggiero and Lapetina, 1985; McNicol et al., 1989).

The apparent paradox of the specific activation of a cell by proteolytic action was explained by the cloning of the moderate-affinity thrombin receptor (Vu et al., 1991a; Rasmussen et al., 1991). Proteolytic cleavage of the terminal portion of the receptor generates a novel amino terminal of amino acid sequence SFLLRNPDKYPF (single amino acid code). This new amino “tail,” termed the tethered ligand, subsequently binds to and autostimulates the receptor (Vu et al., 1991a; Rasmussen et al., 1991; Coughlin, 1993).

Synthetic peptides corresponding to the new amino terminal mimic the effects of thrombin and interact with the receptor (Vu et al., 1991a; Vu et al., 1991b). Such peptides as small as the terminal six amino acids (SFLLRN), termed TRAPs, have been shown to stimulate platelet activation. Several studies have demonstrated TRAP-induced aggregation, ATP release, phospholipase C activity and phosphatidylinositol-3-kinase activity in human platelets (Vu et al., 1991a; Coughlin, 1993; Huang et al., 1991; Seiler et al., 1991) but not in those of some other species (Kinlough-Rathbone et al., 1993).

Additional thrombin binding sites are present on platelet membranes. One such site, GPIb, binds with high affinity, but is not proteolytically cleaved by, thrombin (Okamura et al., 1978; Phillips and Agin, 1977; Harmon and Jamieson, 1986). As a high-affinity receptor, GPIb is believed to be important at low thrombin conditions (Harmon and Jamie-

ABBREVIATIONS: TRAPs, thrombin receptor-activating peptides; GPIb, glycoprotein Ib; TLC, thin-layer chromatography; MAP kinase, mitogen-activated protein kinase.
son, 1986). For example, platelets from individuals with Bernard Soulier syndrome lack GPIb, have normal levels of the moderate-affinity thrombin receptor (Greco et al., 1996b; McNicol et al., 1996) and have abnormal responses to low, but not high, thrombin concentrations (Jandrot-Perrus et al., 1990; Greco et al., 1996b; McNicol et al., 1996). It has been suggested that GPIb plays an active role in thrombin-induced platelet aggregation, including stimulating, or potentiating, specific intracellular pathways (De Marco et al., 1991; Yamamoto et al., 1991; Greco et al., 1996a; Greco et al., 1996b). This is controversial, however, and the role of GPIb in thrombin-induced platelet activation remains elusive.

A major aspect of the platelet response is the release of arachidonic acid from cell membrane phospholipids. Arachidonic acid is greatly enriched in the SN-2 position of platelet phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine (Lands, 1979). Released arachidonic acid is converted by the cyclooxygenase pathway to thromboxane A2, which, through a positive feedback loop, causes full platelet activation.

Several enzymatic pathways have been implicated in the release of arachidonic acid. Although a role for diacylglycerol lipase has been suggested, a cytosolic form of phospholipase A2 is believed to play the major role in arachidonic acid release in platelets (Kramer et al., 1993; Kramer et al., 1995). Both thrombin and TRAP stimulated the phosphorylation of phospholipase A2, which in turn activates the enzyme (Kramer et al., 1995). There is, however, evidence that the two agonists act by different intracellular pathways (Kramer et al., 1995).

In the present study inhibitors of cyclooxygenase and proteolysis have been used to examine specific roles of proteolytic and non-proteolytic receptors in thrombin-induced arachidonic acid release. Further, the ability of TRAP to stimulate the release of arachidonic acid and the role which thromboxane plays in TRAP-induced platelet aggregation have been examined.

Materials and Methods

Preparation of platelets. Blood was collected into acid citrate dextrose (3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 1.9 ml of anticoagulant per 8.1 ml of blood) by venipuncture of healthy human volunteers who had not taken medication known to interfere with platelet function within the previous 2 weeks. Platelet-rich plasma was obtained by centrifugation at 800 g for 15 min (McNicol et al., 1991).

Platelet aggregation. Plasma-free platelet suspensions were obtained by centrifugation of platelet-rich plasma at 800 × g for 15 min, and the resultant pellet was resuspended in the plasma volume of HEPES-buffered Tyrode’s solution (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM MgCl2, 10 mM HEPES, 5 mM dextrose, 0.3% bovine serum albumin; pH 7.4) (Murayama et al., 1990). Aliquots (0.4 ml) containing 1 mM CaCl2 were incubated with 1 ml of acid citrate dextrose and centrifugation at 800 × g for 15 min. The resultant pellet was resuspended in the plasma volume of HEPES-buffered Tyrode’s (see above). Aliquots (0.4 ml) containing 1 mM CaCl2 were incubated for 2 min with the inhibitor, or with the appropriate vehicle control, before the addition of agonist.

Release was terminated by transferring the entire sample to 2 ml of chloroform/methanol/10 N HCl (25:50:4). Arachidonic acid was extracted by adding 0.625 ml of chloroform and 0.625 ml of water, and the organic phase was removed and evaporated under nitrogen. The samples were resuspended in 50 µl of chloroform/methanol (1:1, v/v), applied to heat-activated Silica gel 60 TLC plates and separated by a mobile phase of chloroform: methanol/acetate water (90:0.8:0.2, v/v/v). The plates were subjected to radiochromatographic scanning. Arachidonic acid was identified by comparison with a known standard and was consistent with the published Rf value of 0.78 (Salmon and Flower, 1982). [14C]-arachidonate-containing neutral lipids (dilgycerides, triglycerides) traveled with the solvent front, and [14C]-arachidonate-containing phospholipids (including phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylserine, phosphatidic acid and cardiolipin) remained at the origin. The [14C]-arachidonic acid is expressed as a percentage of total radioactivity per sample.

Materials. Thrombin from bovine plasma, trypsin and leupeptin were obtained from Sigma (St. Louis, MO). TRAP, single-letter code SFLLLRN, was synthesized by Dr. D. Litchfield (University of Manitoba) with an Applied Biosystems Model 431A peptide synthesizer using Fmoc chemistry. All agonists and inhibitors were resuspended in isotonic saline. [1-14C]-arachidonic acid was obtained from Amer sham (Oakville, Ont.). Before each experiment, an aliquot (1 µCi) was removed and the solvent evaporated under nitrogen. Silica gel 60 TLC plates were obtained from VWR Canlab (Edmonton, Ab.). All other laboratory supplies were of the highest available grade.

Results

Effects of inhibitors on thrombin-induced platelet aggregation. The effects of cyclooxygenase and proteolytic inhibitors on agonist-induced platelet aggregation were monitored. Thrombin (0.04–0.1 U/ml) caused full platelet aggregation.

Preincubation for 2 min with the proteolytic inhibitor leupeptin (10 µg/ml) decreased thrombin-induced aggregation (fig. 1A). The inhibitory effects of leupeptin were dependent on the thrombin concentration. Low thrombin concentrations (0.04 U/ml) were particularly susceptible to inhibition by leupeptin, but this tendency was overcome by increasing the concentration of thrombin added (0.1 U/ml) (fig. 1A).

Platelets were pretreated at 37°C for 2 min with the dual cyclooxygenase/lipoxygenase inhibitor BW755C (80 µM) before the addition of thrombin. BW755C had no effect on thrombin-induced aggregation at any agonist concentration (fig. 1B).

Effects of inhibitors on trypsin-induced platelet aggregation. The serine protease trypsin has been reported to stimulate full platelet aggregation (Davey and Luscher, 1967; Martin et al., 1975; Ruggiero and Lapetina, 1985). In a result consistent with these studies, the addition of trypsin (15–24 nM) elicited aggregation as monitored by light transmission.

When the platelets were pretreated for 2 min with leupeptin (10 µg/ml) trypsin-induced aggregation at all concentrations (20–24 nM) was abolished (fig. 2A), as has been previously reported (Ruggiero and Lapetina, 1985). These data confirm the proteolytic nature of trypsin-induced platelet aggregation.
BW755C (80 μM) delayed the onset of, but did not abolish, trypsin-induced platelet aggregation (fig. 2B). This effect was more pronounced at lower trypsin concentrations.

Effects of inhibitors on TRAP-induced platelet aggregation. Numerous studies have reported TRAP-induced platelet activation using synthetic peptides as small as six amino acids in length (Vu et al., 1991a; Coughlin, 1993; Huang et al., 1991; Seiler et al., 1991). In the present study, TRAP (SFLLRN) caused platelet aggregation. There was a large amount of donor variability for the optimal TRAP concentration; however, aggregation was never observed below 4 μM.

Pretreatment of the platelets for 2 min with 10 μg/ml leupeptin (fig. 3A) did not affect TRAP-induced (4–6 μM) aggregation. This is consistent with TRAP activating the moderate-affinity, proteolytic thrombin receptor by a nonproteolytic mechanism.

In contrast, 80 μM BW755C partially inhibited aggregation in response to TRAP. Some donor variability was observed, but, as seen in figure 3B, lower concentrations of TRAP (4 μM and 5 μM) were less sensitive to inhibition than higher ones (6 μM).
Effects of leupeptin on thrombin-, trypsin- and TRAP-induced \(^{14}\)C-arachidonic acid release. We examined the release of \(^{14}\)C-arachidonic acid from platelets incubated for 2 min with thrombin, trypsin or TRAP. Thrombin (0.1 U/ml), trypsin (20 nM) and TRAP (6 \(\mu\)M) stimulated the release of \(^{14}\)C-arachidonic acid by 4.6 ± 0.4-, 4.5 ± 1.2- and 2.3 ± 0.2-fold respectively (\(n = 3–5\)) (fig. 4a, b, d and f), which is consistent with the activation of phospholipase A\(_2\) by each of these agonists. In the case of thrombin and trypsin, the release was accompanied by a decrease of 17 ± 4% and 19 ± 3% (\(n = 4\)) respectively, in the \(^{14}\)C-arachidonic acid content of phosphatidylcholine (data not shown).

The effects of the proteolytic inhibitor leupeptin on agonist-induced \(^{14}\)C-arachidonic acid were examined. Pretreatment of the platelets for 2 min with leupeptin (10 \(\mu\)g/ml) returned trypsin-induced \(^{14}\)C-arachidonic acid release to control levels (fig. 4, d and e), and this effect was accompanied by the complete inhibition of phosphatidylcholine breakdown (data not shown). Leupeptin also partially inhibited both thrombin-induced release (fig. 4, b and c) and thrombin-induced phosphatidycholine breakdown (data not shown) but had no effect on TRAP-induced \(^{14}\)C-arachidonic acid release (fig. 4, f and g).

**Fig. 3.** The effects of inhibitors on TRAP-induced platelet aggregation. Washed human platelets were preincubated for 2 min with A) 10 \(\mu\)g/ml leupeptin or B) 80 \(\mu\)M BW755C before the addition (\(\uparrow\)) of TRAP at the concentrations indicated. Aggregation was monitored continuously as an increase in light transmission. Bar corresponds to 60 sec. Tracings are representative of 4 to 5 experiments using platelets from different donors.

**Fig. 4.** The effects of leupeptin on agonist-induced \(^{14}\)C-arachidonic acid release. Human platelets were prelabeled with \(^{14}\)C-arachidonic acid. Aliquots were pretreated with saline (a, b, d, f) or 10 \(\mu\)g/ml leupeptin (c, e, g) before the addition of saline (a), 0.1 U/ml thrombin (b, c), 20 nM trypsin (d, e) or 6 \(\mu\)M TRAP (f, g). Released \(^{14}\)C-arachidonic acid was extracted, separated by TLC and visualized by radiochromatogram (\(\downarrow\)). \(^{14}\)C-arachidonate-containing neutral lipids traveled with the solvent front (left), and \(^{14}\)C-arachidonate-containing phospholipids remained at the origin (right). Tracings are representative of 3 to 5 separate experiments.
Thrombin is the most powerful stimulant of platelet activation in vitro (McNicol and Gerrard, 1993). Several thrombin binding sites have been identified on platelet membranes, including the GPIb/V/IX complex and a moderate-affinity serpine-tyne receptor (Vu et al., 1991a; Okamura et al., 1978; Phillips and Agin, 1977). The relative contributions of these sites to platelet activation remain unclear.

A potential dual mechanism of platelet activation in response to thrombin has been proposed that has both nonproteolytic and proteolytic components (Martin et al., 1975; Harmon and Jamieson, 1986; McNicol et al., 1989; Yamamoto et al., 1991). The high-affinity receptor may constitute the former, and the moderate-affinity thrombin receptor clearly fulfills the proteolytic requirements (Vu et al., 1991a; Rasmussen et al., 1991; Coughlin, 1993). Proteolytic cleavage by thrombin, of the moderate-affinity receptor generates a new amino terminal called the tethered ligand. The tethered ligand in turn autostimulates the receptor. In the present study, the arachidonic acid-liberating abilities of two agonists, which specifically act on the moderate-affinity receptor, have been compared with that of thrombin.

Trypsin resembles thrombin not only as a serine protease but also in its effects on platelets. In platelets, trypsin has been shown to activate GTPase activity (Jakobs and Akto-rics, 1988), phosphoinoside hydrolysis (Ruggiero and Lapetina, 1985; McNicol et al., 1989; McNicol et al., 1993a), calcium changes (Zavoico et al., 1985), inhibition of adenylate cyclase (Jakobs and Grandt, 1988), arachidonic acid release (Rehm et al., 1988; present study) and aggregation (Davey and Luscher, 1967; Martin et al., 1975; present study). Furthermore, trypsin and thrombin desensitize each other’s responses in HEL cells (Brass et al., 1991). Taken together, these observations suggest that thrombin and trypsin act on the same receptor/substrate, presumably the moderate-affinity thrombin receptor, and that they share a common postreceptor pathway.

The moderate-affinity thrombin receptor can be stimulated directly by peptides (TRAPs) that correspond to the amino terminal generated by the proteolytic action of thrombin on the receptor. TRAP has previously been shown to cause platelet aggregation, ATP and arachidonic acid release, phospholipase C activity, calcium changes, cytosolic acidification and phosphatidylinositol-3-kinase activity (Huang et al., 1991; Seiler et al., 1991; Lau et al., 1994; Nieuwland et al., 1994). Again, this is consistent with thrombin and TRAP sharing common post-receptor processes. However several studies have noted that the platelet response to TRAP differs from that observed with thrombin (Seiler et al., 1991; Lau et al., 1994; Nieuwland et al., 1994; Lasne et al., 1995).

In the present study, both trypsin and TRAP stimulated the release of arachidonic acid, and aggregation in response to both agonists was susceptible to inhibition by the dual cyclooxygenase/lipoxygenase inhibitor BW755C. These data are consistent with activation of the moderate-affinity (proteolytic) thrombin receptor leading to arachidonic acid release. Further, the subsequent conversion of arachidonic acid to thromboxane A2 is important to the proteolytic component of platelet aggregation.

Thrombin-induced aggregation was unaffected by cyclooxygenase inhibition but was partially inhibited by leupeptin. Similarly, thrombin-induced arachidonic acid release and phosphatidylinositol breakdown were partially inhibited by leupeptin. These data are consistent with the presence of both proteolytic and nonproteolytic mechanisms of thrombin-induced arachidonic acid release. Consequently, this additional nonproteolytic mechanism may account for the relative insensitivity of thrombin, when compared with trypsin and TRAP, to BW755C.

Similar differences between thrombin- and TRAP-induced platelet activation have been previously attributed to a more sustained activation of the moderate-affinity receptor by thrombin than by TRAP (Huang et al., 1991; Lau et al., 1994; Liu et al., 1995; Kramer et al., 1995). However, the similarity between activation by TRAP and by trypsin, which also proteolytically cleaves the receptor and would presumably produce a thrombin-like prolonged activation, argues against this concept.

Thrombin, trypsin and TRAP all stimulate arachidonic acid release through the moderate-affinity receptor. Whether this is activation of phospholipase A2, either directly or as a consequence of an intermediate step (such as calcium elevation), or by an alternative pathway (such as diglyceride lipase) remains to be clarified (Brass et al., 1993). Both thrombin and TRAP have been shown to phosphorylate the 85-kDa cytosolic form of phospholipase A2 (Kramer et al., 1993; Kramer et al., 1995), which has been linked to stimulation of the arachidonic acid-liberating actions of the enzyme (Kramer et al., 1993). However, differential pathways have been implicated to account for this effect of the two agonists. Thrombin-induced cytosolic phospholipase A2 phosphorylation is associated with the action of MAP kinase(s), whereas TRAP-induced phosphorylation is not (Kramer et al., 1995). These data are consistent with the results of the present study and with the concept of different signaling events distal to the high-affinity and moderate-affinity thrombin receptors.

The high-affinity thrombin receptor has not been positively identified. Similarly, its role in thrombin-induced platelet activation is unclear, although the high-affinity receptor may have a signal transduction role distinct from that of the moderate-affinity G-protein-mediated receptor. Phospholipase C- is activated by its interaction with rap1b/GAP in thrombin-stimulated platelets (Torti and Lapetina, 1991; Peterson and Lapetina, 1994). This interaction might result from occupancy of the high affinity, nonproteolytic receptor. Such a mechanism would be consistent with thrombin activating both types of receptors and, consequently, with multiple intracellular pathways.

Studies in TRAP-desensitized platelets using γ-thrombin, which does not interact with the putative high-affinity receptor GPIb, argue against a role for GPIb in platelet aggregation (Lau et al., 1994). In contrast, type IIIB von Willebrand factor causes cytosolic calcium changes and arachidonic acid release in human platelets, and these effects are abrogated by pretreatment with antibodies against GPIb (Francesconi et al., 1995). This study suggests that signaling pathways distal to GPIb occupancy lead to platelet arachidonic acid release. It is consistent with this concept that a form of phospholipase A2, a member of the 14-3-3 protein family, is associated with GPIb/IX in platelets (Du et al., 1994). Although, the phospholipase activity of 14-3-3 has been chal-
lenged (Robinson et al., 1994) and its activation by thrombin has not been demonstrated to date, it does provide evidence for a potential specific intracellular pathway associated with a putative high-affinity thrombin receptor. In addition, it provides a potential additional mechanism of arachidonic acid release in thrombin-stimulated platelets.

In conclusion, this study provides additional evidence for the dual nature of thrombin-induced platelet activation. Trypsin and TRAP stimulate arachidonic acid release via the moderate-affinity, proteolytic thrombin receptor. Thrombin also causes arachidonic acid release by an action on the moderate-affinity receptor, but thrombin has an additional mechanism of action distal to a nonproteolytic receptor.

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