Neutrophil cathepsin G and thrombin, the only platelet agonists that are proteases, exhibit a mandatory requirement for catalytic activity to induce platelet aggregation and signal transduction. The thrombin receptor is a G-protein-coupled receptor which undergoes proteolysis to generate a tethered ligand that causes self-activation. Since cathepsin G strongly resembles thrombin in its ability to activate platelets, we have attempted to determine whether cathepsin G and thrombin function through the same or different receptors. Evidence that thrombin and cathepsin G act at different receptors was as follows: (a) an antibody directed against the thrombin receptor blocked thrombin-induced but not cathepsin G-induced platelet responses; (b) human fibroblasts responded to thrombin and to a synthetic thrombin receptor peptide (comprising residues 42–55 of the thrombin receptor) by exhibiting an elevation in cytosolic Ca\(^{2+}\) concentration but did not respond to cathepsin G; and (c) platelets pretreated with neutrophil elastase failed to respond to thrombin but responded when rechallenged by cathepsin G. Thrombin and cathepsin G exhibit heterologous desensitization that is potentiated by okadaic acid and is attenuated by staurosporine, indicating that phosphorylation of serine/threonine residues is important for desensitization and that protein kinase C may be involved. Since catalytic activity of cathepsin G is required for platelet stimulation, it is probable that platelet activation by cathepsin G requires receptor proteolysis and that a tethered ligand mechanism is involved, suggesting that platelets may possess a family of protease receptors.

**INTRODUCTION**

We have previously demonstrated that neutrophil cathepsin G, like thrombin, is a strong platelet agonist which exhibits saturable, reversible binding to platelets, characteristic of the involvement of a specific receptor [1,2]. In addition, cathepsin G and thrombin are the only platelet agonists that are proteases, and most of the biological actions of both cathepsin G and thrombin require the catalytic activity of the enzymes. In particular, aggregation and stimulus–response coupling in platelets are abolished when the proteolytic activity of either cathepsin G or thrombin is blocked or neutralized [1–4]. The thrombin receptor has been cloned and sequenced and shown to possess seven transmembrane domains characteristic of all members of the family of G-protein-linked receptors [5]. However, unlike any other receptor yet described, proteolysis of the extracellular N-terminal receptor domain is required for activation. Cleavage of the thrombin receptor at Arg\(^{161}\) exposes a new N-terminus which then functions as a tethered ligand to activate cells by binding to a site on the receptor [5,6]. The binding site for thrombin includes a region of the N-terminus of the receptor distal to the cleavage site which bears a strong resemblance to hirudin and functions to bind thrombin via its anion exosite [6–8]. A synthetic peptide composed of receptor residues Ser\(^{42}\)-Phe\(^{65}\) (thrombin receptor peptide; TRP\(^{42-65}\) has been shown to function as a full platelet agonist [5,9]. However, only the first six amino acids of this 14-residue peptide are required for activity [10,11]. TRP\(^{42-65}\) has also been shown to activate human endothelial cells [12] and hamster fibroblasts [13], suggesting that endothelial cells, fibroblasts and platelets share a structurally similar thrombin receptor.

Activation of the thrombin receptor by this novel intramolecular rearrangement raises the question of whether a family of similar G-protein-coupled protease receptors might exist. Since the catalytic activity of cathepsin G is mandatory for platelet stimulation, it is highly probable that platelet activation by cathepsin G requires receptor proteolysis and that a tethered ligand mechanism is involved. While our previous results indicate that cathepsin G strongly resembles thrombin in its ability to activate platelets [1], data presented here suggest that these two agonists function through distinct receptors rather than through a single receptor which is differentially cleaved to yield two different tethered ligands. Evidence indicating that platelets possess different receptors for thrombin and cathepsin G suggests that platelets possess a family of protease receptors.

**MATERIALS AND METHODS**

**Materials**

Monoclonal antibody ATAP138, directed against the SFLLRNPNKYPF sequence of the thrombin receptor (Ser\(^{42}\)-Phe\(^{65}\)), was a gift from Dr. Lawrence Grass (University of Pennsylvania, Philadelphia, PA, U.S.A.), and \(\alpha\)-thrombin was a generous gift from Dr. John Fenton (Albany Medical College, Albany, NY, U.S.A.). Okadaic acid was obtained from Gibco or from Kamiya Biomedical Company, and staurosporine was purchased from Kamiya Biomedical Company. TRP\(^{42-65}\) was purchased from Bacham Bioscience Inc., Philadelphia, PA, U.S.A.

**Preparation and labelling of platelets**

Human blood was obtained from healthy volunteers and collected into acid–citrate dextrose. The platelet-rich plasma, obtained by centrifugation at 180 g for 20 min at room temperature, was re-centrifuged at 800 g for 15 min at room temperature to obtain a platelet pellet. The platelet pellet was resuspended in 10 ml of plasma and the platelets were incubated at 37 °C with 3 \(\mu\)M fura-2-acetoxyethyl ester for 45 min and with 1 \(\mu\)M 5-hydroxy[\(^{14}\)C]tryptamine and 1 mM aspirin for 30 min (45 min total incubation time). Fura-2 acetoxyethyl ester and aspirin were dissolved in dimethyl sulfoxide and, when added to cells,
the final dimethyl sulphoxide concentration never exceeded 0.5%. Platelets were gel-filtered using Sepharose CL-2B columns equilibrated and eluted with calcium-free Tyrode’s buffer supplemented with 0.2% fatty acid-free BSA and 10 mM glucose. Imipramine (1 µM) was added to 5-hydroxy[14C]tryptamine-labelled cells.

**Purification of neutrophil cathepsin G and elastase**

Buffcoat leucocytes were used to isolate cathepsin G from neutrophil granules by the method of Baugh and Travis [14]. Granule extracts were subjected to sequential aprotinin-Sepharose and CM-Sephadex chromatographies [15]. Protein concentration was determined using the BCA protein assay (Pierce) with BSA as standard, as well as by absorbance at 280 nm. The purity of cathepsin G and elastase was assessed by specific substrate hydrolysis and by both SDS and non-denaturing gel electrophoresis as described previously [1]. Based on these criteria, cathepsin G was totally devoid of neutrophil elastase and vice versa. Cathepsin G and elastase enzymic activities were measured spectrophotometrically at 37 °C using N-succinyl-(Ala)2-Pro-Phe-p-nitroanilide and methoxy-succinyl-(Ala)2-Pro-Val-p-nitroanilide respectively as substrates, according to the method of Nakajima et al. [16].

**Measurement of 5-hydroxytryptamine release**

Dense granule secretion was measured in 5-hydroxy[14C]tryptamine-labelled platelets supplemented with imipramine. Secretion was stopped in formaldehyde–EDTA according to the method of Costa and Murphy [17] and samples of the supernatants were added to scintillation fluid. In each set of experiments the total 5-hydroxytryptamine content of the platelets was measured by adding platelet suspension to the stopping solution, from which a sample was then transferred directly to the counting solution. Release of 5-hydroxytryptamine was expressed as a percentage of the total 5-hydroxy[14C]tryptamine content.

**Measurement of the cytosolic free Ca2+ concentration ([Ca2+]i) with fura-2**

Fura-2 fluorescence was monitored continuously in platelets loaded with both fura-2 and 5-hydroxy[14C]tryptophan using settings of 340 nm (excitation) and 510 nm (emission). Fmax was determined by the addition of 2 mM EGTA and 20 mM Tris base in the presence of 40 µM digitonin, and Fmax was determined by subsequent addition of 10 mM CaCl2. [Ca2+], was calculated using a Kd of 224 nM for fura-2 [18] after correction for extracellular dye.

**Culture and fura-2 loading of fibroblasts**

Monolayers of GM 06167 normal human lung fibroblasts were grown in 8% CO2/air in 150 cm2 flasks in Eagle’s minimal essential medium with Earle’s salts containing 20% (v/v) fetal calf serum, non-essential amino acids, 100 µg/ml streptomycin and 100 units/ml penicillin. Monolayers of normal human lung HS-27 fibroblasts were grown in Dulbecco’s minimal essential medium with 4.5 mg/ml glucose, 10% (v/v) fetal calf serum, 100 µg/ml streptomycin and 100 units/ml penicillin. Fura-2-acetoxymethyl ester (2 µM) was added to culture flasks and incubated at 37 °C for 45 min. The medium was decanted and replaced with an equal volume of culture medium without fetal calf serum and the cells were incubated for another 30 min at 37 °C (75 min total incubation time). The medium was decanted, Tyrode’s buffer containing 10 mM glucose and 5 mM EDTA was added, and the cells were incubated for 10 min at 37 °C. Cells were harvested mechanically with a cell scraper, dispersed by trituration, washed once and resuspended in Tyrode’s buffer containing 10 mM glucose and 1 mM CaCl2. Fibroblasts were handled as described above for platelets.

**RESULTS**

Platelet responsiveness to cathepsin G and thrombin was examined by measuring the extent of dense granule secretion and the amount of calcium discharged from internal stores, as estimated from the size of calcium transients in fura-2- and 5-hydroxy[14C]tryptophan-loaded cells. To exclude the involvement of endoperoxides/thromboxane A2 and ADP, all measurements were performed using aspirinated platelets incubated in the presence of phosphocreatine/creatine kinase (PC/CK). Controls verified that the cells were unresponsive to ADP and arachidonic acid. Platelet calcium and secretion responses were routinely measured in the presence of EGTA to eliminate repletion of internal pools due to agonist-induced calcium influx through the plasma membrane. Though not always shown, ionomycin was added to each sample to verify that depletion of intracellular calcium was not responsible for attenuated responses following the second challenge with agonist. Since ionophore- and agonist-sensitive internal calcium stores need not necessarily be the same, a failure to observe an elevation in [Ca2+]i, by a second addition of agonist in the presence of EGTA could indicate selective depletion of internal calcium stores. However, parallel experiments conducted in the presence of extracellular calcium demonstrated that the presence or absence of external calcium had no effect on patterns of desensitization and thus was not responsible for diminished second agonist responses. Finally, since ionomycin does not elicit 5-hydroxytryptamine release in the presence of PC/CK and aspirin, the ionophore could also be used to assess the amount of residual intracellular calcium without contributing to agonist-induced dense granule secretion (see Figure 5; [19]). Data shown for each set of experiments are representative of results obtained with platelets from five separate donors.

**Effect of thrombin receptor antibody on cathepsin G- and thrombin-induced platelet responses**

To address the question of whether the thrombin and cathepsin G receptors are the same or different, fura-2-loaded platelets were preincubated with monoclonal antibody ATAP138 directed against TRP421'3', a synthetic peptide composed of Ser421-Phe56 of the new N-terminus exposed by thrombin cleavage of its receptor [5,6]. As noted in Figure 1, ATAP138 prevented an elevation in platelet [Ca2+]i induced by thrombin but had no effect on cathepsin G stimulation when cathepsin G was used as either primary or secondary agonist. Furthermore, ATAP138 had no effect on platelet responses elicited by 2–10 µg/ml cathepsin G. While the differential effects of ATAP138 could indicate that cathepsin G and thrombin have different receptors, they do not exclude the possibility of a single receptor with two separate ligand binding sites.

**Effect of cathepsin G and thrombin on fibroblast [Ca2+]i**

Since thrombin receptors might be expected to be the same regardless of the cell in which they appear, the effects of thrombin and cathepsin G on another cell type known to respond to thrombin were examined. Normal human fibroblasts loaded with fura-2 responded to both thrombin and TRP421'3', as indicated by
50 and Aspirin-treated platelets cathepsin G-induced platelet responses

Platelets treated with neutrophil elastase are known to exhibit diminished responses to thrombin [20,21]. On the other hand, we [22] have observed that neutrophil elastase potentiates platelet responses elicited by low (suboptimal) concentrations of cathepsin G where platelet activation by cathepsin G is partially dependent on amplification by released ADP and thromboxane A2. We have exploited the effects of elastase on thrombin- and cathepsin G-induced platelet responses to examine differences between the receptor(s) for these two agonists. (Since the experiments described here were performed in the presence of PC/CK and aspirin, potentiation of cathepsin G-induced responses by neutrophil elastase was not observed.) As seen in Figure 3, platelets which were unresponsive to thrombin following elastase pretreatment responded when rechallenged with cathepsin G. Elastase had no effect on responses to U46619 and, similar to fibroblasts, elastase had no effect on TRP4255-induced platelet responses. Both potentiation of cathepsin G and inhibition of thrombin-induced responses require catalytically active elastase since phenylmethanesulphonyl fluoride-inhibited elastase had no effect on cell activation.

Desensitization is the regulatory aspect of receptors that decreases responsiveness to further stimulation following prior exposure to an agonist. As shown here for cathepsin G and thrombin, and as previously demonstrated for thrombin [23,24], prior exposure of platelets to cathepsin G or to thrombin makes the cells less sensitive to subsequent activation by the same agonist (Figure 4a). Increasing concentrations of thrombin or cathepsin G elicited a dose-dependent rise in platelet [Ca^{2+}]i. At 3.5 min following addition of the first agonist, i.e. when the [Ca^{2+}]i had returned to resting levels, the cells were challenged by a second, constant, concentration of the same agonist. The rate and amplitude of the elevation in [Ca^{2+}]i induced by the second addition of agonist progressively decreased as the initial concentration of either thrombin or cathepsin G was raised, demonstrating that the platelets exhibited agonist-specific desensitization. As stated earlier, ionomycin was always used to confirm that the decrease in [Ca^{2+}]i observed following the second addition of agonist did not reflect depletion of intracellular pools.

Role of protein phosphorylation in platelet desensitization

To determine whether phosphorylation plays a role in cathepsin G-induced cathepsin G desensitization and in thrombin-induced thrombin desensitization, okadaic acid was used to inhibit protein phosphatases. As seen in Figure 4(b), okadaic acid potentiated desensitization mediated by either thrombin or cathepsin G, suggesting that desensitization is the result of increases in phosphorylation. Stauorospine was used to evaluate the contribution of protein kinase C in the development of desensitization. Control experiments showed that stauorospine inhibited phorbol ester-induced 5-hydroxytryptamine release, with maximal inhibition (87%) observed at 200 nM; this concentration of

Figure 1 Effect of anti-(thrombin receptor) antibody on thrombin- and cathepsin G-induced platelet responses

Aspirin-treated platelets were incubated at 37°C while stirring in the presence of 20 mM EGTA and 50 units/ml CK, plus 1 mM EGTA. Platelets were preincubated with 50 µg/ml ATAP138 or an equivalent volume of buffer for 5 min prior to agonist addition. α-Thrombin (Thr) was used at 0.15 unit/ml, cathepsin G (CG) at 2.5 µg/ml and ionomycin (Iono) at 2 µM. Shown are the 340 nm fura-2 fluorescence signals.
Cross-desensitization of cathepsin G and thrombin responses in platelets

Platelets were examined for heterologous desensitization either by stimulating cells with thrombin followed by restimulation with cathepsin G or vice versa. Concentrations were selected such that the cells exhibited specific desensitization to the initial agonist. As seen in Figure 5, pretreatment of platelets with cathepsin G attenuated cell responses induced by thrombin; conversely, prior exposure to thrombin diminished platelet responses induced by cathepsin G. Both agonists also attenuated platelet responses to TRP42V55 and the thromboxane A2 mimic U46619, although when used as the initial stimulus both TRP42V55 and U46619 elicited large rises in $[Ca^{2+}]_i$. By contrast, pretreatment of platelets with U46619 had no significant effect on subsequent thrombin- or cathepsin G-induced responses. Response patterns similar to those for U46619 were observed with platelet-activating factor (PAF) (results not shown). As with agonist-specific desensitization, development of heterologous desensitization depended on the initial agonist concentration and was potentiated by okadaic acid and attenuated by staurosporine.

DISCUSSION

Cathepsin G, like thrombin, is a strong or full platelet agonist which induces platelet activation independent of thromboxane A2 and ADP, and additionally stimulates release of lysosomal enzymes [1]. Thrombin, a trypsin-like enzyme, is extremely specific and, with rare exceptions, cleaves peptide bonds to arginine in both natural and synthetic substrates [28]. Cathepsin G is approximately one to two orders of magnitude less active than leukocyte elastase and other serine proteases, including chymotrypsin and trypsin, in its ability to degrade numerous proteins [29,30], suggesting that the physiological action of cathepsin G may also be highly selective and may be limited to distinct sites of the protein substrate. The uniqueness of cathepsin
Figure 4 Desensitization of platelet responses to thrombin and cathepsin G

(a) Thrombin-induced thrombin and cathepsin G-induced cathepsin G platelet desensitization. Aspirin-treated platelets were incubated at 37 °C while stirring in the presence of 1 mM EGTA, 20 mM PC and 50 units/ml CK. At 3.5 min after addition of the indicated concentration of thrombin (top series of traces) or cathepsin G (bottom series of traces), platelets were challenged with either 0.5 unit/ml thrombin or 10 µg/ml cathepsin G. Lonomycin (lono) was used at 2 µM. Shown are the 340 nm fura-2 fluorescence signals. (b) Effect of okadaic acid and staurosporine on thrombin- and cathepsin G-induced platelet responses. Aspirin-treated platelets were incubated at 37 °C while stirring in the presence of 20 mM PC and 50 units/ml CK, plus 1 mM EGTA. At 3.5 min after addition of 1 unit/ml α-thrombin (Thr) or 10 µg/ml cathepsin G (CG), platelets were challenged with the same concentration of either thrombin or cathepsin G. Okadaic acid (OA) was preincubated with platelets for 90 s and used at 1.25 µM; staurosporine (Stauro) was preincubated with platelets for 60 s and used at 200 nM. Shown are the 340 nm fura-2 fluorescence signals. The percentage of total 5-hydroxytryptamine released is indicated above each trace. PMA, phorbol 12-myristate 13-acetate.

G is further underscored by the fact that, although cathepsin G exhibits chymotrypsin-like specificity, it differs from chymotrypsin in that it can activate platelets whereas chymotrypsin cannot [3]. Thus, although thrombin and cathepsin G are proteases, they both appear to exert regulatory effects through limited proteolysis of specific peptide bonds in their respective protein substrates. A highly selective substrate specificity is a necessity for agonists whose ability to activate platelets relies on enzymic activity and involves receptor proteolysis [5].

Thrombin and cathepsin G are the only protease platelet agonists; in both cases proteolytic activity is necessary for platelet activation, and both enzymes exhibit strong similarity in their ability to initiate platelet responses. In the light of the many similarities between these two agonists, the question arises as to whether or not the enzymes share the same platelet receptor. To address this issue, platelet responsiveness was examined by measuring calcium mobilization from internal stores under conditions where amplification by ADP and thromboxane A2 was blocked. Preincubation of platelets with a thrombin receptor-specific antibody inhibited stimulation of platelets induced by thrombin but had no effect on platelet responses elicited by a range of cathepsin G concentrations. In addition, platelets pretreated with neutrophil elastase failed to respond to thrombin but responded when rechallenged by cathepsin G. Two different normal human fibroblast cell lines loaded with fura-2 responded to thrombin and to TRP435, as indicated by an elevation in
Aspirin-treated platelets were incubated at 37 °C while stirring in the presence of 20 mM PC and 50 units/ml CK. At 3.5 min after addition of 1 unit/ml Fenton thrombin (Thr) or 20 μg/ml cathepsin G (CG), platelets were challenged with the same concentration of either thrombin or cathepsin G. U46619 was used at 5 μM and ionomycin (Iono) at 2 μM. Shown are the 340 nm fura-2 fluorescence signals. The percentage of total 5-hydroxytryptamine released is indicated above each trace.

Figure 5  Effect of thrombin and cathepsin G on platelet responses

[Ca\(^{2+}\)]
, but both failed to respond to cathepsin G. TRP\(^{42,55}\) has also been shown to activate hamster fibroblasts [13], suggesting that the fibroblast thrombin receptor is activated by a tethered ligand mechanism and that fibroblasts and platelets share a structurally similar receptor. Consistent with this conclusion is the observation here that pretreatment of human fibroblasts with leucocyte elastase attenuated fibroblast responses to thrombin and, also similar to platelets, elastase had no effect on TRP\(^{42,55}\)-induced responses.

The inhibition of thrombin-induced calcium mobilization by pretreatment with neutrophil elastase is consistent with the demonstration by other investigators that leucocyte elastase is associated with hydrolysis of glycoprotein Ib (GPIb) and with a decrease in the number of high-affinity thrombin binding sites [20,21]. Although it is not known how GPIb and the cloned thrombin receptor relate in terms of platelet signal transduction, it may be relevant that residues 269–287 in GPIb are rich in negatively charged amino acids [31]. It is possible that thrombin binding to these residues via its anion exosite enhances stimulus-response coupling mediated by the thrombin receptor. Elastase cleavage of GPIb on the C-terminal side of this binding region would eliminate the high-affinity thrombin binding domain, thereby leading to diminished thrombin responsiveness. It is also noteworthy that elastase had no effect on TRP\(^{42,55}\)-induced platelet or fibroblast responses, suggesting that, in addition to cleaving GPIb, elastase may also possibly cleave the thrombin receptor on the C-terminal side of Ser\(^{40}\), thereby precluding formation of the tethered ligand.

Homologous desensitization decreases the responsiveness to a second round of stimulation by the same agonist and is diagnostic of different receptors; heterologous desensitization decreases the response to multiple agonists but does not indicate whether the receptors are the same or different [32]. Exposure of platelets to thrombin is known to make them less sensitive to subsequent stimulation by thrombin [23,24]. We demonstrate here that, as with thrombin, platelets pretreated with cathepsin G become unresponsive when re-exposed to the same ligand. In addition, platelets incubated with cathepsin G exhibited attenuated calcium and secretion responses when rechallenged by thrombin, and vice versa. Desensitization to cathepsin G and thrombin was dependent on the concentration of the initial agonist, consistent with a proteolytic event. Moreover, desensitization was increased by okadaic acid and was decreased by staurosporine, suggesting that phosphorylation of serine/threonine residues is important and that protein kinase C may play a role in desensitization. These results are consistent with the observations of Brass [33] that protein kinase C plays a limited role in thrombin desensitization in human erythrolemukaemia (HEL) cells. Although staurosporine is a potent protein kinase C inhibitor, it can also inhibit other protein kinases [34]. Consequently, the effect of staurosporine suggests, but does not prove, that protein kinase C is involved in the desensitization of cathepsin G- and thrombin-induced platelet responses. Cathepsin G and thrombin also attenuated platelet responses to the thromboxane A\(_{2}\)-mimetic U46619 and to PAF although, when used as initial stimuli, U46619 and PAF elicited large rises in [Ca\(^{2+}\)]. The reduced ability of U46619 and PAF to induce an elevation in [Ca\(^{2+}\)], following prior stimulation with either thrombin or cathepsin G suggests that both thrombin and cathepsin G mediate global changes in cell responsiveness by protein phosphorylations which lead to a functional uncoupling of many different receptors from their effector systems.

It is important to note that the cathepsin G and thrombin receptors must desensitize rapidly or they would not be activated in a dose-dependent fashion. Furthermore, since the thrombin receptor, and possibly the cathepsin G receptor, appears to be activated via a tethered ligand mechanism, tight regulation (desensitization) is mandatory because the ligand is part of the receptor itself. Different receptors for cathepsin G and thrombin are suggested by results demonstrating that platelets can respond to cathepsin G under conditions where the cells are unresponsive to thrombin. Collectively, the observations presented here suggest that the thrombin receptor may be the prototype for a family of G-protein-coupled protease receptors and suggest that the cathepsin G receptor is a member of this family, indicating that platelets possess different protease receptors.

I am indebted to Dr. J. Bryan Smith for his support of the research presented here and to Dr. Lawrence Brass for generously supplying ATAP138. I also thank Dr. Barrie Ashby for his comments and suggestions during the preparation of this paper. This
work was supported in part by grants from the American Heart Association and by New Hampshire Agricultural Experimental Station Hatch Project no. 359. This is a scientific contribution no. 1808 from the New Hampshire Agricultural Experimental Station.

REFERENCES

15 Travis, J., Bowen, J. and Baugh, R. (1978) Biochemistry 17, 5651–5656

Received 16 March 1993/10 August 1993; accepted 1 September 1993