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Cathepsin G is a strong platelet agonist released by neutrophils

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The present studies were undertaken to characterize a serine protease released by *N*-formyl-L-Met-L-Leu-L-Phe (fMet-Leu-Phe)-stimulated neutrophils that rapidly induces platelet calcium mobilization, secretion and aggregation. The biological activity associated with this protease was unaffected by leupeptin, was only weakly diminished by *N*-*p*-tosyl-L-Lys-chloromethane, but was strongly inhibited by α_1-antitrypsin, soyabean trypsin inhibitor, *N*-tosyl-L-Phe-chloromethane and benzoyloxycarbonyl-Gly-Leu-Phe-chloromethane (Z-Gly-Leu-PheCH_2Cl). These observations indicated that the biological activity of neutrophil supernatants could be attributed to a chymotrypsin-like enzyme such as cathepsin G. Furthermore, platelet aggregation and 5-hydroxytryptamine release induced by cell-free supernatants from fMet-Leu-Phe-stimulated neutrophils were found to be blocked by antiserum to cathepsin G in a concentration-dependent manner but were unaffected by antiserum to elastase. The biological activity present in neutrophil supernatants co-purified with enzymic activity for cathepsin G during sequential Aprotinin–Sepharose affinity chromatography and carboxymethyl–Sephadex chromatography. SDS/polyacrylamide-gel electrophoresis of the reduced, purified protein, demonstrated three polypeptides with apparent M_r values of 31500, 29000 and 28000 and four polypeptides were resolved on acid-gel electrophoresis. Purified cathepsin G from neutrophils cross-reacted with anti-(cathepsin G) serum in a double immunodiffusion assay and elicited platelet calcium mobilization, 5-hydroxytryptamine secretion and aggregation. Calcium mobilization and secretion induced by low concentrations of cathepsin G were partially dependent on arachidonic acid metabolites and ADP, while stimulation by higher enzyme concentrations was independent of amplification pathways, indicating that cathepsin G is a strong platelet agonist. These results suggest that pathological processes which stimulate neutrophils and release cathepsin G can in turn result in the recruitment and activation of platelets.

INTRODUCTION

Previously we have demonstrated that human neutrophils stimulated by *N*-formyl-L-Met-L-Leu-L-Phe (fMet-Leu-Phe) in the presence of cytochalasin B release a soluble factor which is capable of rapidly activating platelets (Chignard *et al.*, 1986). This factor was shown not to be thromboxane A_2, arachidonic acid, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor), ADP, thrombin, superoxide ions or peroxide but, rather, appeared to be a serine protease based on its sensitivity to phenylmethanesulphonyl fluoride. These initial observations provided direct evidence for secondary signal transmission to platelets subsequent to primary stimulation of neutrophils and they excluded the above mentioned classical mediators as the active agent. In the present work, studies were undertaken to characterize the properties of and to identify the enzyme responsible for inducing platelet activation. The results presented here demonstrate that the mediator released by neutrophils is a strong platelet agonist (Holmsen & Day, 1970) that appears to be identical to cathepsin G (EC 3.4.21.20).

MATERIALS AND METHODS

Materials

Cytochalasin B, fMet-Leu-Phe, fibrinogen, adrenaline, Histopaque 1077, leupeptin, imipramine, soyabean trypsin inhibitor, trypsin (bovine pancreas, 13000 units/mg of protein), TPCK, Aprotinin, Protein A–Sepharose CL-4B, creatine kinase, phosphocreatine, benzoyl-tyrosine ethyl ester, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and methyoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide were obtained from Sigma. α_1-Antitrypsin (bovine) and TLCK were purchased from Boehringer Mannheim. Z-Gly-Leu-PheCH_2Cl was purchased from Enzyme Systems Products (Livermore, CA, U.S.A.). Carboxylmethyl–Sephadex, Sephadex G-25 and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia and 5-hydroxy [*side-chain*-2-^{14}C]-tryptamine creatinine sulphate was obtained from Amersham. Anti-(human cathepsin G), -elastase and -α-fetoprotein sera were purchased from ICN Immunobiologicals. All chloromethyl ketone solutions were prepared fresh daily and dissolved in dimethyl sulphoxide. Aprotinin was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions.

Preparation of platelets

Human blood was collected into acid/citrate/dextrose as anticoagulant from healthy volunteers who had not taken aspirin during the previous ten days (Aster & Jandl, 1964). Platelet-rich plasma was prepared by centrifugation at 180 *g* for 20 min and incubated for 30 min at 37 °C with 1 μM-5-hydroxy[^{14}C]tryptamine

Abbreviations used: fMet-Leu-Phe, *N*-formyl-L-Met-L-Leu-L-Phe; TPCK, *N*-tosyl-L-Phe-chloromethane; TLCK, *N*-*p*-tosyl-L-Lys-chloromethane; Z-Gly-Leu-PheCH_2Cl, benzyloxycarbonyl-Gly-Leu-Phe-chloromethane.

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(55 mCi/mmol). In some experiments, two-fold concentrated platelet-rich plasma was incubated for 30 min with 5-hydroxy[^{14}C]tryptamine and 5 μM-fura-2 acetoxymethyl ester dissolved in dimethyl sulphoxide. The volume of dimethyl sulphoxide never exceeded 0.5% (v/v). When labelling was complete, the platelets were collected by centrifugation at 500 *g* for 20 min and gently resuspended in Tyrode's buffer composed of 145 mM-NaCl/5 mM-KCl/1 mM-$MgSO_4$/0.5 mM-NaH_2PO_4/10 mM-glucose/10 mM-Hepes, pH 7.4. Where indicated, aspirin was added to the platelet-rich plasma at a final concentration of 1 mM during the 30 min labelling period. The platelet concentration was adjusted to 2×10^8 or 4×10^8 cells/ml and kept at ambient temperature after addition of 2 μM-imipramine to the final cell suspension.

Measurement of platelet aggregation and secretion

5-Hydroxy[^{14}C]tryptamine-loaded platelets were incubated in Tyrode's buffer at 37° C while stirring for 2 min in the presence of 1.3 mM-$CaCl_2$ and 160 μg of fibrinogen/ml. Portions of supernatant from fMet-Leu-Phe-stimulated neutrophils were added to radiolabelled platelets and aggregation monitored by determining changes in light transmission using a Payton aggregometer. Secretion was stopped in formaldehyde-EDTA according to the method of Costa & Murphy (1975) and samples of the supernatants added to scintillation fluid (Liquiscint, National Diagnostics). In each set of experiments the total 5-hydroxytryptamine content of platelets was measured by adding platelet suspension to the stopping solution from which a sample was transferred directly to the counting solution. Release of 5-hydroxytryptamine was expressed as a percentage of the total 5-hydroxytryptamine content and aggregation was expressed as a percentage of the maximal light transmission.

Preparation of neutrophils

Human blood was collected using acid/citrate/dextrose as anticoagulant (Aster & Jandl, 1964) and neutrophils were prepared according to the method of Boyum (1968) as previously described (Chignard *et al.*, 1986). Cell number and purity were determined by phase microscopy following dilution in Turk's solution and viability was assessed by Trypan Blue dye exclusion. Microscopic examination following differential staining with Giemsa or Wright stain was additionally used to evaluate the purity of the cell preparation. The cells were resuspended in Hanks' balanced salt solution at a concentration of 10^8/ml and the cell preparation maintained at room temperature in the presence of 1 mM-$MgCl_2$. Cells prepared in this manner routinely contained 98 ± 1% neutrophils and were 97 ± 2% viable. Cell suspensions were preincubated for 5 min at 37 °C in the presence of 1.3 mM-$CaCl_2$ and 5 μg of cytochalasin B/ml before the addition of 1 μM-fMet-Leu-Phe. Cell-free supernatants (13000 *g* for 2 min) were prepared 3 min after the addition of the chemotactic peptide and were concentrated by ultrafiltration where indicated.

Fluorescence measurements and estimation of cytosolic calcium

Portions of fura-2- and 5-hydroxy[^{14}C]tryptamine-loaded platelets were incubated for 3 min in the presence of 1.3 mM-$CaCl_2$ or 1 mM-EGTA before addition of cathepsin G. Fluorescence measurements were conducted in a Perkin–Elmer Model LS-5 fluorometer using quartz cuvettes thermostatically controlled at 37 °C and continuously stirred. Fura-2 fluorescence signals were obtained using an excitation wavelength of 340 nm (5 nm slit width) and an emission wavelength of 510 nm (10 nm slit width). $F_{min.}$ was determined following the addition of digitonin in the presence of EGTA and Tris base and $F_{max.}$ was achieved by the subsequent addition of excess calcium. Cytosolic free calcium concentrations were calculated using a K_d of 224 nM (Grynkiewicz *et al.*, 1985) after correction for extracellular dye.

Antibody specificity studies

Anti-(human cathepsin G), anti-(human elastase) and anti-(human α-fetoprotein) sera were reconstituted according to the manufacturer's instructions and dialysed extensively at 4 °C against phosphate-buffered saline (pH 7) to remove azide which could interfere with platelet function (Stibbe & Holmsen, 1977). After centrifugation at 13000 *g* to remove particulate material, protein concentration was estimated by absorption at 280 nm and the antisera were used at equivalent protein concentrations. Portions of concentrated neutrophil supernatant or supernatant cathepsin G, purified according to Travis *et al.* (1978), were incubated for 5 min at ambient temperature with each of the three antisera followed by addition of Protein A–Sepharose CL-4B to immobilize immunoglobulin G which was subsequently removed by centrifugation at 13000 *g*. Portions of the resulting supernatants were then tested for their ability to induce platelet responses. Inhibition was expressed as a percentage of 5-hydroxytryptamine released compared with corresponding control samples in which phosphate-buffered saline was used in place of antiserum. Additional controls were performed to verify that Protein A–Sepharose CL-4B completely removed immunoglobulin G which could non-specifically activate platelets by binding to Fc receptors (Ishizaka & Ishizaka, 1962). Antibody specificity studies were performed using double immunodiffusion in agar by the method of Garvey *et al.* (1977).

Purification of cathepsin G

Pooled supernatants from fMet-Leu-Phe-stimulated neutrophils were dialysed against 0.8 M-NaCl/0.05 M-Tris/HCl, pH 8, and then applied to an Aprotinin–Sepharose column (1 cm × 19 cm) equilibrated with the same buffer. The column was washed with the same buffer until the A_{280} was less than 0.02 according to the method of Travis *et al.* (1978). The column was subsequently washed with 0.8 M-NaCl/0.05 M-sodium acetate, pH 4.5. Column fractions were tested for their ability to induce platelet secretion and for cathepsin G activity. Active fractions obtained by affinity chromatography were dialysed against 0.15 M-NaCl/0.05 M-sodium acetate, pH 5.5, and applied to a carboxymethyl–Sephadex column (1 cm × 2.5 cm) overlaid with 0.5 cm of Sephadex G-25. Elastase and cathepsin G were eluted by a gradient from 0.46–0.78 M-NaCl. Column fractions were assayed for elastase and cathepsin G activity and were tested for their ability to induce platelet secretion, the latter in the presence of 2 μM adrenaline. Ion-exchange chromatography fractions containing either elastase or cathepsin G were pooled, dialysed against 0.15 M-NaCl/0.01 M-Hepes, pH 7.4, and concentrated by ultrafiltration. The con-

centrated dialysates were assayed for enzymic and biological activity. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Measurement of enzymic activity

Cathepsin G activity was measured spectrophotometrically at 37 °C and either *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide or benzoyl-tyrosine ethyl ester and elastase was measured with methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide according to previously published methodologies (Rick, 1974; Nakajima *et al.*, 1979).

SDS/polyacrylamide- and acid-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis (10%) was conducted according to the procedure of Laemmli (1970) and 15% non-denaturing acid gels were performed as described by Blackshear (1984). Proteins used for calibration standards for SDS/polyacrylamide-gel electrophoresis were myosin (100000), β-galactosidase (116250), phosphorylase *b* (97400), bovine serum albumin (66200), ovalbumin (42699), carbonic anhydrase (31000) and soyabean trypin inhibitor (21500). Both gel systems were stained with Coomassie R-250 except for those experiments in which proteins were to be extracted from acid gels for subsequent assay. Unstained strips from acid-gel electrophoresis corresponding to each of the four cathepsin G isozymes were minced and homogenized in Tyrode's buffer without glucose. After 24 h at 4 °C, the gel slurry was centrifuged to obtain supernatants which were then concentrated at 4 °C using polyethylene glycol 20000. The gel concentrates were dialysed overnight against 150 mM-NaCl/10 mM-Hepes, pH 7.4.

RESULTS

Effect of protease inhibitors and antisera on neutrophil supernatant-induced platelet dense-granule secretion

The protease released by activated neutrophils was characterized using the different classes of specific enzyme inhibitors shown in Table 1. Each inhibitor was preincubated with concentrated neutrophil supernatant at the indicated concentration(s) before testing portions of treated supernatants for their ability to induce 5-hydroxytryptamine release from platelets. The lack of a direct effect of each inhibitor on platelets was excluded by testing platelet responsiveness to other appropriate agonists (thrombin or arachidonic acid). Finally, where appropriate, the inhibitors were tested for their ability to block 5-hydroxytryptamine release induced by trypsin. Biological activity was unaffected by leupeptin, only weakly inhibited by TLCK, inhibited by α_1-antitrypsin and soyabean trypsin inhibitor in a concentration-dependent fashion and strongly inhibited by TPCK and Z-Gly-Leu-PheCH_2Cl.

The inhibitor profile presented in Table 1 suggested that the biological activity present in neutrophil supernatants could be attributed to a chymotrypsin-like enzyme. In particular, the sensitivity of platelet activation to the cathepsin G-selective inhibitor, Z-Gly-Leu-PheCH_2Cl, implicated cathepsin G as the protease responsible for the observed responses. Thus, the effect of anti-(cathepsin G) serum on platelet responsiveness was tested. An antiserum to human neutrophil cathepsin G abolished platelet aggregation and 5-hydroxytryptamine release induced by neutrophil supernatant while equivalent protein concentrations of antisera to human elastase and α-fetoprotein had no effect on platelet responses (Fig. 1). A constant volume of neutrophil supernatant was incubated with various concentrations of anti-(cathepsin G) serum and the ability of the treated supernatants then tested for their ability to elicit 5-hydroxytryptamine release from platelets. As seen in Fig. 1(*b*), the 5-hydroxytryptamine-releasing activity of neutrophil supernatants was blocked in a concentration-dependent manner by the antiserum. The specificities of anti-(cathepsin G) and anti-elastase sera were confirmed by double immunodiffusion subsequent to purification of neutrophil cathepsin G and elastase.

Table 1. Inhibition of platelet 5-hydroxytryptamine release by potential inhibitors

Concentrated cell-free neutrophil supernatant or trypsin were preincubated for 5 min at 37 °C with the indicated inhibitor concentration(s). The percentage inhibition was calculated by comparison with the corresponding control platelet 5-hydroxytryptamine release conducted under identical conditions in the presence of the appropriate solvent. Shown are the means of multiple determinations conducted with at least three different donors. For each set of experiments, a volume of neutrophil supernatant and a concentration of trypsin were chosen that induced full aggregation. Trypsin was incubated with inhibitors at 200 μg/ml and tested at a final concentration of 2–3 μg/ml.

Inhibitor	Neutrophil supernatant (%)	Trypsin (%)
Leupeptin		
500 μM	0	100
TLCK		
20 mM	13.4	89.4
α-Antitrypsin		
125 μM	99.4	100
100 μM	40.9	100
10 μM	0	100
Soyabean trypsin inhibitor		
100 μg/ml	100	100
50 μg/ml	96.4	86.4
25 μg/ml	4.7	1.4
TPCK		
20 mM	75.6	0
Z-Gly-Leu-PheCH_2Cl		
10 mM	81.4	5
0.5 mM	81.2	0
0.1 mM	79.1	0
0.02 mM	60.2	0

Co-purification of cathepsin G and 5-hydroxytryptamine-releasing activities from neutrophil supernatants

A pool of cell-free supernatants from fMet-Leu-Phe-stimulated neutrophils was applied to an Aprotinin–Sepharose column. Cathepsin G and the 5-hydroxytryptamine-releasing activity were completely retained by the column and elution of both activities coincided with a decrease in buffer pH. Active fractions from the

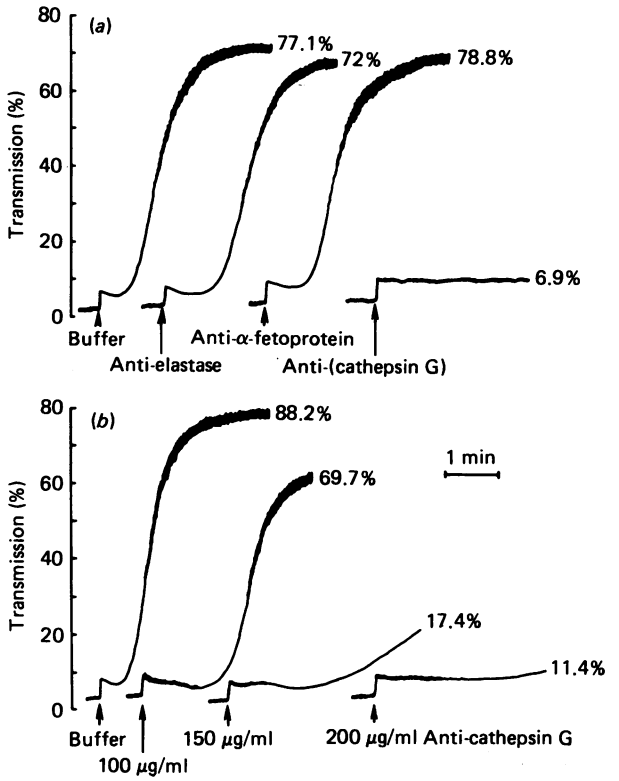

Fig. 1. Effect of different antisera on neutrophil supernatant-induced platelet aggregation and secretion

(*a*) Effects of anti-(cathepsin G), anti-elastase and anti-α-fetoprotein sera. Platelets (2×10^8/ml) were preincubated in Tyrode's buffer supplemented with 1.3 mM-$CaCl_2$ and 160 μg of fibrinogen/ml for 2 min before addition of portions of neutrophil supernatant treated with equivalent protein concentrations (350 μg/ml) of anti-(human cathepsin G), anti-elastase, or anti-α-fetoprotein sera or with phosphate-buffered saline. The percentage 5-hydroxytryptamine release was determined 3 min after addition of treated neutrophil supernatant and is indicated at the end of each aggregation trace. (*b*) Concentration-dependent effect of anti-(cathepsin G) serum. Neutrophil supernatant was treated with buffer, and 100 μg, 150 μg and 200 μg of anti-(cathepsin G) protein/ml as indicated.

affinity column were pooled, dialysed and chromatographed on carboxymethyl–Sephadex. Fig. 2 shows that elastase and cathepsin G were separated by the ion-exchange step and elution of the latter occurred concurrently with elution of biological activity. Fractions from the carboxymethyl–Sephadex column containing either elastase or cathepsin G were pooled, dialysed and concentrated by ultrafiltration. The purity of each fractionation step was assessed by SDS/polyacrylamide-gel electrophoresis run under reducing conditions. Cathepsin G was found to migrate as three polypeptide chains with apparent M_r values of 31500, 29000 and 28000. Electrophoresis conducted under non-denaturing acid conditions revealed four polypeptides for cathepsin G. Extracts of each of the four isozymes present in the acid-gel system hydrolysed *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, a cathepsin G-specific substrate (Nakajima *et al.*, 1979).

Immunodiffusion of purified neutrophil cathepsin G and elastase

Neutrophil supernatant cross-reacted with anti-(cathepsin G) serum and the immunoprecipitin band formed a line of complete identity with the precipitin band formed by the isolated neutrophil supernatant cathepsin G and the antiserum. Antiserum to elastase cross-reacted with neutrophil supernatant and isolated neutrophil elastase. On the other hand, cathepsin G did not cross-react with anti-elastase serum and elastase did not cross-react with anti-(cathepsin G) serum. The lack of sharp precipitin bands observed with anti-elastase serum may be a consequence of the glycoprotein nature of the antigen (Baugh & Travis, 1976).

Effect of purified neutrophil cathepsin G on platelet secretion and calcium mobilization

As seen in Fig. 3, increasing concentrations of purified cathepsin G induced progressively greater extents of calcium mobilization and 5-hydroxytryptamine release from platelets in the presence or absence of extracellular calcium. At low cathepsin G concentrations (5–10 μg/ml) inhibition of arachidonic acid metabolism by aspirin pretreatment decreased the extent of calcium mobilization and dense-granule secretion and the subsequent addition of phosphocreatine/creatine kinase further decreased both responses. At higher cathepsin G concentrations, however, aspirin and/or phosphocreatine/creatine kinase had negligible effects on the extents of calcium mobilization and secretion. On the other hand, while platelets remained totally refractory to the addition of high concentrations of purified neutrophil elastase (25 μg/ml), addition of elastase 60 s before cathepsin G enhanced the degree of aggregation and secretion induced by the chymotrypsin-like enzyme (results not shown).

DISCUSSION

This paper describes the characterization and identification of an enzyme released by fMet-Leu-Phe-stimulated neutrophils that was previously demonstrated capable of inducing platelet calcium mobilization, secretion and aggregation (Chignard *et al.*, 1986). The sensitivity to specific inhibitors was initially utilized in the present studies to classify the proteolytic activity in supernatants from stimulated neutrophils. The lack of inhibition by leupeptin indicates that the enzyme is not a cysteine protease (Umezawa, 1972). The biological activity present in neutrophil supernatants was only weakly diminished by TLCK, showed a concentration-dependent inhibition by α_1-antitrypsin and soyabean trypsin inhibitor and was strongly inhibited by TPCK. The naturally occurring inhibitors, α_1-antitrypsin and soyabean trypsin inhibitor, inhibit both trypsin and chymotrypsin-like activities (Rindler-Ludwig & Braunsteiner, 1975). In contrast, TLCK inactivates trypsin by stoichiometric alkylation of an active-centre histidine (Shaw *et al.*, 1965) and TPCK acts similarly in chymotrypsin (Schoellmann & Shaw, 1963). The results presented here, in conjunction with the earlier observation demonstrating the sensitivity of the enzyme to phenylmethanesulphonyl fluoride (Chignard *et al.*, 1986), indicate that the protease in neutrophil supernatants possesses both reactive serine (Gold & Fahrney,

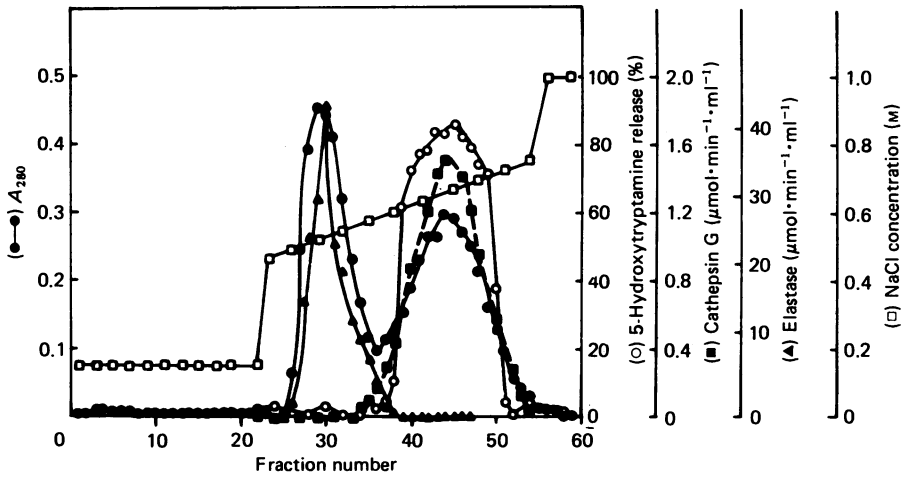

Fig. 2. Carboxymethyl–Sephadex chromatography of neutrophil cathepsin G

Active fractions from the Aprotinin–Sepharose column (39 ml; 7.7 mg of protein) were pooled, dialysed against 0.15 M-NaCl/0.05 M-sodium acetate, pH 5.5, and applied to a carboxymethyl–Sephadex column (1 cm × 2.5 cm), equilibrated with the same buffer. After loading the sample, during which time 2 ml fractions were collected, elastase and cathepsin G were eluted by a linear gradient of 0.46–0.78 M-NaCl and 1 ml fractions were collected. Subsequent to assay, fractions 26–34 and 41–52 containing elastase and cathepsin G, respectively, were pooled, dialysed and concentrated by ultrafiltration. Cathepsin G and elastase were assayed in each of the fractions using benzoyltyrosine ethyl ester and methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide as substrates, respectively. Absorbance at 280 nm (●); 5-hydroxytryptamine release (○); cathepsin G activity (■); elastase activity (▲); NaCl concentration (□).

1964) and histidine (Schoellmann & Shaw, 1963) residues. Furthermore, leupeptin has been shown to competitively inhibit trypsin but not chymotrypsin (Umezawa, 1972). Taken together, these observations indicate that a chymotrypsin-like enzyme was responsible for the platelet activation elicited by neutrophil supernatants. The aggregating and 5-hydroxytryptamine-releasing activity of neutrophil supernatants was almost completely inhibited by 100 μM-Z-Gly-Leu-PheCH_2Cl. Since Z-Gly-Leu-PheCH_2Cl is an active-site-directed irreversible inhibitor specific for neutrophil cathepsin G and chymotrypsin (Powers *et al.*, 1977), these results strongly suggested that the enzyme was cathepsin G.

Corroborating evidence suggesting that the neutrophil protease is cathepsin G was obtained by characterizing its immunological cross-reactivity with specific antisera. Neutrophil supernatant-induced platelet activation was blocked by commercially obtained anti-(cathepsin G) serum but was unaffected by antiserum generated against elastase or α-fetoprotein.

Direct evidence that cathepsin G in supernatants from stimulated neutrophils was responsible for platelet activation was shown by the co-purification of biological and cathepsin G activities. The purified enzyme is free of contaminants as judged by electrophoretic and immunological techniques and substrate specificity studies. Although benzoyl-Tyr-ethyl ester was the substrate used to monitor the elution profile of cathepsin G during the two chromatography steps, samples from each purification step were also assayed using *N*-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. Approximately 3-fold greater specific activities were attained with the nitroanilide due to its similarity to the extended substrate-binding domain of cathepsin G (Nakajima *et al.*, 1979). Cathepsin G prepared in the representative isolation protocol shown here had a specific activity of 9.5 μmol of nitroanilide · min^{-1} · mg^{-1} of protein and was recovered with a 30-fold enrichment and a 40% yield.

The cathepsin G purified from stimulated neutrophil supernatants induced platelet calcium mobilization, secretion and aggregation. Platelet responses were first observed at a cathepsin G concentration of 0.5 μg/ml (17 nM assuming an M_r of 30000) with half-maximal increases in 5-hydroxytryptamine release observed at an enzyme concentration of 3 μg/ml. At cathepsin G concentrations below approx. 10 μg/ml, aspirin pretreatment lowered the extent of platelet calcium mobilization and dense-granule secretion and the further addition of phosphocreatine/creatine kinase further decreased both responses. At higher cathepsin G concentrations, however, aspirin and/or phosphocreatine/creatine kinase had a negligible effect. These observations suggest that arachidonic acid metabolites and ADP can potentiate platelet activation by cathepsin G but that higher enzyme concentrations render platelet responses independent of secondary amplification pathways.

The first demonstration of an effect of cathepsin G on platelets was made by Hallgren & Venge (1976). These investigators observed that purified cathepsin G potentiated agonist-induced platelet 5-hydroxytryptamine release but their enzyme alone failed to exhibit a direct effect on secretion. Purified cathepsin G was subsequently shown to be capable of eliciting platelet aggregation and secretion by Bykowska *et al.* (1983). These authors, however, found that cathepsin G induced platelet aggregation that exhibited a prolonged lag phase and occurred only in the absence of apyrase, suggesting that it was a weak agonist that depended on ADP for its action. The present work with neutrophil supernatant cathepsin G indicates that it is a strong platelet agonist that does not depend on ADP release or thromboxane synthesis for its action.

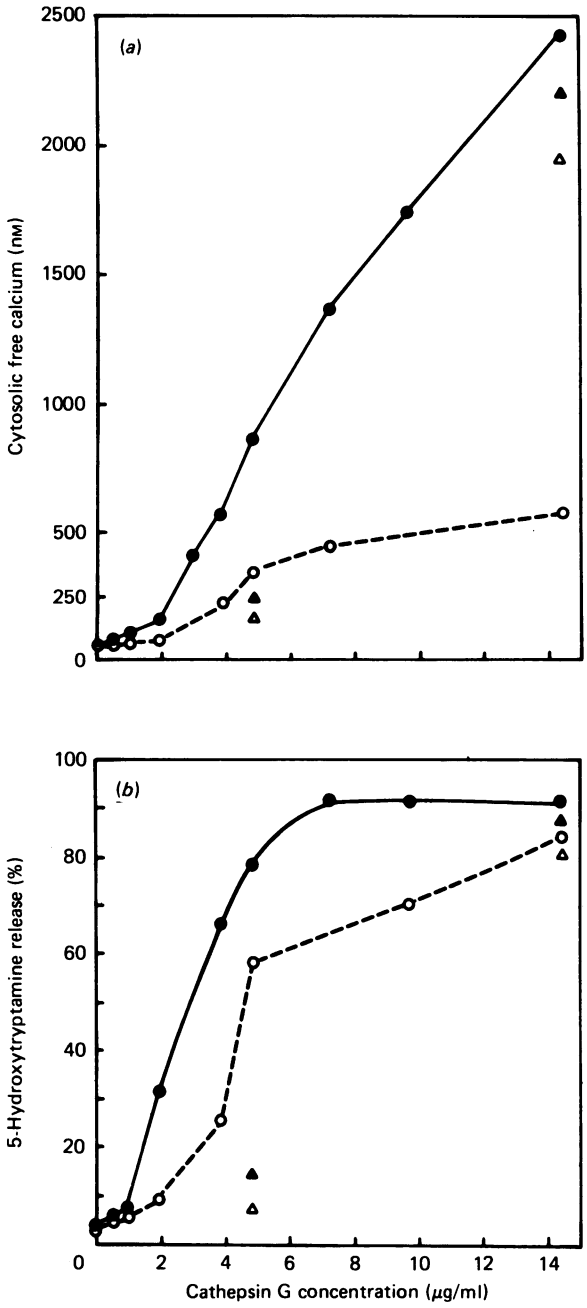

Fig. 3. Effect of cathepsin G on platelet calcium mobilization and secretion

Fura-2- and 5-hydroxy[^{14}C]tryptamine-loaded platelets (2×10^8/ml) were preincubated for 3 min at 37 °C in the presence of either 1.3 mM-$CaCl_2$ (●) or 1 mM-EGTA (○) before addition of the indicated protein concentration of purified neutrophil cathepsin G determined by the method of Lowry *et al.* (1951). Percent 5-hydroxy[^{14}C]tryptamine secretion refers to the extent of release after 3 min and cytosolic free calcium refers to the peak concentration elicited following enzyme addition. Where indicated, platelets were incubated for 30 min in platelet-rich plasma in the presence of 1 mM-aspirin (▲) during fura-2 and 5-hydroxytryptamine loading. Creatine phosphate was used at a final concn. of 20 mM and creatine kinase at 10 units/ml (△) and added during the 3 min preincubation period to aspirin-treated platelets. The values represent the means of at least three different experiments using different donors.

The cathepsin G content of human neutrophils was determined and found to be in the range 1.3–3.8 μg/10^6 cells (Senior & Campbell, 1984; Heck *et al.*, 1986), and this enzyme is known to be discharged under physiological conditions following phagocytosis or cell death (Baggiolini & Dewald, 1985). Secretion of only 10% of the cathepsin G contained in 10^7 neutrophils/ml (i.e. 2.5 μg/ml) would thus be sufficient to cause platelet activation. This calculation, however, may underestimate the actual local concentration resulting from massive infiltration of neutrophils at inflammatory sites even in the presence of naturally occurring antiproteases (Travis & Salvesen, 1983).

Although cathepsin G exhibits a chrymotrypsin-like specificity (Powers *et al.*, 1985), the uniqueness of cathepsin G is underscored by the fact that chymotrypsin is unable to activate platelets (Davey & Luscher, 1967; Bykowska *et al.*, 1983). It is possible that at least one difference between the two enzymes results from (as was recently demonstrated by Salvesen *et al.*, 1987) a difference in the sequence homology of residues lining the primary specificity pocket, a difference that could alter the geometry and thereby affect the substrate specificity of the proteases. In addition to causing direct tissue damage, the present results demonstrate a second mechanism by which cathepsin G can participate in inflammatory diseases, i.e. by recruitment and activation of platelets. The present studies thus expand the possible physiological roles of neutrophil cathepsin G to include that of a strong platelet agonist.

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