The phospholipase C/protein kinase C pathway is involved in cathepsin G-induced human platelet activation: comparison with thrombin

Mustapha SI-TAHAR,*‡, Patricia RENESTO,* Hervé FALET,† Francine RENDU† and Michel CHIGNARD*

*Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur/INSERM U285, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, and †INSERM U428, UFR des Sciences Pharmaceutiques et Biologiques, Université René Descartes, 4, avenue de l'Observatoire, 75006 Paris, France

Cathepsin G, an enzyme released by stimulated polymorphonuclear neutrophils, and thrombin are two human proteinases which potently trigger platelet activation. Unlike thrombin, the mechanisms by which cathepsin G initiates platelet activation have yet to be elucidated. The involvement of the phospholipase C (PLC)/protein kinase C (PKC) pathway in cathepsin G-induced activation was investigated and compared with stimulation by thrombin. Exposure of 5-[¹⁴C]hydroxytryptamine-labelled platelets to cathepsin G, in the presence of acetylsalicylic acid and phosphocreatine/creatine kinase, induced platelet aggregation and degranulation in a concentration-dependent manner (0.1–3.0 μ M). Time-course studies (0–180 s) comparing equivalent concentrations of cathepsin G (3 μ M) and thrombin (0.5 unit/ml) resulted in very similar transient hydrolysis of phosphatidylinositol 4,5-bisphosphate and steady accumulation

of phosphatidic acid. In addition cathepsin G, like thrombin, initiated the production of inositol phosphates. The neutrophil-derived proteinase also induced phosphorylation of both the myosin light chain and pleckstrin, a substrate for PKC, to levels similar to those observed in platelets challenged with thrombin. Inhibition of PKC by GF 109203X, a specific inhibitor, suppressed platelet aggregation and degranulation to the same extent for both proteinases. Using fura 2-loaded platelets, the rise in the cytosolic free Ca²⁺ concentration induced by cathepsin G was shown to result, as for thrombin, from both mobilization of internal stores and Ca²⁺ entry across the plasma membrane. These findings provide evidence that cathepsin G stimulates the PLC/PKC pathway as potently as does thrombin, independently of thromboxane A₂ formation and ADP release, and that this pathway is required for platelet functional responses.

INTRODUCTION

Cathepsin G (EC 3.4.21.20), an enzyme stored in the azurophilic granules of polymorphonuclear neutrophils and released upon stimulation, and thrombin (EC 3.4.21.5) are two human serine proteinases with one main common feature, i.e. their ability to activate platelets [1,2]. While it has been clearly established that the functional catalytic activity of these proteinases is required for platelet activation [1,3], the mechanisms responsible for such activation are better known for thrombin than for cathepsin G. Although cathepsin G exhibits saturable and reversible binding to platelets, a specific receptor has not yet been identified [4]. The thrombin receptor has been isolated and characterized as belonging to the family of G-protein-coupled receptors [5,6]. Its activation requires an unusual mechanism by which thrombin cleaves the receptor to create a new N-terminus domain that functions as a tethered ligand to activate the cells [5]. Selak [7] has shown that cathepsin G and thrombin act through distinct receptors, suggesting that platelets possess a family of proteinase receptors. In this regard, elegant studies have recently described the ability of cathepsin G to cleave, without activating, the platelet and endothelial cell thrombin receptor [8]. Two proteolytic sites were identified: at the known thrombin cleavage site between Arg-41 and Ser-42, and between Phe-55 and Trp-56. Cathepsin G was shown to activate the thrombin receptor only if Phe-55 was mutated to Ala or protected with a targeted antibody. These results suggest that the effects of cathepsin G may not be due to the well described form of thrombin receptor [5,6] but rather to a variant receptor with differences in glycosylation sites or local structural constraints preventing access of cathepsin G to Phe-55–Trp-56.

Over the past few years, detailed information has been obtained concerning the signal transduction induced by thrombin (for reviews, see [9,10]). The mechanisms involve the stimulation of PtdIns(4,5)P₂ breakdown by phospholipase C (PLC), generating two messengers, 1,2-diacylglycerol (DAG) and Ins(1,4,5)P₃ [11]. DAG is subsequently phosphorylated to form phosphatidic acid (PtdOH) [12]. Ins(1,4,5)P₃ mobilizes Ca²⁺ from the dense tubular system, although cytosolic Ca²⁺ is also increased through an agonist-induced transient permeability of the plasma membrane, allowing extracellular Ca²⁺ to enter the cells [13]. DAG acts synergistically with Ca²⁺ to activate protein kinase C (PKC), leading to the phosphorylation of pleckstrin, a 47 kDa protein (P47), which is the main substrate for this kinase in platelets [14–16]. The increase in cytosolic Ca²⁺ also activates Ca²⁺-dependent enzymes such as (i) myosin light chain kinase (MLCK), which phosphorylates the myosin light chain (P20) [17], and (ii) phospholipase A₂, which releases arachidonic acid from membrane phospholipids. The production of free arachidonic acid initiates the synthesis of cyclic endoperoxides, thromboxane A₂ (TxA₂) and prostaglandins through the cyclo-oxygenase pathway [18].

Limited information is available concerning the signal transduction pathways triggered by cathepsin G. Moreover, the data reported are quite controversial. Molino and co-workers [19] failed to detect Ins(1,4,5)P₃ production and reported that cathepsin G induced an increase in cytosolic free Ca²⁺ via a massive influx of extracellular Ca²⁺ across the plasma membrane, probably due to the opening of receptor-operated channels. By contrast, Selak [20] suggested that receptor occupation by cathepsin G stimulates PLC-mediated hydrolysis of phosphoinositides. In addition, platelets stimulated by cathepsin G have

Abbreviations used: PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; Tx, thromboxane; PtdOH, phosphatidic acid; DAG, 1,2-diacylglycerol; InsPs, inositol phosphates; 5-HT, 5-hydroxytryptamine; ASA, acetylsalicylic acid; CP, phosphocreatine; CK, creatine kinase; P20, myosin light chain; P47, pleckstrin; MLCK, myosin light chain kinase.

‡ To whom correspondence should be addressed.

been shown to translocate cytoplasmic PKC to the inner plasma membrane [21].

To obtain a better understanding of cathepsin G-induced platelet activation, the present studies were undertaken to (i) analyse the early biochemical events induced by cathepsin G by monitoring $\text{PtdIns}(4,5)\text{P}_2$ breakdown, formation of PtdOH and inositol phosphates (InsPs) production, as well as the later events by studying profiles of protein phosphorylation; (ii) investigate the contribution of intra- and extracellular Ca^{2+} to cytosolic Ca^{2+} mobilization; and (iii) compare cathepsin G- to thrombin-induced responses in order to determine whether these proteinases share similar transducing mechanisms for platelet activation.

The present study clearly identifies cathepsin G as an agonist which triggers activation of the PLC/PKC pathway to a level comparable with that induced by thrombin. Moreover, this activation is compulsory for platelet response in terms of aggregation and degranulation. Finally, reasons for discrepancies [19,20] in the field of signal transduction induced by cathepsin G are discussed and clarified.

EXPERIMENTAL

Materials and reagents

Blood was obtained from the Centre National de Transfusion Sanguine (Paris, France). Aprotinin, prostacyclin, Hepes, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, phosphocreatine (CP), creatine kinase (CK) and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Imipramine was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Acetylsalicylic acid (ASA) was obtained from Synthelabo (Paris, France). Purified human thrombin was from Diagnostica Stago (Asnières-sur-Seine, France). BSA was purchased from Euromedex (Strasbourg, France). Heparin was from Choay (Paris, France). CNBr-activated Sepharose 4B and CM-Trisacryl M were obtained from Pharmacia (Uppsala, Sweden) and IBF Biotechnics (Villeneuve-la-Garenne, France) respectively. Biodegradable Counting Scintillant, 5- ^{14}C hydroxytryptamine (5- ^{14}C HT) (1.85 MBq/ml), *myo*-[2- ^3H]inositol (37 MBq/ml) and ^{32}P phosphoric acid (370 MBq/ml) were from Amersham International. Glycerol (anhydrous), ammonium peroxodisulphate and isobutanol were purchased from Fluka (Buchs, Switzerland), and other reagents for SDS/PAGE were from Bio-Rad (Hercules, CA, U.S.A.). Oxalate-treated (1%) silica gels were from Merck (Darmstadt, Germany). Fura 2-acetoxymethyl ester was obtained from Calbiochem Corp. (La Jolla, CA, U.S.A.). GF 109203X was kindly donated by Dr. J. Kirilovsky (Glaxo, Les Ulis, France). This compound was dissolved in DMSO (final concentration less than 0.5%, v/v). All other products were of the highest purity grade.

Purification of neutrophil cathepsin G

Cathepsin G was purified from human neutrophils by the method of Baugh and Travis [22], as modified by Martodam et al. [23]. Briefly, leucocytes were washed and erythrocytes were subsequently lysed by hypotonic shock. Neutrophil granules were obtained by disrupting neutrophils by a nitrogen cavitation procedure. Following ultracentrifugation at 30000 *g* for 30 min at 4 °C, the granule pellets were subjected to crushing with an Ultraturax in order to extract the proteinases. After centrifugation at 1000 *g* for 15 min at 4 °C, the cathepsin G-containing supernatants were pooled and submitted to ultracentrifugation at 30000 *g* for 45 min at 4 °C. The resulting supernatant underwent a two-step chromatographic procedure (aprotinin-Sepharose affinity and CM-Trisacryl ion-exchange). Elution was

monitored by spectrophotometric measurement at 280 nm and fractions corresponding to cathepsin G were pooled and stored at -80 °C. The purity of the neutrophil proteinase was assessed by SDS/PAGE. Moreover, it was verified that the cathepsin G preparation was devoid of elastase enzymic activity, a contaminant highly likely to be found. The enzymic activity of purified cathepsin G was determined spectrophotometrically at 410 nm by monitoring the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, a specific substrate, in the presence of increasing amounts of titrated α 1-antitrypsin. Extrapolation of the active-site concentration of cathepsin G was performed using linear regression analysis.

Preparation and labelling of platelets

Blood was obtained from healthy human volunteers and collected in SAG-M solution (composition, mM: NaCl, 187.6; dextrose, 71; adenine, 1950; mannitol, 10.7; pH 5.1). The platelet-rich plasma was isolated by centrifugation at 180 *g* for 20 min and incubated with 5- ^{14}C HT (0.05 mCi/ml) for 30 min at 37 °C. For protein phosphorylation and phospholipid metabolism studies, the platelet-rich plasma was centrifuged (1600 *g*, 10 min) and the resulting pellet was concentrated 10-fold in platelet-rich plasma and incubated with ^{32}P phosphoric acid (0.05 mCi/ml) for 90 min at 37 °C.

Labelled platelets were subsequently washed by centrifugation (1600 *g*, 10 min)/resuspension cycles in Tyrode's buffer (composition, mM: NaCl, 137.0; KCl, 2.68; NaHCO_3 , 11.9; NaH_2PO_4 , 0.42; CaCl_2 , 2.0; MgCl_2 , 1.0; glucose, 5.5; Hepes, 5.0; plus 0.35% BSA, pH 7.4) supplemented with prostacyclin (0.25 μM) and heparin (50 units/ml). Prior to the final centrifugation, the cell pellet was resuspended in the same buffer without heparin. Cells were resuspended in a volume of Tyrode's buffer such that the final platelet concentration was 4×10^8 /ml. The entire procedure was performed at 37 °C and isolated platelets were maintained at this temperature until use.

Aggregation measurements

Platelet aggregation (in 500 μl aliquots) was recorded at 37 °C using a Dual Aggro-Meter (Chrono-Log-Corp., Hevertown, PA, U.S.A.) under constant stirring (1100 rev./min). Samples for aggregation were preincubated without or with ASA (100 μM) or a CP (300 μM)/CK (25 units/ml) system for 5 min and 2 min respectively before stimulation. Activation was initiated by addition of cathepsin G or thrombin and the variations in light transmission were continuously recorded. At the designated times, the 5- ^{14}C HT- or ^{32}P -labelled platelets were transferred to tubes containing 125 μl of a stopping solution of EDTA (77 mM)/NaCl (155 mM)/formaldehyde (33%) (1:9:8, by vol.) or 1.8 ml of ice-cold chloroform/methanol/HCl (12 M)/EDTA (0.1 M) (20:40:1:2, by vol.) respectively to terminate the reaction. Aggregation was expressed as the percentage change in light transmission.

5-HT release measurements

Platelets were incubated with imipramine (1 μM) for 2 min before stimulation to prevent the re-uptake of released 5-HT. Supernatants containing released 5- ^{14}C HT were mixed with scintillation fluid for radioactivity counting (1212 Rackbeta Counter; LKB. Wallac, Stockholm, Sweden). 5- ^{14}C HT release was expressed as a percentage of the total platelet 5- ^{14}C HT content.

Figure 1 Concentration-dependent platelet aggregation induced by (A) cathepsin G and (B) thrombin, in the presence or absence of CP/CK/ASA

Washed human platelets (500 μ l aliquots; 4×10^8 cells/ml) stirred at 37 °C were incubated with or without ASA (100 μ M) and CP (300 μ M)/CK (25 units/ml) for 5 min and 2 min respectively before addition of various concentrations of cathepsin G (A) or thrombin (B). Aggregation was followed for 3 min. Results are expressed as a percentage of maximal light transmission, and are means $\pm$ S.E.M. of four experiments conducted with cells from different donors.

Protein phosphorylation and polyphosphoinositide metabolism

Chloroform/distilled water (0.5 vol. of each) was added to 32 P-labelled platelets diluted in the stopping organic solution (see above). This suspension was vigorously shaken and centrifuged (600 g) for 10 min at 10 °C. The upper aqueous phase was discarded. Proteins, concentrated at the interphase, were solubilized according to the procedure of Laemmli [24] in a mixture containing 2% (w/v) SDS, 0.0625 M Tris, 20% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue under reducing conditions with 5% (v/v) β -mercaptoethanol and heating at 100 °C for 5 min. Radiolabelled proteins were then subjected to SDS/PAGE using a 12.5% resolving gel and a 5% stacking gel. After staining with Coomassie Blue-R250, dried gels were exposed to a Molecular Dynamics (MD; Z. I. Les Bordes, Evry, France) phosphorimaging screen, and the PKC activity was evaluated by quantifying radioactive bands (using an area integration procedure) using the MD phosphorimager and ImageQuant software (version 3.3).

The lower chloroform phase was evaporated, washed according to Jolles et al. [25] and resuspended in chloroform. Phosphoinositides and PtdOH were separated by TLC on an oxalate-treated (1%) silica gel using chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7, by vol.) as the migration solvent. Individual 32 P-labelled phospholipids were located by autoradiography with Kodak X-OMAT AR films, and identified bands were removed by scraping. The amount of radioactivity in each fraction was quantified by liquid scintillation counting.

Measurement of InsPs production

Platelet suspensions were labelled with *myo*-[3 H]inositol (50 μ Ci/ml) for 180 min. Following resuspension in a *myo*-[3 H]inositol-free buffer, platelets were processed as described in [26]. Briefly, in order to inhibit the conversion of InsPs into free inositol, platelets were incubated with 15 mM LiCl for 5 min in the aggregometer cuvette before stimulation for 5 min by cathepsin G or thrombin. Reactions were stopped by transfer of the reaction medium (2 $\times$ 0.5 ml for each sample), using a silicone-treated pipette, to glass tubes containing 1 ml of chloroform/methanol/1 M HCl (50:50:1, by vol.). After vortexing, 0.5 ml of chloroform was added. Samples were centrifuged (800 g, 10 min,

4 °C) and the aqueous upper phase containing [3 H]InsPs was separated on 1 ml columns of Dowex anion-exchange resin 1 $\times$ 8.

Calcium flux measurements

Platelets were prepared as described above, with slight modifications. Following resuspension in Tyrode's buffer supplemented with prostacyclin and heparin, platelets were incubated for 30 min at 37 °C with 3 μ M fura 2. After centrifugation to remove extracellular fura 2 in the presence of prostacyclin, the final platelet concentration was adjusted to 4×10^8 /ml in Tyrode's buffer and platelets were then kept at 20 °C in order to minimize fura 2 leakage from cells. The basal fluorescence of 1 ml aliquots of cell suspension was monitored with a Jobin Yvon JY 3D spectrofluorimeter thermostatted at 37 °C, under agitation. Fluorescence excitation and emission wavelengths were 340 nm and 510 nm respectively. CP/CK/ASA-treated platelets, pre-incubated or not with 5 mM EDTA, were stimulated by cathepsin G or thrombin, and the changes in fluorescence were recorded for 2 min.

Statistics

Results are expressed as means $\pm$ S.E.M. for the indicated number of independently performed experiments. Statistical significance between the different values was analysed by Student's *t* test for unpaired data with a threshold of $P < 0.05$.

RESULTS

Aggregation and 5-HT release

Study as a function of proteinase concentration

Addition of cathepsin G (0.1–3.0 μ M) to washed human platelets initiated a concentration-dependent aggregation (Figure 1A) which was maximal from 0.55 μ M. 5-[14 C]HT release induced by this concentration of proteinase ($86.7 \pm 4.9\%$, $n = 4$) was not significantly different from that observed with 3 μ M cathepsin G ($89.6 \pm 3.7\%$, $n = 4$, $P > 0.05$). In order to delineate the role of the two secondary mediators, cyclic endoperoxides/TxA₂ and ADP, on the measured parameters, experiments were performed in the presence of a cyclo-oxygenase inhibitor, ASA, and an ADP scavenging system, CP/CK. The addition of CP/CK

Figure 2 Time-dependent platelet degranulation induced by cathepsin G (A) and thrombin (B) in the presence or absence of CP/CK/ASA

5-[14 C]HT-labelled platelets were preincubated at 37 °C with or without CP/CK/ASA as indicated in the legend to Figure 1, and stimulated with 3 μ M cathepsin G (A) or 0.5 units/ml thrombin (B). Reactions were stopped at different times and degranulation was evaluated in terms of 5-HT release. Results are expressed as a percentage of the total granule contents, and are means $\pm$ S.E.M. of three or four experiments conducted with cells from different donors.

together with ASA had no effect on maximal platelet aggregation and only very slightly shifted the curve to the right. As shown in Figure 1(B), similar results were obtained when the concentration-dependent aggregation curve was established with thrombin.

Study as a function of time

The time course for 5-HT secretion and subsequently analysed parameters was measured with the optimal concentrations determined from the above study, i.e. 3 μ M cathepsin G and 0.5 unit/ml thrombin. These two concentrations were considered equipotent as deduced from the concentration-dependent aggregation curves depicted in Figure 1. Indeed, 3 μ M cathepsin G and 0.5 unit/ml thrombin are both 10 times higher than the EC_{50} values (i.e. 0.30 ± 0.03 μ M and 0.05 ± 0.02 unit/ml for cathepsin G and thrombin respectively) and 5 times greater than the concentration required to produce maximal aggregation (about 0.55 μ M and 0.1 unit/ml for cathepsin G and thrombin respectively).

Upon addition of 3 μ M cathepsin G, the maximal 5-[14 C]HT release occurred within 30 s (Figure 2A). The addition of CP/CK together with ASA slightly reduced degranulation to 84.5 ± 2.3 % ($n = 4$, $P > 0.05$). Rapid 5-HT secretion was also observed for thrombin, with near-maximal secretion within 30 s (Figure 2B). In addition, 5-HT release was not affected by CP/CK/ASA treatment, since the maximal 5-[14 C]HT release fell from 98.6 ± 0.7 % to 92.6 ± 0.6 % ($n = 3$, $P > 0.05$) in control and CP/CK/ASA conditions respectively.

Although ADP and/or cyclic endoperoxides/TxA₂ did not appear to participate in the aggregation and degranulation of platelets upon cathepsin G challenge, CP/CK/ASA treatment was performed throughout the remaining experiments in order to prevent even discrete amplifying effects induced by these secondary mediators.

Phospholipid metabolism

Figure 3 illustrates the changes in PtdIns(4,5) P_2 and PtdOH as a function of time in response to 3 μ M cathepsin G and 0.5 unit/ml thrombin. The breakdown of PtdIns(4,5) P_2 induced by cathepsin G became maximal within 20 s (18 % of the basal value). This decrease was comparable with the loss of 27 % triggered by

Figure 3 PtdIns(4,5) P_2 hydrolysis and variations in PtdOH in platelets challenged with cathepsin G or thrombin

32 P-labelled platelets were preincubated at 37 °C with CP/CK/ASA as indicated in the legend to Figure 1 and stimulated by 3 μ M cathepsin G (○) or 0.5 unit/ml thrombin (●). Reactions were stopped at different times, and PtdIns(4,5) P_2 and PtdOH were extracted and quantified. Results are expressed as the ratio of radioactivity present in a lipid species at a given time relative to that at time zero. Data are expressed as means $\pm$ S.E.M. of three experiments conducted with cells from different donors.

Figure 4 Time course of protein phosphorylation in platelets challenged with cathepsin G or thrombin

³²P-labelled platelets were preincubated at 37 °C with CP/CK/ASA as indicated in the legend to Figure 1, and stimulated by 3 μM cathepsin G or 0.5 unit/ml thrombin. Reactions were stopped at the indicated times and protein phosphorylation was analysed and quantified after SDS/PAGE as described in the Experimental section. (A) Phosphorimager images of ³²P-labelled proteins in platelets stimulated by cathepsin G and thrombin. Each image is representative of three distinct experiments. (B) Quantification of cathepsin G- and thrombin-induced P47 phosphorylation after the indicated times. Data are expressed as the ratio of radioactivity in P47 at a given time relative to that at time zero. Results are expressed as means ± S.E.M. of three experiments conducted with cells from different donors.

thrombin under the same experimental conditions. Hydrolysis of PtdIns(4,5)P₂ induced by cathepsin G or thrombin was followed by a rapid resynthesis (approx. 50% and 60% increase respectively above the control value at 180 s). The influence of cathepsin G on the formation of InsPs after platelet labelling with *myo*-[³H]inositol was next examined. Addition of cathepsin G increased the amount of InsPs to a level similar to that observed with thrombin (370 and 440% respectively above the control values at 300 s; *n* = 3). Concomitantly, cathepsin G induced a steady accumulation of PtdOH which was as rapid (in less than 10 s) and intense (around 20-fold over control values at 180 s) as the accumulation observed in thrombin-stimulated platelets.

Protein phosphorylation

Time course of P20 and P47 phosphorylation

The patterns of phosphorylation of P20 and P47 induced by 3 μM cathepsin G were very similar to those initiated by 0.5 unit/ml thrombin under the same conditions. P20 phosphorylation was observed within 10 s for cathepsin G, as for thrombin (Figure 4A). Maximum P47 phosphorylation was reached at 20 s after thrombin stimulation (Figure 4B) and was not significantly different from that measured at 120 s (2.6 ± 0.4 and 3.2 ± 0.5 arbitrary units respectively, *n* = 3, *P* > 0.05). Similarly, P47 phosphorylation induced by cathepsin G progressively increased up to 20 s, and then plateaued at a maximal level similar to that reached in platelets challenged with thrombin (Figure 4B).

Effect of a specific inhibitor of PKC

As illustrated in Figure 5, the PKC inhibitor GF 109203X [27] reduced aggregation (Figure 5A) and 5-HT secretion (Figure 5B) induced by cathepsin G or thrombin, in a concentration-dependent manner. Moreover, the inhibition exerted on the neutrophil-proteinase-induced platelet activation was similar to that exerted on thrombin-induced activation. Indeed, in the presence of 15 μM GF 109203X, inhibition of 5-HT release was $85.8 \pm 6.3\%$ and $82.6 \pm 2.8\%$ (*n* = 4, *P* > 0.05) for cathepsin G and thrombin respectively, and inhibition of aggregation was 92.0 ± 4.6 and $92.3 \pm 5.2\%$ (*n* = 4, *P* > 0.05) respectively. As a specific inhibitor of PKC, GF 109203X (7.5 μM) abolished cathepsin G-induced P47 phosphorylation, but P20 phosphorylation was not reduced (Figure 5C).

Measurements of Ca²⁺ movements

Changes in cytosolic Ca²⁺ were measured in platelets incubated with two different concentrations of cathepsin G (0.55 or 3 μM) and compared with those observed with 0.5 unit/ml thrombin (Figure 6). Upon stimulation of platelets with 3 μM of the neutrophil proteinase, an immediate and marked elevation of cytosolic free Ca²⁺ was induced. The profile of the fluorescence peak and its susceptibility to EDTA were similar to those observed with the equipotent concentration of thrombin. Interestingly, the submaximal concentration of cathepsin G (i.e. 0.55 μM, cf. Figure 1) triggered an elevation of cytosolic Ca²⁺ close to that induced by 0.5 unit/ml thrombin. However, pre-

Figure 5 Effect of GF 109203X on platelet activation induced by cathepsin G or thrombin

(A) and (B) Dose–response curves of the effect of GF 109203X on aggregation and 5-[14 C]HT release induced by cathepsin G or thrombin. 5-[14 C]HT-labelled platelets were preincubated at 37 °C with CP/CK/ASA as indicated in the legend to Figure 1 and various concentrations of GF 109203X for 1 min, before stimulation by 3 μ M cathepsin G or 0.5 unit/ml thrombin for 3 min. Aggregation (A) was estimated by the increase in light transmission, and 5-[14 C]HT release (B) was measured in supernatants as described in the Experimental section. Results are expressed as percentage inhibition compared with control experiments performed without the PKC inhibitor but in the presence of 0.5% (v/v) DMSO. Results are expressed as means $\pm$ S.E.M. of four experiments conducted with cells from different donors. (C) Phosphorimager image showing the effect of GF 109203X on cathepsin G-induced protein phosphorylation. 32 P-labelled platelets were preincubated at 37 °C with CP/CK/ASA as indicated in the legend to Figure 1 and with 7.5 μ M GF 109203X for 1 min before stimulation by 3 μ M cathepsin G (CATH. G) for 3 min. Radiolabelled proteins were analysed as described in the Experimental section. NS, non-stimulated cells in the presence of 0.5% (v/v) DMSO; –, cathepsin G in the presence of 0.5% (v/v) DMSO; GF, GF 109203X. This image is representative of three distinct experiments.

Figure 6 Effects of cathepsin G and thrombin on Ca^{2+} movements in platelets

Fura 2-loaded platelets stirred at 37 °C were preincubated with CP/CK/ASA as indicated in the legend to Figure 1, in the presence of either vehicle or 5 mM EDTA for 2 min, prior to stimulation with 0.55 μ M or 3 μ M cathepsin G, or 0.5 unit/ml thrombin. The changes in fluorescence, reflecting changes in Ca^{2+} concentration, were monitored for 2 min. Tracings are representative of three distinct experiments.

incubation of platelets with EDTA severely inhibited the Ca^{2+} rise produced by this concentration of cathepsin G, while responses induced by supramaximal concentrations of thrombin or cathepsin G were only slightly affected.

DISCUSSION

Activation of human platelets by stimulated polymorphonuclear neutrophils was initially shown by Chignard et al. [28] and confirmed by others [29]. Cathepsin G, a serine proteinase stored in the azurophilic granules of neutrophils, has been established as the main mediator of this cell-to-cell interaction [30–32]. However, few attempts have been made to characterize the mechanisms by which cathepsin G initiates platelet activation. The present investigation demonstrates that the interrelated

elements of the PLC/PKC pathway act in concert to mediate such a cell response.

Cathepsin G initiated platelet aggregation and degranulation in a concentration-dependent manner, as also observed with thrombin. In order to rigorously compare the effects of these two stimuli for all measured parameters, experiments were performed using 3 μ M cathepsin G and 0.5 unit/ml thrombin. These concentrations were determined to be equivalent in terms of aggregation ($56.8 \pm 0.5\%$ and $62.4 \pm 3.7\%$ respectively; Figure 1) and degranulation ($89.6 \pm 3.7\%$ and $92.6 \pm 0.6\%$ respectively; Figure 2). More importantly, these concentrations were comparable in terms of potency, since both corresponded to 5 times the concentration required to induce maximal aggregation and 10 times the EC_{50} . The contribution of secondary mediators, i.e. ADP and cyclic endoperoxides/ TxA_2 , released by cathepsin G

activated platelets was evaluated by CP/CK/ASA pretreatment. Under these experimental conditions, aggregation and degranulation were only weakly affected. These data indicate that cathepsin G is a strong platelet agonist whose effects do not depend on cyclic endoperoxide/TxA₂ formation and/or ADP release. However, to prevent even slight consequences of these secondary mediators and to analyse the specific action of cathepsin G, all further experiments were performed in the presence of ASA and CP/CK.

The simultaneous analysis of aggregation, 5-[¹⁴C]HT release and different biochemical events showed that cathepsin G-induced platelet activation occurs through the PLC/PKC pathway. First, this proteinase triggered an early and transient decrease in ³²P-labelled PtdIns(4,5)P₂. In theory, this decline could be the result of PtdIns(4,5)P₂ dephosphorylation, inhibition of PtdInsP phosphorylation, activation of PtdIns(4,5)P₂ hydrolysis or a combination of these reactions. However, in thrombin-stimulated platelets this decrease has been identified to be a result of phosphodiester cleavage of PtdIns(4,5)P₂ into DAG and Ins(1,4,5)P₃ by PLC [33,34]. Thus we assumed that the same pathway operated under cathepsin G activation. Indeed, the fall in PtdIns(4,5)P₂ triggered by this neutrophil proteinase was comparable with that obtained with thrombin under similar experimental conditions (Figure 3 and [35,36]), as was the ensuing relabelling of the phospholipid.

More direct evidence of PLC activation was obtained by the measurement of sustained InsPs accumulation in platelets challenged with cathepsin G. These data indicate a potent activation of PLC by the neutrophil proteinase and thus are inconsistent with the results of Molino and co-workers [19]. While these authors detected Ins(1,4,5)P₃ in thrombin-stimulated platelets, it was not apparent in platelets challenged with cathepsin G, providing no evidence of cathepsin G-dependent PLC stimulation. This apparent discrepancy may be explained by the relatively low concentrations of cathepsin G tested by these authors (up to 0.8 µM). Consequently it is likely that, due to the low activation of PLC, the Ins(1,4,5)P₃ concentration was below the detection limit of the assay. In our hands, 0.55 µM cathepsin G (a submaximal concentration; Figure 1) induced only weak activation of PLC compared with that initiated by 3 µM cathepsin G (a supramaximal concentration; Figure 1) under the same experimental conditions. This feature is reflected by (i) a low degree of breakdown of PtdIns(4,5)P₂ [9 % of the basal value (not shown), compared with 18 % induced by 3 µM cathepsin G (Figure 3)]; (ii) slow and slight PtdOH accumulation, which reached a maximum of 8.2-fold over the control 60 s after stimulation, declining to 5.0-fold at 180 s (results not shown), but increasing steadily with the supramaximal concentration of cathepsin G to 18.3 ± 2.1-fold at 180 s (Figure 3); (iii) the weak DAG-dependent activation of PKC (0.55 µM cathepsin G induced delayed and transient P47 phosphorylation; results not shown) with, however, a maximal level similar to that produced by 3 µM enzyme; and (iv) the small increase in cytosolic Ca²⁺ due to opening of Ins(1,4,5)P₃-sensitive Ca²⁺ intracellular stores [37], as observed in the presence of EDTA (Figure 6). In this case, the lower cytosolic Ca²⁺ increase induced by 3 µM cathepsin G in EDTA buffer did not result from reduced PLC activation. Indeed, neither Ins(1,4,5)P₃ formation (results not shown) nor PtdIns(4,5)P₂ hydrolysis was modified in the presence of 5 mM EDTA [loss of 17 % versus 18 % without EDTA (*n* = 3, *P* > 0.05) respectively]. These results show that PLC activation by cathepsin G does not require the presence of extracellular Ca²⁺. Similarly, the activation of platelet PLC by stimuli such as thrombin [38–40] or collagen [41] was described to be independent of external Ca²⁺, although controversial data have been reported

recently [42]. The reason for this difference remains to be elucidated.

As depicted above, PLC activation in platelets stimulated by cathepsin G was also illustrated by the time-dependent accumulation of PtdOH, generally attributed to the activity of DAG kinase on newly generated DAG. Nonetheless, some studies have demonstrated that PtdOH formation could result from the hydrolysis of phosphatidylcholine by phospholipase D (PLD) [43]. PLD activity can be assessed by the ability of this enzyme to transphosphatidylate phospholipids with ethanol to form phosphatidylethanol. It has been estimated that only 13 % of the PtdOH formed in stimulated platelets is the result of the action of PLD on phosphatidylcholine [44]. In this regard, PLD does not seem to contribute to the generation of PtdOH in response to cathepsin G. Indeed, the formation of phosphatidylethanol was not detected in platelets incubated for 2 min in presence of 0.5 % ethanol before stimulation by 3 µM cathepsin G (results not shown). Taken together, these data demonstrate the ability of cathepsin G to potentially activate PLC.

Two mechanisms have been identified to account for receptor-mediated activation of PLC. One requires G-proteins while the other involves protein tyrosine kinase-mediated phosphorylation of PLCγ, like that triggered by growth factor receptors [45]. Cathepsin G and thrombin share some common characteristics: (i) similar signal transduction pathways (the present paper), and (ii) the maintenance of proteolytic function required to elicit cellular responses [1,3]. In light of these similarities, it is tempting to speculate that, as for thrombin [5,6], cathepsin G-induced PLC activation occurs via a proteolytically activated G-protein-coupled receptor. In this regard, attempts have been made to determine whether cathepsin G and thrombin operate through the same receptor to trigger the platelet response. Selak [7] provided evidence that these proteinases act through distinct receptors by demonstrating that an antibody directed against the thrombin receptor prevents platelet activation by thrombin without inhibiting that induced by cathepsin G. However, more recent studies carried out in Brass's laboratory showed that cathepsin G cleaves the thrombin receptor at at least two different sites: between Phe-55 and Trp-56, deleting the tethered ligand domain, and after Arg-41, thus activating the receptor when the distal site is altered or otherwise protected against proteolysis [8]. Further studies are expected to identify whether the cathepsin G effect on platelets is due to a unique cathepsin G receptor or to a variant thrombin receptor.

We also explored whether cathepsin G might induce the MLCK-dependent phosphorylation of a 20 kDa protein (P20) and the PKC-dependent phosphorylation of P47. The results indicated that the extent of phosphorylation of these two proteins in response to 3 µM cathepsin G was similar to that observed with 0.5 unit/ml thrombin. In order to ascertain whether PKC activation and subsequent P47 phosphorylation were essential for cathepsin G-induced platelet activation, the effect of the selective inhibitor of PKC activity, GF 109203X [27], was investigated. Activation of the PLC/PKC pathway seems to be required for the platelet responses induced by cathepsin G, as it is for the effects of thrombin, since inhibition of P47 phosphorylation led to nearly complete inhibition of platelet aggregation and degranulation. It is of interest to note that 5-HT secretion was more sensitive to PKC inhibition than was platelet aggregation, with maximum inhibition observed with 7.5 µM and 15 µM GF 109203X respectively. This result is consistent with the existence of a close link between PKC activity and the level of platelet secretion [14,46].

Ca²⁺ plays a central role in platelet activation, since it has been established as a key mediator of stimulus-aggregation/

degranulation coupling [13]. Cathepsin G (3 μ M) and 0.5 unit/ml thrombin evoked similar increases in cytosolic free Ca^{2+} in platelets, due both to release of Ca^{2+} sequestered within the dense tubular system and to an increase in Ca^{2+} influx across the plasma membrane. Thus addition of EDTA to platelets prior to activation by cathepsin G reduced, although only moderately, the magnitude of the cytoplasmic Ca^{2+} flux. EGTA was reported to decrease the catalytic activity of this proteinase [19], which is essential for platelet activation. In the present study, EDTA up to 5 mM exerted no inhibitory effect on cathepsin G enzymic activity [change in absorbance of 0.144 ± 0.012 unit/min and 0.146 ± 0.008 unit/min in the absence and presence respectively of EDTA ($n = 6$, $P > 0.05$)]. Therefore the present data are in disagreement with those reported by Molino et al. [19] showing that the main mechanism of cathepsin G-induced increase in Ca^{2+} is a massive entry of this cation from the extracellular medium. Indeed, these authors demonstrated that preventing extracellular Ca^{2+} influx in fura 2-loaded platelets (1×10^8 cells/ml) severely affected the Ca^{2+} transients initiated by 0.1 μ M cathepsin G, unlike the Ca^{2+} mobilization triggered by 0.25 unit/ml thrombin. This concentration of cathepsin G was considered optimal according to aggregation values, TxB_2 formation, 5-HT secretion and the rise in cytosolic Ca^{2+} . However, as no detailed concentration-dependence study was displayed for thrombin, it is difficult to evaluate whether the concentrations of cathepsin G and thrombin were equipotent, this feature being crucial. Thus, as already indicated, 0.55 μ M cathepsin G produced maximal aggregation, substantial 5-HT release and a similar Ca^{2+} mobilization to that triggered by 0.5 unit/ml thrombin, although PLC activation was substantially lower. Under these conditions, and in agreement with Molino's study, preventing entry of external Ca^{2+} dramatically reduced the magnitude of the cathepsin G-induced cytoplasmic rise in Ca^{2+} . In contrast, Ca^{2+} movements produced by the supramaximal concentration of cathepsin G or the equipotent concentration of thrombin were only slightly affected by the presence of EDTA. Consequently, the difference in the requirement for extracellular Ca^{2+} in platelets stimulated by a maximal or submaximal concentration of cathepsin G may result from distinct production of $\text{Ins}(1,4,5)\text{P}_3$ ensuing from activation of the PLC pathway.

In conclusion, the present study clarifies the previous controversy concerning signal transduction induced by cathepsin G, and demonstrates for the first time that this proteinase triggers a strong platelet activation which requires (like that induced by thrombin) an increase in cytosolic Ca^{2+} and activation of PLC and PKC.

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