Cathepsin G binding to human platelets

Evidence for a specific receptor

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We have shown previously that purified human neutrophil cathepsin G is a strong platelet agonist. We now demonstrate that cathepsin G exhibits saturable, reversible binding to human platelets which is characteristic of binding to a specific receptor. At room temperature, cathepsin G displayed apparent positive co-operativity of binding, as indicated by sigmoidal binding curves and a Hill coefficient greater than unity. By contrast, binding curves conducted with native enzyme at 0.5 °C displayed a much smaller degree of sigmoidicity, and binding studies performed with phenylmethanesulphonyl fluoride-treated enzyme at 22–25 °C exhibited hyperbolic binding curves. The concentrations of cathepsin G required to give half-saturation ($S_{0.5}$) with inhibitor-treated enzyme or with native enzyme at either room temperature or 0.5 °C were all similar, suggesting that sigmoidal binding curves did not result from an alteration in the affinity of the binding sites for cathepsin G. However, platelets bound approximately twice as many molecules of native enzyme as molecules of phenylmethanesulphonyl fluoride-treated cathepsin G per cell. From these observations it can be inferred that the apparent positive co-operativity may in part reflect the exposure of binding sites due to the proteolytic activity of cathepsin G. However, this conclusion is not supported by experiments conducted with subsaturating cathepsin G concentrations, which demonstrated that ligand binding did not show an expected increase at longer time intervals. Measurement of Ca^{2+} mobilization and cathepsin G binding in the same platelet suspensions demonstrated that elevations in cytosolic free Ca^{2+} concentration had achieved near-maximal levels in the presence of 15 µg of cathepsin G/ml, whereas maximal binding was observed at approx. 35 µg/ml, indicating that only a fraction of the total binding sites need be occupied to elicit platelet activation. Pretreatment of platelets with forskolin or phorbol 12-myristate 13-acetate (PMA) decreased cathepsin G binding by approx. 60 % and 40 % respectively, indicating that the receptor may be desensitized or down-regulated by phosphorylation due to protein kinases. Since forskolin and PMA could diminish receptor availability by activating negative feedback mechanisms, inhibition of negative signal-transduction pathways could conversely play a role in the up-regulation of cathepsin G binding. In any event, these results show that cathepsin G is an agonist that must bind to platelets to initiate processes associated with cell activation, and suggest a role for cathepsin G in platelet function.

INTRODUCTION

Although thrombin is the most potent known stimulus for platelet activation, the mechanism by which it elicits platelet responses is not completely understood. Stimulus–response coupling appears to require the binding of thrombin to specific receptors and thus resembles an agonist–receptor interaction (Detwiler & Feinman, 1973; Tollefsen *et al.*, 1974; Harmon & Jamieson, 1986; Jandrot-Perrus *et al.*, 1988). On the other hand, thrombin is a proteinase whose interaction with platelets shows characteristics of an enzyme-catalysed reaction (Martin *et al.*, 1975; Detwiler, 1981). We have demonstrated previously that human neutrophil cathepsin G, like thrombin, is a strong platelet agonist that does not depend on either ADP release or thromboxane synthesis for its action (Selak *et al.*, 1988) and is capable of stimulating the secretion of lysosomal enzymes, liberation and metabolism of arachidonic acid and formation of phosphatidic acid (M. A. Selak & J. B. Smith, unpublished work). Because cathepsin G resembles thrombin in

its ability to activate platelets and because both agonists possess enzymic activity, it was of interest to determine if a platelet receptor exists for cathepsin G and, if so, to study the characteristics of binding of the enzyme to its receptor. We have therefore studied the binding of ^{125}I -labelled cathepsin G to platelets by measuring free and bound cathepsin G after separation of platelets by centrifugation through oil. We have observed that cathepsin G exhibits rapid, saturable, reversible binding to platelets, observations characteristic of a specific receptor. We also present data suggesting that the platelet cathepsin G receptor, and hence signal transduction pathways mediated by it, are subject to regulation.

MATERIALS AND METHODS

Materials

Indomethacin, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPPN), methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, benzoyl-Arg-*p*-nitroanilide, trypsin, imi-

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; SAAPPN, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; $S_{0.5}$, concn. required to give half-saturation of binding; B_{max} , maximum binding-site density.

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pramine, phenylmethanesulphonyl fluoride (PMSF) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. 5-Hydroxy[side chain-2- ^{14}C]tryptamine creatinine sulphate, [^{14}C]sucrose and ^{125}I were obtained from Amersham. Dow-Corning silicone oils DC550 and DC200 were purchased from William F. Nye, Inc. (New Bedford, MA, U.S.A.), and Iodo-Gen was purchased from Pierce Chemical Co. Fura-2 acetoxymethyl ester was purchased from Molecular Probes, ZK36374 was from Berlex (a subsidiary of Schering), and forskolin was purchased from Calbiochem.

Purification of cathepsin G and elastase

Human neutrophil cathepsin G and elastase were isolated according to the published methods of Baugh & Travis (1976) and Travis *et al.* (1978) as described previously (Selak *et al.*, 1988) using sequential Aprotinin-Sephadex and carboxymethyl-Sephadex chromatography. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumin as standard, as well as by absorbance at 280 nm.

Preparation of ^{125}I -cathepsin G

Purified cathepsin G was iodinated at 4 °C by an Iodo-Gen procedure followed by gel filtration on a Sephadex G25 column to separate free and protein-bound ^{125}I . Iodination was terminated when the specific radioactivity of the ^{125}I -cathepsin G was approx. 8 $\mu\text{Ci}/\mu\text{g}$. More than 93% of the total radioactivity present in the iodinated cathepsin G was precipitable by 10% trichloroacetic acid. The iodinated product was stored in aliquots at -70 °C and generally used within one month of iodination.

PMSF-inhibited unlabelled and ^{125}I -labelled cathepsin G were prepared by incubating cathepsin G for 90 min at 22 °C in the presence of 1.25 mM-PMSF. After confirming the complete absence of cathepsin G enzymic activity by monitoring the hydrolysis of SAAPPN, free and protein-bound inhibitor were separated by chromatography on a Sephadex G25 column. Column fractions were monitored for the presence of radioactivity by using a gamma counter and for protein by absorbance at 280 nm, and appropriate fractions containing inhibitor-treated enzyme were again assayed for enzymic activity. Inhibitor-treated enzyme was periodically assayed before use in binding assays to confirm that proteolytic activity had not regenerated (Means & Feeney, 1971).

Characteristics of ^{125}I -cathepsin G

Immunoreactivity and biological and enzymic activities were used to evaluate the integrity of ^{125}I -cathepsin G. The immunoreactivity and electrophoretic properties of iodinated cathepsin G were indistinguishable from those of the unlabelled protein. Autoradiography of ^{125}I -cathepsin G following electrophoresis in a non-denaturing acid gel system demonstrated that all four isoenzymes were radiolabelled and comigrated with unlabelled cathepsin G. In a typical iodination, the specific enzyme activity of ^{125}I -cathepsin G for SAAPPN hydrolysis was diminished by approx. 9%. Biological activity, assessed by Ca^{2+} mobilization and dense granule release in fura-2-loaded and 5-hydroxy[^{14}C]tryptamine-labelled platelets, was essentially unchanged. Moreover, no apparent difference was observed between the binding characteristics of native and iodinated cathepsin G when

they were compared using cathepsin G of different specific radioactivities. ^{125}I -Cathepsin G was mixed with unlabelled cathepsin G to obtain solutions of different specific radioactivities, and each solution was used to measure cathepsin G binding. Only if labelled molecules bound in exactly the same way as unlabelled molecules would these solutions show identical binding characteristics.

Preparation of platelets

Human blood was collected into acid/citrate/dextrose from healthy volunteers who had not taken aspirin during the previous 10 days (Aster & Jandl, 1964). Platelet-rich plasma was prepared by centrifugation at 180 g for 15 min. Platelets were collected by centrifugation at 500 g for 20 min and gently resuspended in Hepes-buffered saline composed of 145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 0.5 mM-NaH₂PO₄, 10 mM-glucose, 10 mM-Hepes, pH 7.4, and 5 mg of fatty-acid-free bovine serum albumin/ml. The platelet concentration was adjusted to $2 \times 10^8/\text{ml}$ and the cells were kept at ambient temperature (22–25 °C). In some experiments, 2-fold-concentrated platelet-rich plasma was incubated at 37 °C for 30 min with 3 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 and resuspended at a final concentration of $2 \times 10^8/\text{ml}$ in Hepes-buffered saline with bovine serum albumin.

Binding of cathepsin G to platelets

In a typical binding experiment, 100 μl aliquots of platelets were incubated at 22–25 °C for 5 min with various concentrations of ^{125}I -cathepsin G. Duplicate samples contained a 20–25-fold excess of unlabelled ligand (either native cathepsin G or PMSF-treated cathepsin G) to correct for non-specific binding. All data shown represent specific binding curves obtained after subtraction of non-specific binding from the total bound radioactivity. In time-course experiments, after incubation of appropriately expanded volumes for a specified time, 100 μl aliquots were removed and centrifuged in a Microfuge through 150 μl of a fresh mixture of silicon oils (5 parts DC550/1 part DC200) in microsediment tubes with narrow bore extended tips (Sinha *et al.*, 1984). The tips containing the platelet pellets were cut off and the supernatants and tips were counted for radioactivity separately in a gamma counter. The amount of trapped unbound radioligand in the pellets was estimated using [^{14}C]sucrose and found to represent less than 0.1% of the total radioactivity. In some experiments, platelets were preincubated for 5 min either with 10 μM -forskolin or with 100 ng of PMA/ml. In the assays performed on treated cells, the concentration of inhibitor was maintained during the binding assays.

Fluorescence measurements and estimation of cytosolic Ca^{2+}

Portions of fura-2-loaded platelets in Hepes-buffered saline were routinely used to monitor Ca^{2+} mobilization at ambient temperature in the presence of nominal exogenous Ca^{2+} . Fluorescence measurements were conducted at 22–23 °C in a Perkin-Elmer Model LS-5 fluorimeter using quartz cuvettes. Platelet suspensions were magnetically stirred for 10 s after addition of

cathepsin G and for the final 15 s before removal of aliquots for binding analysis 5 min after adding agonist. 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 Ca^{2+} . Cytosolic free Ca^{2+} concentrations were calculated using a K_d of 224 nM (Grynkiewicz *et al.*, 1985) after correction for extracellular dye.

Measurement of platelet secretion

5-Hydroxy[^{14}C]tryptamine- and fura-2-loaded platelets were collected 5 min after addition of cathepsin G. Secretion was stopped in formaldehyde/EDTA according to the method of Costa & Murphy (1975), and samples of the supernatants were 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.

Measurement of cathepsin G, elastase and trypsin enzymic activity

Cathepsin G, elastase and trypsin activity were measured spectrophotometrically at 37 °C using SAAPPN, methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide and benzoyl-Arg-*p*-nitroanilide respectively as substrates, according to the methods of Nakajima *et al.* (1979) and Erlanger *et al.* (1961). In some cases, the enzymic activity of cathepsin G was also measured at 0.5 °C.

SDS/polyacrylamide-gel and acid-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis (12% gels, non-reduced) was conducted according to the procedure of Laemmli (1970) and acid-gel electrophoresis (15% gels, non-denaturing) was performed as described by Blackshear (1984). Proteins used as calibration standards for SDS/polyacrylamide-gel electrophoresis were obtained from Bethesda Research Laboratories Life Technologies, Inc. and included lysozyme (M_r 14300), β -lactoglobulin (18400), carbonic anhydrase (29000), ovalbumin (43000), bovine serum albumin (68000), phosphorylase *b* (97400) and myosin heavy chain (200000).

RESULTS

Characteristics of cathepsin G binding at 22–25 °C

Initial experiments were conducted to determine if ^{125}I -cathepsin G binds to platelets and to establish the time required for maximum binding. Shown in Fig. 1 is a representative time course for cathepsin G binding to platelets. These experiments established that maximal binding was achieved within 5 min at room temperature (22–25 °C). (Unless otherwise indicated, all subsequent binding assays were conducted at room temperature for 5 min.) The reversibility of cathepsin G binding to platelets was examined by measuring the dissociation of bound ^{125}I -cathepsin G when a high concentration of unlabelled cathepsin G was added to the reaction mixture. The dissociation rate constant (k_{-1} for the representative

Fig. 1. Time course and reversibility of ^{125}I -cathepsin G binding to platelets

Binding was initiated by addition of 25 μg of ^{125}I -cathepsin G/ml to platelets ($2 \times 10^8/\text{ml}$) suspended in Hepes-buffered saline containing 0.5% fatty-acid-free bovine serum albumin and maintained at 23 °C. At 10 min after addition of the radioligand (arrow), 625 μg of unlabelled cathepsin G/ml (○) or an equal volume of buffer (●) was added. Portions of the cell suspension were removed at the times indicated and centrifuged in microsediment tubes through a mixture of silicon oils. The tips containing the platelet pellet were cut and both tips and supernatants were counted separately for radioactivity in a gamma counter.

data shown in Fig. 1) was determined to be 0.1 min^{-1} on addition of a 25-fold excess of unlabelled enzyme to the reaction mixture 10 min after addition of the radiolabelled ligand. These observations suggest that freely dissociable equilibrium binding of cathepsin G to platelets occurs under our experimental conditions. As seen in Fig. 1, the amount of cathepsin G bound in the absence of excess unlabelled ligand showed a tendency to decrease with time. Following a 5 min incubation with platelets, free cathepsin G co-migrated with control enzyme in a non-denaturing acid-gel-electrophoresis system, suggesting that the ligand had undergone no apparent modification or destruction to account for the decline in the binding curves. However, cathepsin G may be altered during longer platelet incubation times, or, alternatively, the decline in the binding curves may reflect a loss of receptor integrity.

Binding of cathepsin G to platelets was measured as a function of ligand concentration. As seen in Fig. 2(a), binding appeared to approach saturation and displayed a pronounced sigmoidicity. Sigmoidal binding curves prohibited a precise estimation of the number of cathepsin G binding sites from Scatchard plot analysis, since such plots yield concave-downward curves. However, extrapolation from the position of the maximum in Scatchard plots to the abscissa at high saturation yielded a value for the $B_{\max}$ (maximum binding site density) of 2.32×10^7 binding sites/platelet. As seen in the Hill plot in Fig. 2(b), the slope for specific binding is greater than 1 ($h = 3.97 \pm 0.58$) and the x -intercept gave an average

Fig. 2. Binding of cathepsin G to platelets at 23 °C as a function of cathepsin G concentration

(a) Effect of cathepsin G concentration on ^{125}I -cathepsin G binding to platelets. Portions of platelets ($2 \times 10^8/\text{ml}$) were incubated for 5 min at 23 °C with the indicated concentrations of cathepsin G followed by centrifugation through a silicone oil barrier as described in the Materials and methods section. (b) Hill plot of cathepsin G binding to platelets at 23 °C.

value for $S_{0.5}$ (the concn. giving half-saturation of binding) of $(663 \pm 159) \times 10^{-7} \text{ M}$ -cathepsin G. Sigmoidal binding curves are characteristic of positive co-operativity, indicating that the first molecule(s) of ligand binds with a lower affinity than the subsequent molecule(s) (Cantor & Schimmel, 1980). In the case where an iodinated ligand binds less tightly than the native protein, Scatchard plots are concave downward (Taylor, 1975). However, as discussed in the Materials and methods section, we were able to exclude ligand heterogeneity as the cause for the pronounced deviation from a simple equilibrium. Sigmoidal binding kinetics might also be observed if new binding sites on the platelet surface are exposed due to the proteolytic activity of cathepsin G.

Fig. 3. Time course of cathepsin G binding at 23 °C as a function of cathepsin G concentration

Binding experiments were conducted as described in the legend to Fig. 1 in the presence of 5 μg of cathepsin G/ml ($\blacktriangle$), 25 μg of cathepsin G/ml ($\bullet$) or 45 μg of cathepsin G/ml ($\circ$).

To test the possibility that the enzymic activity of cathepsin G is responsible for the sigmoidal binding curves, binding studies were conducted for various periods of time with 5 μg , 25 μg or 45 μg of cathepsin G/ml. If this supposition is valid, it would be expected that the binding of native cathepsin G would increase as a function of time when platelets are exposed to a sub-saturating ligand concentration (i.e. 5 $\mu\text{g}/\text{ml}$). However, as shown in Fig. 3, this was observed not to be true. These data indicate that exposure of new binding sites is not a simple kinetic process but one that is also dependent on absolute ligand concentration. This suggests either that a critical threshold of receptors must be occupied before new binding sites are uncovered, or that two different topologically distinct binding sites exist on the platelet for cathepsin G. Alternatively, these observations may suggest that while the cathepsin G receptor is being slowly digested, the rate of hydrolysis is slower than the rate of receptor exposure at this ligand concentration, and therefore binding neither decays nor increases with time.

Binding of PMSF-inhibited cathepsin G

To further address the question of proteolytic exposure of binding sites as the cause for the apparent positive co-operativity, ^{125}I -cathepsin G was rendered inactive by treatment with PMSF, a treatment that totally abolishes platelet Ca^{2+} mobilization, 5-hydroxytryptamine release and aggregation. When binding studies were carried out using PMSF-treated ^{125}I -cathepsin G and compared with native radioligand, the inhibited form exhibited diminished binding with hyperbolic binding curves (Fig. 4a). Dissociation studies demonstrated that native cathepsin G could displace PMSF-treated cathepsin G bound

Fig. 4. Binding of PMSF-treated cathepsin G to platelets: comparison with native enzyme

(a) Effect of PMSF-treated cathepsin G concentration on binding of PMSF-treated ^{125}I -cathepsin G (○) to platelets. Binding experiments were conducted as described in the legend to Fig. 2, except excess unlabelled PMSF-treated cathepsin G was used to assess non-specific binding. Shown for comparison is the binding curve obtained using native ^{125}I -cathepsin G (●) with autologous platelets. The binding curves are the means of three different experiments conducted with different donors. Native and PMSF-treated radioligands were used at similar specific activities. (b) Time course of PMSF-treated cathepsin G binding to platelets and reversibility by native cathepsin G. Binding was initiated by addition of $25 \mu\text{g}$ of PMSF-treated ^{125}I -cathepsin G/ml. At 10 min after addition of the radioligand, $325 \mu\text{g}$ of unlabelled native cathepsin G/ml was added (arrow).

to platelets (Fig. 4b), signifying that native and PMSF-treated cathepsin G can bind to the same sites on the platelet. Scatchard (1949) analysis yielded a straight line, indicating that PMSF-treated cathepsin G binds to a single class of identical and independent sites. A single non-interacting site was also suggested by a value of 1.07 ± 0.1 for the Hill coefficient. Hill plots gave an average value of $(592 \pm 96) \times 10^{-7} \text{ M}$ for $S_{0.5}$ and Scatchard plots gave a value for B_{max} of 0.91×10^7 molecules of PMSF-treated cathepsin G bound/platelet. Although the binding of PMSF-treated ^{125}I -cathepsin G was hyperbolic and was diminished relative to that of the

native enzyme, the binding curves were shifted to the left relative to those of the uninhibited enzyme at low ligand concentrations, suggesting that the PMSF-inhibited enzyme bound to platelets with a higher affinity than did the native form (Figs. 2 and 3a).

To ascertain if PMSF treatment of cathepsin G had altered the conformation and thereby the affinity of the ligand, native ^{125}I -cathepsin G and PMSF-treated ^{125}I -cathepsin G were compared for their susceptibility to trypsin digestion. Portions of the tryptic digests were subjected to SDS and non-denaturing acid polyacrylamide-gel electrophoresis followed by autoradiography. [A similar approach has been used by Staatz *et al.* (1988) to ascertain if different conformations result when platelet glycoproteins Ia and IIa form complexes with either calcium or magnesium.] Interestingly, PMSF-treated cathepsin G was found to be more resistant to trypsin digestion than was native enzyme, indicating that differences exist between the native and inhibitor-treated forms of the enzyme that may account for the observed binding curves (results not shown). A trivial explanation for this finding is that trypsin is inhibited by non-protein-bound PMSF trapped within a hydrophobic pocket of inhibitor-treated cathepsin G. This explanation cannot be entirely excluded, but seems unlikely for the following reasons. First, ongoing substrate (SAAPPN) hydrolysis by native cathepsin G was totally unaffected by addition of PMSF-treated cathepsin G. Secondly, PMSF-treated cathepsin G had no effect on the enzymic activity of trypsin as measured with a synthetic trypsin substrate (benzoyl-Arg-p-nitroanilide). Both of these observations suggest that our preparations of active-site-inhibited cathepsin G lack free PMSF.

Fig. 5. Binding of cathepsin G to platelets at 0.5 °C as a function of cathepsin G concentration: comparison with binding at 23 °C

Experiments were conducted as described in the legend to Fig. 2 after temperature equilibration of platelets to 0.5 °C (○). Shown for comparison is the 23 °C binding curve (●) conducted with autologous platelets. The values shown are the means of three different experiments conducted with different donors.

Binding of native cathepsin G at 0.5 °C

To circumvent potential differences between PMSF-inhibited and native enzyme, binding studies were also conducted in an ice bath at 0.5 °C (actual temperature of binding assay) using native enzyme and compared with ligand binding at 23 °C in autologous platelets. As seen in Fig. 5, substantially less native cathepsin G bound to platelets at 0.5 °C than at 23 °C, and the curves were much less obviously sigmoidal. Hill plots of the low-temperature binding data revealed an $S_{0.5}$ of $(858 \pm 161) \times 10^{-7}$ M and a Hill coefficient of 1.93 ± 0.47 . The value of h is lower than that observed at 23 °C, indicating a lower degree of apparent co-operativity. That any co-operativity remains probably reflects the fact that cathepsin G exhibits a very low but nonetheless significant enzymic activity at 0.5 °C. At 37 °C, cathepsin G hydrolysed 11.6 μmol of SAAPPN/min per mg, whereas at 0.5 °C it exhibited a specific activity of 0.186 $\mu\text{mol}/\text{min}$ per mg.

Cathepsin G receptor occupation and platelet responses

The extent of ^{125}I -cathepsin G binding and the stimulation of changes in cytosolic free Ca^{2+} were used to correlate the relationship between binding and platelet activation. As seen in Fig. 6, near-maximal Ca^{2+} mobilization was achieved at 15 μg of cathepsin G/ml, and maximal radioligand binding was observed at approx. 35 $\mu\text{g}/\text{ml}$.

Fig. 6. Correlation of cathepsin G binding with platelet functional changes

Cathepsin G binding and Ca^{2+} mobilization are shown as a function of cathepsin G concentration. Fura-2-loaded platelets were stimulated by addition of the indicated concentrations of ^{125}I -cathepsin G at 22 °C in the presence of nominal exogenous Ca^{2+} . Fluorescence changes (●) were monitored continuously and cathepsin G binding (○) was determined 5 min after agonist addition following centrifugation of an aliquot of the platelet suspensions through a silicone oil barrier as described in the Materials and methods section. The results shown are representative of three experiments conducted with different donors.

Fig. 7. Effect of forskolin or PMA on cathepsin G binding to platelets

Platelets were preincubated for 5 min at 22–24 °C with either 10 μM -forskolin (▲) or 100 ng of PMA/ml (○) before initiating binding studies as described in the legend to Fig. 2. Control platelets (●) were untreated. The binding curves are the means of four experiments conducted with different donors.

Effect of forskolin and PMA on cathepsin G binding

Agents which increase cyclic AMP and promote the activity of cyclic AMP-dependent protein kinase inhibit platelet responses to a wide range of agonists (Haslam, 1987). We have observed that cathepsin G-induced platelet stimulation is potentiated by adrenaline, an inhibitor of platelet adenylate cyclase, and is inhibited by the prostacyclin analogue ZK36374, an activator of platelet adenylate cyclase (results not shown). PMA also inhibits signal generation by agonists when preincubated with platelets, suggesting that the activation of protein kinase C could have important negative feedback effects on platelet function. The effect of activation of protein kinase A or protein kinase C on cathepsin G binding was thus examined. As seen in Fig. 7, pretreatment of platelets with forskolin under conditions known to elevate cyclic AMP (Daly, 1984) decreased the overall binding of ^{125}I -cathepsin G by over 60 %. Similarly, preincubation of platelets with PMA, a direct activator of Ca^{2+} -activated phospholipid-dependent protein kinase C, decreased cathepsin G binding by approx. 40 %.

DISCUSSION

The results of the present study demonstrate that neutrophil cathepsin G exhibits specific, saturable, reversible binding to platelets which is characteristic of binding to a distinct receptor. Binding of native cathepsin G at 22–25 °C exhibited sigmoidal binding, concave-downward Scatchard plots and a Hill coefficient of 4. The binding of PMSF-inhibited enzyme at room tem-

perature was hyperbolic, whereas the binding curves of native enzyme at 0.5 °C exhibited decreased sigmoidicity, suggesting that enzymic activity is required for the observed positive co-operativity. Although the concentrations of cathepsin G required to give half saturation estimated from binding data obtained with native enzyme at either 23 °C or 0.5 °C or with PMSF-treated enzyme at room temperature were all similar, the data do suggest that the inhibitor-treated ligand differed from the native enzyme. On the other hand, analysis of the data showed that human platelets bind approx. 10^7 molecules of PMSF-treated cathepsin G/cell and approx. 2×10^7 molecules of native enzyme/platelet. The similarities in $S_{0.5}$ values and the large differences in $B_{\max}$ imply that sigmoidal binding may be a consequence of proteolytic exposure of additional binding sites rather than a decreased affinity of the binding sites for cathepsin G. Exposure of sites by proteolysis would be expected to occur even at low concentrations of cathepsin G in a time-dependent manner. However, binding studies conducted with subsaturating concentrations of cathepsin G for variable periods of time showed that binding did not increase, suggesting that a critical threshold of receptors must be occupied before new binding sites are uncovered.

The binding of cathepsin G and the stimulation of Ca^{2+} mobilization in fura-2-loaded platelets were monitored in the same platelet samples to correlate receptor occupation with a functional change. The elevation in cytosolic free Ca^{2+} had achieved near-maximal levels by 15 μg of cathepsin G/ml, whereas maximal binding did not occur until approx. 35 μg /ml. These observations suggest that only a fraction of the total cathepsin G receptors need to be occupied for platelet activation to occur.

Treatment of platelets with forskolin or with PMA was found to decrease overall cathepsin G binding by approx. 60% and 40% respectively. A major effect of cyclic AMP seems to be the suppression of generation of second messengers, e.g. cyclic AMP inhibits agonist-induced hydrolysis of inositol phospholipids (Rittenhouse & Sasson, 1985) as well as the rise in cytosolic Ca^{2+} (MacIntyre *et al.*, 1985). More recently, Lerea *et al.* (1987) have observed that agents which elevate platelet cyclic AMP decrease thrombin binding. Although the mechanism by which cyclic AMP exerts this effect is unknown, these investigators suggest that increased levels of cyclic AMP might lead to a direct modification of the receptor or might alter its interaction with a protein(s) that regulates it. Treatment with phorbol ester has been shown to reduce the amount of inositol 1,4,5-trisphosphate formed (Rittenhouse & Sasson, 1985) and to inhibit the elevation in cytosolic Ca^{2+} and phosphatidic acid formation (MacIntyre *et al.*, 1985) on agonist stimulation of platelets, suggesting that protein kinase C activation can exert negative feedback effects on platelet function. Although the mechanism by which cyclic AMP and PMA attenuate cathepsin G binding remains to be elucidated, the present results suggest that activation of either protein kinase A or protein kinase C may desensitize or down-regulate the cathepsin G receptor by phosphorylation and perhaps by internalization. In this regard, receptor phosphorylation in many different cell systems has been shown to be associated with a decrease in receptor function and distribution (Sibley *et al.*, 1988). Our observations suggest that the cathepsin G receptor, and hence signal transduction pathways mediated by it,

are subject to regulation. Whereas both kinases could alter platelet responsiveness to cathepsin G at numerous stages, data shown here demonstrate that a primary effect may be exerted at the receptor level.

Having established that the binding of cathepsin G to platelets exhibits characteristics indicative of a distinct receptor, we have attempted to determine the mechanism responsible for the apparent positive co-operativity of binding. Although our data show that cathepsin G appears to up-regulate its own binding to human platelets, from the data we are unable to define clearly the mechanism by which this up-regulation occurs. Proteolytic exposure of additional binding sites would appear to be the simplest explanation for the apparent positive co-operativity of cathepsin G binding; however, this conclusion is incompatible with the failure to observe increased binding of cathepsin G after longer time intervals in the presence of low ligand concentrations. The latter observation suggests that a critical threshold of cathepsin G receptors must be occupied before up-regulation is observed, and further suggests that cathepsin G can function as a homotropic effector to regulate its own binding. Since PMA and forskolin diminish receptor availability by activating negative feedback mechanisms, inhibition of negative signal transduction pathways could conversely result in up-regulation of cathepsin G binding. However, modulation of cathepsin G binding by protein kinase A or protein kinase C cannot solely account for up-regulation, since neither PMA nor forskolin pretreatment of platelets completely eliminated the sigmoidal binding curves.

As a serine proteinase, cathepsin G has esterolytic and amidolytic activities, either or both of which may play a role in platelet activation. Although cathepsin G exhibits chymotrypsin-like specificity (Powers *et al.*, 1985), it differs from chymotrypsin in that it can activate platelets whereas chymotrypsin cannot (Davy & Luscher, 1967). The difference in the ability to activate platelets may be related to the sequence of residues lining the substrate specificity pocket as well as to the absence of the disulphide bond linking residues Cys-191 and Cys-220 (Salvesen *et al.*, 1987). These changes may alter the geometry of the binding pocket and affect the substrate specificity (Woodbury *et al.*, 1978). Glycine-226 in chymotrypsin has been replaced by a glutamyl residue in cathepsin G and this alteration, along with the absence of the disulphide bridge, may confer trypsin-like properties on cathepsin G, and the similarity to trypsin may account for the ability of cathepsin G to activate platelets. Since cathepsin G resembles thrombin and trypsin in its ability to activate platelets, and since cathepsin G and thrombin are both strong platelet agonists that are also proteinases, it will be of interest to determine how their respective target proteins on the platelet membrane differ. We believe that the demonstration that cathepsin G is a strong platelet agonist that binds to specific receptors provides an insight into the potential physiological role of this neutral proteinase and has important implications for the understanding of both thrombosis and the critical events surrounding the initiation and propagation of the inflammatory response.

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REFERENCES

- Aster, R. H. & Jandl, J. H. (1964) *J. Clin. Invest.* **43**, 834–855
- Baugh, R. J. & Travis, J. (1976) *Biochemistry* **15**, 836–841
- Blackshear, P. J. (1984) *Methods Enzymol.* **104**, 237–255
- Cantor, C. R. & Schimmel, P. R. (1980) *Biophysical Chemistry Part III: The Behavior of Biological Macromolecules*, W. H. Freeman, San Francisco
- Costa, J. L. & Murphy, D. L. (1975) *Nature (London)* **255**, 407–408
- Daly, J. W. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 81–89
- Davy, M. G. & Luscher, E. F. (1967) *Nature (London)* **216**, 857–858
- Detwiler, T. C. (1981) *Ann. N.Y. Acad. Sci.* **370**, 67–71
- Detwiler, T. C. & Feinman, R. D. (1973) *Biochemistry* **12**, 282–289
- Erlanger, B. F., Kowosky, N. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271–278
- Gryniewicz, G., Poenie, M. & Tsein, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Harmon, J. T. & Jamieson, G. A. (1986) *J. Biol. Chem.* **261**, 13224–13229
- Haslam, R. J. (1987) in *Thrombosis and Haemostasis* (Verstraete, M., Vermeylen, J., Lijnen, H. R. & Arnout, J., eds.), pp. 147–174, International Society on Thrombosis and Haemostasis and Leuven University Press, Leuven
- Jandrot-Perrus, M., Didry, D., Guillin, M. C. & Nurden, A. T. (1988) *Eur. J. Biochem.* **174**, 359–367
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lerea, K. M., Glomset, J. A. & Krebs, E. G. (1987) *J. Biol. Chem.* **262**, 282–288
- MacIntyre, D. E., McNicol, A. & Drummond, A. H. (1985) *FEBS Lett.* **180**, 160–164
- Martin, B. M., Feinman, R. D. & Detwiler, T. C. (1975) *Biochemistry* **14**, 1308–1314
- Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, Inc., San Francisco
- Nakajima, K., Powers, J. C., Ashe, B. M. & Zimmerman, M. (1979) *J. Biol. Chem.* **254**, 4027–4031
- Powers, J. C., Tanaka, T., Harper, J. W., Minematsu, Y., Barker, L., Lincoln, D., Crumley, K. V., Fraki, J. E., Schechter, N. M., Lazarus, G. G., Nakajima, K., Nakashino, K., Neurath, H. & Woodbury, R. G. (1985) *Biochemistry* **24**, 2048–2058
- Rittenhouse, S. E. & Sasson, J. P. (1985) *J. Biol. Chem.* **260**, 8657–8660
- Salvesen, G., Farley, D., Shuman, J., Przbyla, A., Reilly, C. & Travis, J. (1987) *Biochemistry* **26**, 2289–2293
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Selak, M. A., Chignard, M. & Smith, J. B. (1988) *Biochem. J.* **251**, 293–299
- Sibley, D. R., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1988) *Endocr. Rev.* **9**, 38–56
- Sinha, D., Seaman, F. S., Koshy, A., Knight, L. C. & Walsh, P. N. (1984) *J. Clin. Invest.* **73**, 1550–1556
- Staatz, W. D., Peters, K. J. & Santoro, S. A. (1988) *J. Cell Biol.* **197**, 148a
- Taylor, S. I. (1975) *Biochemistry* **14**, 2357–2361
- Tollefsen, D. M., Feagler, J. R. & Magerus, P. W. (1974) *J. Biol. Chem.* **249**, 2646–2651
- Travis, J., Bowen, J. & Baugh, R. (1978) *Biochemistry* **17**, 5651–5656
- Woodbury, R. G., Katunmuna, N., Kobayashi, K., Titani, K. & Neurath, H. (1978) *Biochemistry* **17**, 811–819

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