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# Intracellular $Ca^{2+}$ rise in human platelets induced by polymorphonuclear-leucocyte-derived cathepsin G

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Cathepsin G, a serine protease released by polymorphonuclear-leucocyte azurophilic granules upon stimulation, activates human platelets, inducing an increase in intra-platelet $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner (50–200 nM). The $[Ca^{2+}]_i$ rises elicited by low (50–80 nM) cathepsin G concentrations in fura-2-loaded platelets showed a biphasic mode, with a first small peak followed by a greater and more prolonged $Ca^{2+}$ transient. Higher (100–200 nM) cathepsin G concentrations induced a monophasic increase in intracellular $Ca^{2+}$. Acetylsalicylic acid, nordihydroguaiaretic acid and ketanserin did not affect platelet activation by cathepsin G, whereas the ADP-scavenger system phosphocreatine/creatine kinase significantly decreased $Ca^{2+}$ mobilization, platelet aggregation and 5-hydroxytryptamine secretion by cathepsin G. Preventing cathepsin G-induced platelet aggregation with the synthetic peptide RGDSP (Arg-Gly-Asp-Ser-Pro) did not significantly affect cathepsin G-induced $Ca^{2+}$ transients. $Ni^{2+}$ (4 mM), a bivalent-cation-channel inhibitor, decreased the cathepsin G-induced fluorescence rise by more than 90%. This effect was reversed by either decreasing $Ni^{2+}$ or increasing cathepsin G concentration. Preventing $Ca^{2+}$ influx across the plasma membrane with 4 mM-EGTA totally abolished $Ca^{2+}$ transients. However, EGTA also strongly decreased catalytic activity of cathepsin G, which is essential for platelet activation. Evidence of a rapid and sustained bivalent-cation channel opening in the platelet membrane was obtained by adding $Mn^{2+}$ to the platelet suspension 30 s or 3 min after cathepsin G. No accumulation of $InsP_3$ could be detected when platelets were stimulated with cathepsin G. All these data indicate that cathepsin G induces a $[Ca^{2+}]_i$ increase mainly through an influx across the plasma membrane. This massive $Ca^{2+}$ entry is probably due to opening of receptor-operated channels and is amplified by endogenous ADP release.

## INTRODUCTION

Previous reports [1,2] demonstrated that activation of polymorphonuclear leucocytes (PMN) with *N*-formylmethionyl-leucyl-phenylalanine resulted in a stimulation of platelets in mixed cell suspensions and that cathepsin G, a serine protease released by activated PMN, is responsible for stimulation of platelet function [3,4]. Cathepsin G was able to induce platelet aggregation, increase in cytoplasmic $Ca^{2+}$, and 5-hydroxytryptamine (5-HT) release [3,4]. A specific platelet receptor for cathepsin G has been described [5]. Recently our group [4] reported that cathepsin G released in a microenvironment of PMN–platelet suspension may be protected from different anti-proteinases. These facts suggest that cathepsin G released from activated PMN plays a crucial role in the local recruitment and stimulation of platelets during pathological processes characterized by PMN accumulation and activation [6]. The mechanism(s) of platelet activation by cathepsin G, however, are still unclear. The present study was undertaken to characterize $Ca^{2+}$ transients induced in human platelets by cathepsin G. In some experiments thrombin was also used in parallel.

We report here that purified cathepsin G, in the range of concentrations comparable with those released from activated PMN, induced a $Ni^{2+}$-inhibitable entry of $Ca^{2+}$ and $Mn^{2+}$ in fura-2-loaded platelets. Cathepsin G, at the concentrations used, only induced a very limited mobilization of $Ca^{2+}$ from internal stores, but no apparent stimulation of phosphatidylinositol metabolism.

## EXPERIMENTAL

### Chemicals

Fura-2 acetoxymethyl ester (fura-2-AM), Hepes, EGTA, prostaglandin $E_1$ ($PGE_1$), phosphocreatine, creatine kinase, the cathepsin G-specific chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, 5-HT, nordihydroguaiaretic acid and human thrombin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cathepsin G purified from human neutrophils and BAPTA (tetrasodium salt) were from Calbiochem (San Diego, CA, U.S.A.). The thrombin-specific chromogenic substrate tosyl-Gly-Pro-Arg *p*-nitroanilide was obtained from Boeringer Mannheim (Milano, Italy). Acetylsalicylic acid (lysine salt; Flectadol) was provided by Maggioni Winthrop, Milano, Italy. The synthetic peptide RGDSP (Arg-Gly-Asp-Ser-Pro) was obtained from Novabiochem (Switzerland). *myo*-[2-$^3$H]Inositol was from Amersham International. AG 1-X8 anion-exchange resin (200–400 mesh; formate form) was from Bio-Rad. Other chemicals were of reagent grade or higher quality.

### Platelet isolation

Human venous blood was drawn by venipuncture from adult volunteers who had not taken any medication for at least 10 days, and mixed with sodium citrate (3.8%, w/v) in the ratio of 9 vol. of blood to 1 vol. of anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 250 *g* for 15 min at room temperature.

Abbreviations used: fura-2 AM, fura-2 acetoxymethyl ester; BAPTA, bis-(*o*-aminophenoxy)-ethane-*NNN'N'*-tetra-acetic acid; $PGE_1$, prostaglandin $E_1$; RGDSP, Arg-Gly-Asp-Ser-Pro; $[Ca^{2+}]_i$, intra-platelet $Ca^{2+}$ concentration; PMN, polymorphonuclear leucocyte; $t_{max.}$, time to reach the maximum; 5-HT, 5-hydroxytryptamine (serotonin); $TxB_2$, thromboxane $B_2$.

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### Fura-2-AM loading

Platelets were loaded with the fluorophore fura-2 as described [7]. Briefly, platelet-rich plasma was centrifuged at 1100 *g* for 15 min and the platelets were resuspended in $Ca^{2+}$-free Hepes/Tyrode buffer (pH 7.4) containing 1 $\mu$M-$PGE_1$ and 1 mM-EGTA. The cell count was adjusted to $3 \times 10^8$/ml, to achieve an optimal loading. Washed platelets were mixed with 1 $\mu$M fura-2-AM and incubated at 37 °C for 45 min. Then 1 $\mu$M-$PGE_1$ and 1 mM-EGTA were added before centrifugation for 15 min at 1100 *g*. Platelets were finally resuspended at $2 \times 10^8$/ml in Hepes/Tyrode buffer: 1 mM-$CaCl_2$, 1–4 mM-EGTA or 1–4 mM-$NiCl_2$ were added to platelet suspensions as specified.

### Measurement of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$)

Fura-2-loaded platelet fluorescence was measured at 37 °C in a Perkin–Elmer LS-5B fluorescence spectrophotometer; monochromator settings of 340 nm (excitation) and 500 nm (emission) were used. A 1.5 ml portion of cell suspension ($1 \times 10^8$ platelets/ml) was preincubated in the presence of 1 mM-$CaCl_2$ at 37 °C for 2 min before addition of the agonist under continuous stirring. $[Ca^{2+}]_i$ was calculated from the equation:

$$[Ca^{2+}]_i = K_d[F - F_{min.})/(F_{max.} - F)]$$

where $F_{max.}$ and $F_{min.}$ are the fluorescence values at saturating and zero $Ca^{2+}$ concentrations respectively; $F_{max.}$ was achieved by lysing the cells with 50 $\mu$M-digitonin in the presence of 1 mM-$Ca^{2+}$; $F_{min.}$ was obtained by exposing the lysed platelets to 12 mM-EGTA after adjusting the pH to 8.5 with 20 mM-Tris base. $F$ is the observed fluorescence under resting conditions, and $K_d$ represents the $Ca^{2+}$-binding dissociation constant (224 nM for fura-2).

### Measurement of [$^3$H]inositol phosphates

Platelets were resuspended in Hepes/Tyrode buffer containing 1 mM-EGTA and *myo*-[2-$^3$H]inositol (33 $\mu$Ci/ml) and incubated at 37 °C for 3 h. They were then washed in Hepes/Tyrode buffer with the addition of 1 mM-EGTA, 1 $\mu$M-$PGE_1$ and 10 mM-LiCl, and finally resuspended ($8 \times 10^8$ cells/ml) in Hepes/Tyrode buffer containing 10 mM-LiCl and 1 mM-$CaCl_2$. The cell suspension (0.5 ml) was placed in aggregometer cuvettes and stirred for 1 min at 37 °C before adding the stimulus. The reaction was stopped with an equivalent volume of ice-cold trichloroacetic acid (15 %, w/v) and samples were centrifuged (7000 *g* for 3 min). The supernatants were then extracted three times with diethyl ether, neutralized with sodium tetraborate and resuspended in 3 ml of distilled water. Samples were applied to AG 1-X8 anion-exchange columns. The columns were washed with $5 \times 2$ ml of distilled water, and the phosphate esters were eluted by stepwise addition of: (A) 5 mM-sodium tetraborate/60 mM-sodium formate; (B) 0.1 M-formic acid/0.2 M-ammonium formate; (C) 0.1 M-formic acid/0.5 M-ammonium formate; (D) 0.1 M-formic acid/1.0 M-ammonium formate. Fractions (2 ml) were collected and radioactivity was determined by scintillation counting.

### Proteolytic-activity measurements

Cathepsin G catalytic activity was measured with the specific chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide. Thrombin catalytic activity was monitored with the specific chromogenic substrate Tos-Gly-Pro-Arg *p*-nitroanilide. Absorbance changes were measured at 410 and 405 nm wavelength respectively, in a Uvikon 860 (Kontron Instruments) spectrophotometer.

### Measurement of 5-HT secretion and thromboxane (Tx) $B_2$ production

5-HT released by activated platelets was measured by h.p.l.c. by using a coulometric detector, as described in [8]. Values were expressed as percentages of total content measured in the supernatant of $10^8$ sonicated platelets. Basal secretion caused by stirring was subtracted.

$TxB_2$ was quantified by radioimmunoassay, as described in [9], and reported as pmol/ml of incubation.

## RESULTS AND DISCUSSION

### $Ca^{2+}$ mobilization in platelets induced by cathepsin G

Purified PMN-derived cathepsin G added to human fura-2-loaded platelets induced a rise in $[Ca^{2+}]_i$ in a concentration-dependent fashion. The range of cathepsin G concentrations (50–200 nM) was similar to that released from activated PMN [3,4,10]. $Ca^{2+}$ transients rose from the resting values of $204.6 \pm 18.0$ nM to $235 \pm 30$, $806 \pm 88$ and $2010 \pm 322$ nM at cathepsin G concentrations of 50, 100 and 200 nM respectively (means ± S.E.M., $n = 6$–20). $TxB_2$ production and 5-HT secretion occurred in parallel to $Ca^{2+}$ mobilization, reaching maximal values of $101.4 \pm 17.8$ pmol/ml and $46.35 \pm 5.34$ % of control respectively at 200 nM-cathepsin G. Irreversible platelet aggregation was recorded at concentrations of cathepsin G higher than 50 nM, whereas lower concentrations only induced shape change (results not shown).

The cathepsin G-evoked large increase in $[Ca^{2+}]_i$ occurred within a few seconds. As shown in Fig. 1, relatively low cathepsin G concentrations (50–80 nM) evoked a $[Ca^{2+}]_i$ rise consisting of two phases: an initial fast phase, reaching the peak in $14.3 \pm 0.75$ s (mean ± S.E.M., $n = 4$) after the onset of response ($t_{max.}$), and a second, sustained, phase which reached the maximum fluorescence rise in $99.8 \pm 6.5$ s (mean ± S.E.M., $n = 4$). Higher cathepsin G concentrations (100–200 nM) induced a monophasic rise in $[Ca^{2+}]_i$ with a $t_{max.}$ of $73.5 \pm 2.7$ s (mean ± S.E.M., $n = 6$).

### Role of amplification mechanisms in cathepsin G-induced platelet activation

Incubation of platelets with acetylsalicylic acid (500 $\mu$M), completely preventing $TxB_2$ formation, did not significantly modify platelet aggregation, $Ca^{2+}$ movements and 5-HT secretion induced by cathepsin G (100 nM), these being decreased by only 30 %. This suggests that cathepsin G-induced platelet activation does not depend on cyclo-oxygenase activation, in agreement with previous work on platelet activation utilizing both purified

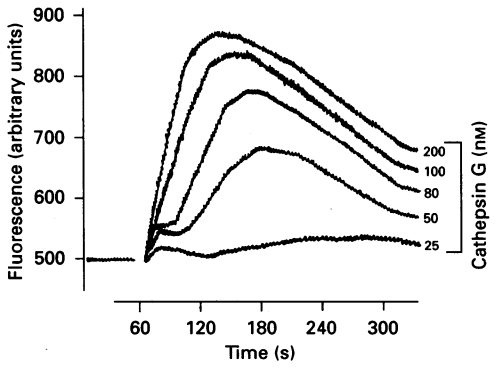


**Fig. 1. Fluorescence measurement in cathepsin G-activated platelets**

Concentration-response of cathepsin G-induced fluorescence increase in fura-2-loaded platelets. Fura-2-loaded human platelets ($1 \times 10^8$ cells/ml; 1.5 ml samples), resuspended in Hepes/Tyrode buffer containing 1 mM-$CaCl_2$, were incubated for 2 min and stimulated, under stirring, with different concentrations of cathepsin G. Tracings are representative of at least four different experiments.

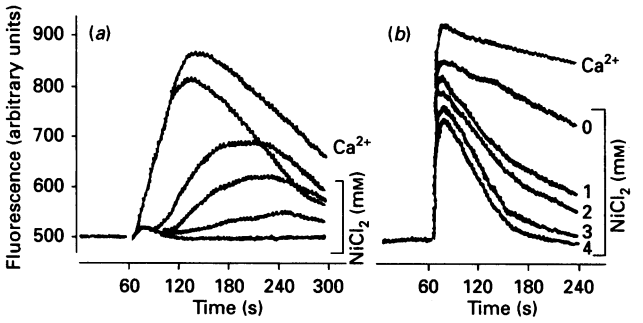


**Fig. 2. Effect of $Ni^{2+}$ on $[Ca^{2+}]_i$ mobilization induced by cathepsin G and thrombin in human platelets**

Fura-2-loaded human platelets ($1 \times 10^8$ cells/ml; 1.5 ml samples) resuspended in Hepes/Tyrode buffer were incubated under stirring for 1 min at 37 °C with 1–4 mM-$NiCl_2$, and then stimulated with 100 nM-cathepsin G (*a*) or 0.25 unit of thrombin/ml (*b*). Tracings are representative of four different experiments.

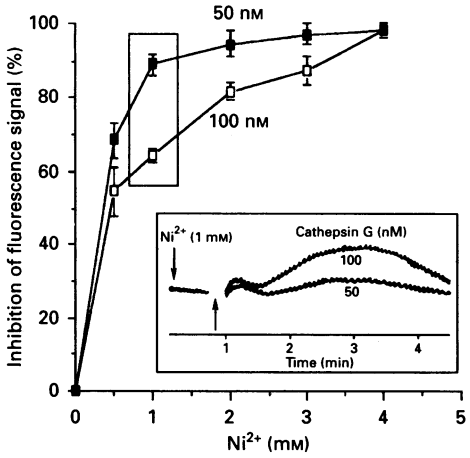


**Fig. 3. Inhibition by increasing concentrations of $Ni^{2+}$ of cathepsin G-induced fluorescence rises**

Fura-2-loaded human platelets ($1 \times 10^8$ cells/ml; 1.5 ml samples) resuspended in Hepes/Tyrode buffer were incubated under stirring for 1 min at 37 °C with $NiCl_2$, and subsequently stimulated with 50 nM- or 100 nM-cathepsin G. Percentage inhibition of fluorescence signal versus $NiCl_2$ concentration is represented. Data are means ± S.E.M. of three different experiments. Inset: effect of cathepsin G (50 or 100 nM) in the presence of 1 mM-$Ni^{2+}$. Tracings are representative of three different experiments.

cathepsin G or activated PMN [1,2,10,11]. Since several examples of opening of ion channels by lipoxygenase metabolites are known [12], the possible involvement of the lipoxygenase pathway was investigated by inhibiting formation of hydroperoxy and hydroxy derivatives with nordihydroguaiaretic acid [12]. Pretreatment of platelets with 10 μM-nordihydroguaiaretic acid did not affect cathepsin G-induced $Ca^{2+}$ movements (results not shown). In contrast, the ADP-scavenger system phosphocreatine (4 mM)/creatine kinase (40 units/ml) inhibited $Ca^{2+}$ mobilization induced by 50, 100 and 200 nM-cathepsin G by 92.2 ± 1.1, 49.4 ± 7.2 and 55.4 ± 7.4% respectively (means ± S.E.M.; $n = 3$). Also 1 mM-ATP, used as an ADP-receptor antagonist, significantly decreased $Ca^{2+}$ mobilization induced by 100 nM-cathepsin G (results not shown). In parallel, phosphocreatine/creatine kinase inhibited platelet aggregation, 5-HT secretion (73.9 ± 2.6% inhibition) and $TxB_2$ production (86.8 ± 10.0% inhibition) induced by 100 nM-cathepsin G. These data strongly suggest that platelet activation induced by cathepsin G may be amplified by ADP release. Similarly, Bykowska *et al.* [13] showed that platelet aggregation induced by cathepsin G was suppressed by apyrase. On the other hand, the 5-HT antagonist ketanserine (0.3 μM) did not affect $Ca^{2+}$ transients induced by cathepsin G (results not shown).

Cathepsin G-induced platelet activation has been reported as being characterized by exposure of the glycoprotein complex GP IIb/IIIa [13]. However, preventing platelet aggregation with the synthetic peptide RGDSP (100 μM) did not significantly modify the $[Ca^{2+}]_i$ rise induced by 100 nM-cathepsin G (687 ± 116 nM, versus 858 ± 164 nM for control samples; mean ± S.E.M., $n = 4$).

### Analysis of the components of $Ca^{2+}$ transients induced by cathepsin G

Stimulation of platelets by several agonists produces $[Ca^{2+}]_i$ elevation due to both mobilization from intracellular stores and/or $Ca^{2+}$ entry from the extracellular space through plasma-membrane channels. In order to assess the relative importance of these pools in $[Ca^{2+}]_i$ increases during cathepsin G activation of platelets, $Ca^{2+}$ influx was prevented by blocking agonist-induced $Ca^{2+}$ entry with $Ni^{2+}$ or chelating external $Ca^{2+}$ with EGTA. In these circumstances, the residual $[Ca^{2+}]_i$ elevation is due to discharge from the internal stores [14,15]. $Ni^{2+}$ (1–4 mM) affected in a concentration-dependent fashion cathepsin G-induced $Ca^{2+}$ transients, abolishing the second peak at the highest $Ni^{2+}$ concentration and leaving a very small initial fluorescence peak ($[Ca^{2+}]_i = 20.0 \pm 2.8$ nM; mean ± S.E.M., $n = 6$), which could correspond to mobilization from intracellular stores (Fig. 2*a*). The $t_{max.}$ of fluorescence rise in the presence of $Ni^{2+}$ was also prolonged (control $t_{max.} = 74.3 \pm 5.9$ s; at 2 mM-$Ni^{2+}$ $t_{max.} = 166 \pm 22.3$ s). When 50 nM-cathepsin G was used, 2–3 mM-$Ni^{2+}$ was enough to block the second fluorescence peak completely (Fig. 3). Moreover, at the fixed concentration of 1 mM-$Ni^{2+}$, inhibition of the fluorescence signal could be overcome by increasing the concentration of cathepsin G (Fig. 3 inset), suggesting the necessity for higher concentrations of $Ni^{2+}$ to block $Ca^{2+}$ influx induced by higher concentrations of cathepsin G. $Ca^{2+}$ mobilization by thrombin is known to be due to both influx and mobilization from internal stores. The fluorescence rise elicited by 0.25 unit of thrombin/ml was decreased by 19.4 ± 2.1% in the presence of 1 mM-$Ni^{2+}$. Increasing concentrations of $Ni^{2+}$ further decreased the fluorescence signal only slightly (33 ± 1% inhibition at 4 mM-$Ni^{2+}$), but increased the rate of return towards the baseline in a marked and concentration-dependent manner (Fig. 2*b*). These data strongly suggest that, at variance with the effect of thrombin, the main component of cathepsin G-induced $[Ca^{2+}]_i$ rise is an influx across the plasma membrane. This $Ca^{2+}$ influx seems to be important for platelet activation, since aggregation induced by 100–200 nM-cathepsin G was completely abolished by pre-incubation with 4 mM-$Ni^{2+}$, whereas $TxB_2$ production was also significantly decreased by 95% (results not shown). EGTA (4 mM) almost totally abolished the cathepsin G (100 nM)-induced $Ca^{2+}$ rise (Fig. 4), but only partially decreased the $Ca^{2+}$ transients induced by 0.25 units of thrombin/ml (results not shown).

Since effects of complete removal of external $Ca^{2+}$ by EGTA on receptor–ligand binding or on subsequent transduction processes have been suggested [15], we examined the possibility of additional effects of EGTA on platelet activation induced by cathepsin G. In fact, EGTA decreased by 49.5 ± 4.2% (mean ± S.E.M., $n = 3$) the catalytic activity of 200 nM-cathepsin G, as measured by a chromogenic test. The enzymic activity of cathepsin G followed typical Michaelis–Menten kinetics and was inhibited by EGTA in a non-competitive manner (Fig. 5). Since the proteinase activity of cathepsin G is essential for platelet activation [5], the observed decrease in $Ca^{2+}$ mobilization by

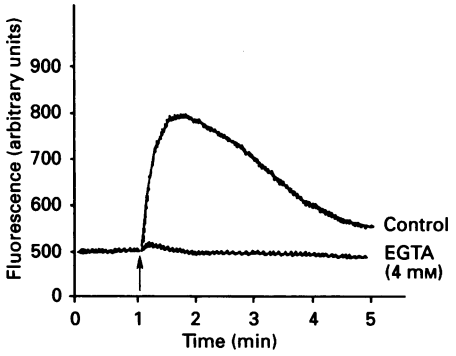


**Fig. 4. Effect of EGTA on $[Ca^{2+}]_i$ mobilization induced by cathepsin G in human platelets**

Fura-2-loaded human platelets ($1 \times 10^8$ cells/ml; 1.5 ml samples) resuspended in Hepes/Tyrode buffer were incubated under stirring for 1 min at 37 °C with 4 mM-EGTA, and subsequently stimulated with 100 nM-cathepsin G. Tracings are representative of four different experiments.

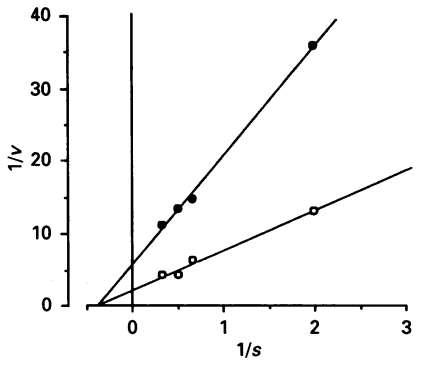


**Fig. 5. Lineweaver–Burk plot of cathepsin G activation in the presence (●) or absence (○) of 4 mM-EGTA**

The catalytic activity of 200 nM purified cathepsin G was measured as described in the Experimental section. $1/v$ $(\Delta A_{410}/\text{min})^{-1}$ represents the reciprocal of the rate of increase in $A_{410}$ owing to release of nitroanilide from the cathepsin G-specific chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; $1/s$ $(\text{mM}^{-1})$ represents the reciprocal of the concentration of substrate.

EGTA could partially be due to the decreased ability of cathepsin G to activate platelets. This effect does not seem to be related to removal of bivalent cations, since BAPTA at a concentration (4 mM) that completely prevents $Ca^{2+}$ mobilization induced by 100 nM-cathepsin G does not affect the catalytic activity of this enzyme. $Ni^{2+}$ (4 mM) did not affect cathepsin G catalytic activity. On the other hand, EGTA (4 mM) did not affect thrombin catalytic activity (results not shown). These findings suggest that EGTA should be used with caution to analyse $Ca^{2+}$ movements induced by cathepsin G.

### Analysis of cathepsin G-evoked bivalent-cation influx

The external influx was further examined in experiments performed in the presence of $Mn^{2+}$. This ion has been widely used as a marker of agonist-induced bivalent-cation entry into platelets [14,16] and in other cell types [17–19]; in fact, when $Mn^{2+}$ is added to the resuspending medium, it penetrates across the plasma membrane through $Ca^{2+}$ channels opened by stimulation, and binds to fura-2 inside the cells, quenching the fluorescence signal. At an excitation wavelength of 360 nm, which is the isosbestic point (when fura-2 emission is not influenced by $Ca^{2+}$),

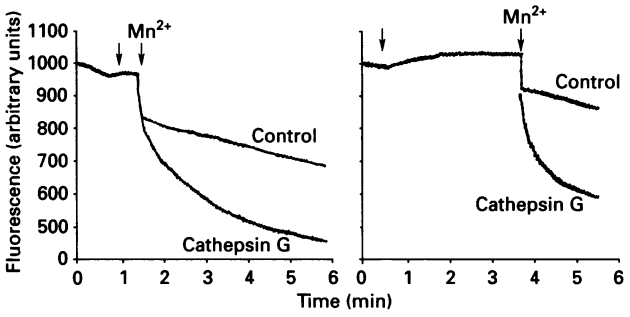


**Fig. 6. Analysis of $Ca^{2+}$ influx in cathepsin G-activated platelets**

Washed platelets ($1 \times 10^8$/ml) were incubated under stirring for 2 min at 37 °C; 100 nM-cathepsin G (unlabelled arrow) and 1 mM-$MnCl_2$ were subsequently added. Tracings of fluorescence obtained at monochromator settings for excitation of 360 nm in the presence of cathepsin G or vehicle are represented. $MnCl_2$ was added to platelets 30 s (left) or 3 min (right) after cathepsin G. Similar results (not shown) were obtained when $MnCl_2$ was added to platelets 4 min after cathepsin G.

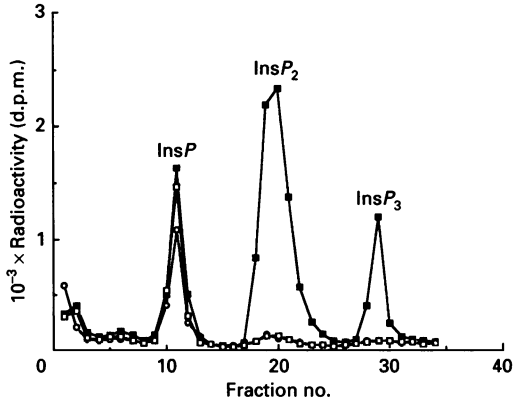


**Fig. 7. Analysis of $[^3H]InsP_3$ accumulation in platelets stimulated with cathepsin G**

Samples (0.5 ml) of *myo*-[2-$^3$H]inositol-labelled human platelets ($8 \times 10^8$/ml) were incubated under stirring at 37 °C with 600 nM-cathepsin G (□) or thrombin (2 units/ml) (■) or saline (○, control) for 15 s. Incubations were stopped and samples applied, for determination of inositol phosphates, to AG 1-X8 anion-exchange columns as described in the Experimental section. Results are representative of 15 different samples of platelets activated with 200–600 nM-cathepsin G for 15 s–3 min.

addition of $Mn^{2+}$ to the platelet suspension caused a consistent dose-dependent fall in the fluorescence signal. This effect was observed when $Mn^{2+}$ was used either 30 s or 3 min after stimulation of platelets with cathepsin G (Fig. 6). The cathepsin G-induced $Mn^{2+}$ uptake was prevented by $Ni^{2+}$ (results not shown).

These results confirm the existence of a fast bivalent-cation entry pathway activated by cathepsin G in human platelets, and indicate that the activation of such a pathway is sustained. The contribution of endogenous ADP, which also mobilizes $Ca^{2+}$ mainly from the extracellular milieu [20], cannot completely account for cathepsin G-evoked $[Ca^{2+}]_i$ increase. The nature of $Ca^{2+}$ channels opened by cathepsin G remains to be established. Since voltage-operated channels [14,20–22] or $Ca^{2+}$-sensitive $Ca^{2+}$ channels opened by $Ca^{2+}$ discharged from intracellular stores are not likely to be involved in platelets [15,16], a receptor-mediated $Ca^{2+}$ entry mechanism has to be considered. Coupling of cathepsin G receptor to the opening of a plasma-membrane

channel might be direct, but might also involve a GTP-binding protein or the generation of a diffusible messenger.

### Analysis of $InsP_3$ production induced by cathepsin G

The discharge of the intracellular $Ca^{2+}$ stores by several agonists is usually linked to generation of Ins(1,4,5)$P_3$ [23]. Hydrolysis of PtdIns$P_2$ is one of the earliest events when platelets are stimulated with thrombin [24] and is accompanied by the accumulation of inositol phosphates [25]. Such a production has been reported in cathepsin G-stimulated human endothelial cells [26]. Since a very small $Ca^{2+}$ mobilization is still evident in the presence of $Ni^{2+}$, in order to verify the involvement of phospholipase C activation we measured inositol triphosphates Ins$P_3$ accumulation. Platelet stimulation with thrombin resulted in the production of Ins$P_3$ at 4 times the basal value within 15 s, whereas no accumulation of this second messenger could be detected when platelets were stimulated for 15 s with 200–800 nM-cathepsin G (Fig. 7). Longer incubation times (up to 3 min) were also used, with similar negative results (not shown).

We were thus unable to obtain evidence of an involvement of Ins$P_3$ in $Ca^{2+}$mobilization induced by cathepsin G, at least in the ranges of concentrations and at the times used; the mechanism(s) by which cathepsin G activates platelets and endothelial cells may be therefore different.

In conclusion, the main mechanism of cathepsin G-induced increase of $[Ca^{2+}]_i$ in human fura-2-loaded platelets is massive $Ca^{2+}$ entry across $Ni^{2+}$-sensitive channels of the plasma membrane. Whether opening of receptor-operated and/or second-messenger-operated channels is involved in cathepsin G stimulation of platelets remains to be established.

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