Activation of calpain I in thrombin-stimulated platelets is regulated by the initial elevation of the cytosolic Ca^{2+} concentration

Hidemi ISHII,*† Youichi SUZUKI,* Masaaki KUBOKI,* Masako MORIKAWA,† Michiko INOUE† and Mutsuyoshi KAZAMA*

*Department of Clinical Biochemistry, Faculty of Pharmaceutical Sciences, Teikyo University, 1091-1 Suarashi, Sagamiko, Tsukui-gun, Kanagawa 199-01, Japan, and †Tokyo College of Pharmacy, Hachioji, Tokyo 192-03, Japan

The source and concentration of Ca^{2+} required to activate calpain I were investigated in thrombin-stimulated platelets. The concentration of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) was measured in platelets containing fura-2-AM, and exhibited a biphasic response after stimulation with 0.05, 0.1 or 0.5 NIH units of thrombin/ml. An initial transient elevation, which was predominantly dependent upon Ca^{2+} released from the internal stores into the cytosol, peaked at 15 s after stimulation, and a secondary sustained elevation, which was due to Ca^{2+} influx, was observed following the initial elevation. Calpain I was present at about 540 ng/ 10^8 unstimulated platelets, as measured by immunoblotting using rabbit anti-(human calpain I) IgG. Calpain I was activated 10 s after thrombin stimulation, as determined by the appearance of the 78 kDa and 76 kDa forms on immunoblots. The activation ratio of calpain I was calculated as the amount of the 78 + 76 kDa forms as a percentage of the total (80 + 78 + 76 kDa), and was influenced by the extent of the initial transient $[\text{Ca}^{2+}]_i$ elevation after stimulation. An initial increase in $[\text{Ca}^{2+}]_i$ of 300 nM was required to achieve the maximal activation (60%) of calpain I, and half-maximal activation occurred at 160 nM- $[\text{Ca}^{2+}]_i$. These results suggest that the activation of calpain I in platelets is regulated by the initial elevation in $[\text{Ca}^{2+}]_i$ after thrombin stimulation, and does not necessarily require a Ca^{2+} influx.

INTRODUCTION

Calpain (EC 3.4.22.17) is a cytosolic thiol proteinase in mammalian cells whose activity is controlled by Ca^{2+} and by an endogenous calpain inhibitor, calpastatin [1–6]. There are two distinct types of calpain [1–6]. One enzyme, calpain I, requires micromolar Ca^{2+} concentrations for its activation and proteinase activity *in vitro*, and the other, calpain II, requires millimolar Ca^{2+} concentrations. Calpain is a heterodimer composed of 80 kDa and 30 kDa subunits. The catalytic site of the enzyme is located in the 80 kDa subunit, and the 30 kDa subunit is involved in regulation of the enzyme activity [7]. A calpain structure composed of the 80 kDa subunit is the proenzyme (procalpain), which does not have enzyme activity. Procalpain undergoes a conformational change after binding Ca^{2+} , and this Ca^{2+} -procalpain conformer is responsible for autoproteolysis resulting in an intermediate 78 kDa form and the active 76 kDa enzyme [8–13]. It has been proposed that the activation of procalpain I in cells takes place at the inner surface of the plasma membrane after binding with proteins on the surface in a Ca^{2+} -dependent manner [8–11, 14–16].

A physiological role for calpain has not yet been clearly identified in any cell, but there is some evidence to suggest that it contributes to platelet aggregation. It is known that (a) many agonists increase the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in platelets [17, 18]; (b) calpain is a major neutral proteinase in platelets [3, 4]; (c) hydrolysis of various supposed calpain substrates (actin-binding protein, calmodulin-binding proteins, protein kinase C, aggregin and others) accompanies platelet aggregation [19–23]; (d) platelet activation is inhibited by pretreatment with a membrane-permeable thiol protease inhibitor, EST [L-*trans*-epoxysuccinyl-leucylamide(3-methyl)butane ethyl ester] [24]; and (e) calpain-proteolysed von Willebrand factor is highly reactive with platelets, and participates in formation of

platelet aggregates [25]. It therefore seems likely that calpain is involved in platelet aggregation.

The role of Ca^{2+} in calpain activation, however, remains unclear. It is not known whether calpain can be activated by Ca^{2+} released from internal stores into the cytosol or if Ca^{2+} influx is required, nor how much $[\text{Ca}^{2+}]_i$ is required for the activation of calpain in platelets. Furthermore, although a lot of calpain substrates have been proposed, the relationship between substrate hydrolysis by calpain and the rapid events of the platelet response remain uncertain.

The activation and action of calpain are frequently inferred from the disappearance of its supposed substrates or the appearance of breakdown products of the substrates, although such methods provide only indirect data supporting the conclusion of calpain activity. The activation of calpain I can be observed directly by immunoblot detection of the active 76 kDa form and the intermediate 78 kDa form [8, 10, 26]. Therefore we have applied this method to platelets stimulated with thrombin in the presence of Ca^{2+} or EGTA in the medium, in order to relate calpain I activation to $[\text{Ca}^{2+}]_i$, as measured by fura-2 fluorescence.

MATERIALS AND METHODS

Materials

Reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan, except where indicated. Fura-2-AM and fura-2 were purchased from Dojin Chemical Institute Ltd., Kumamoto, Japan. Human thrombin was kindly provided by Green Cross Co. Ltd., Osaka, Japan. Molecular mass markers for electrophoresis (LMW Kit E) were purchased from Pharmacia-LKB, Uppsala, Sweden. Vectastain ABC Kit for detection of rabbit anti-(human calpain I) IgG was from Vector Laboratories, Burlingame, CA, U.S.A. Konica Immunostain

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concn.; PRP, platelet-rich plasma; WPS, washed platelet suspension; Me_2SO , dimethyl sulphoxide.

† To whom correspondence and reprint requests should be addressed.

HRP IS-50B for immunostaining was from Konica Co., Tokyo, Japan. Nitrocellulose membrane and Tween-20 were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Auro Dye was from Janssen Pharmaceutica Life Sciences Products, Beerse, Belgium. Distilled and deionized water was further purified with a Milli Q II Water Purification System (Millipore, Bedford, MA, U.S.A.), and the purified water was used in all experiments.

Platelet preparation

Blood was drawn from adult volunteers who had not taken any medication for at least 2 weeks prior to venesection, and then mixed immediately with 3.8% sodium citrate (9 vol. of blood/1 vol. of citrate). The citrated blood was centrifuged at 150 *g* for 15 min at room temperature. The platelet-rich plasma (PRP) obtained was incubated with or without 1 mM-fura-2 AM in dimethyl sulphoxide (Me_2SO), adjusted to a final concentration of 3 μM , for 40 min at 37 °C. The PRP was mixed with $\frac{1}{8}$ vol. of ACD solution (1.3% citrate, 2.5% sodium citrate and 2% dextrose) and centrifuged at 530 *g* for 15 min at room temperature. The pellet obtained was suspended in buffer A (26 mM-Tes, 130 mM-NaCl, 25 mM-dextrose, 1 mM-KCl and 0.1% human serum albumin, pH 6.8) and then the suspension was further mixed with $\frac{1}{8}$ vol. of ACD. The suspension was centrifuged at 530 *g* for 15 min at room temperature. The resulting pellet was resuspended in modified Tyrode/Hepes buffer (134 mM-NaCl, 12 mM- NaHCO_3 , 2.9 mM-KCl, 0.36 mM- NaH_2PO_4 , 1 mM- MgCl_2 , 10 mM-dextrose, 10 mM-Hepes, pH 7.4) and the concentration of washed platelet suspension (WPS) was adjusted to $(2.0\text{--}2.22) \times 10^9$ platelets/ml with the same buffer.

Calcium concentrations

The $[\text{Ca}^{2+}]_i$ was measured in fura-2 AM-loaded WPS (2×10^9 /ml; 500 μl) which was incubated with 10 μl of 52 mM- CaCl_2 (final concn. 1 mM) or with various concentrations of EGTA for 2 min at 37 °C. The WPS was stirred at 37 °C in a Jasco CAF-100 Ca^{2+} Analyzer (Japan Spectroscopic Co., Tokyo, Japan) and $[\text{Ca}^{2+}]_i$ was measured immediately after addition of 10 μl of various concentrations of thrombin into the fluorimeter. The $[\text{Ca}^{2+}]_i$ was calculated from the ratio of fluorescence emission measured at 500 nm with excitation wavelengths of 340 and 380 nm, as described [27].

The concentration of external Ca^{2+} in WPS was measured in the Jasco CAF-100 Ca^{2+} Analyzer after the addition of 2 μM -fura-2 into fura-2-AM-unloaded WPS in the presence of various concentrations of EGTA. The Ca^{2+} concentration was calculated as described [27].

Preparation of rabbit anti-(human calpain I) antibody

Calpain I was purified from human erythrocytes by ion-exchange chromatography on DE-52 DEAE-cellulose/Blue-Sepharose CL-6B column chromatography and phenyl-Sepharose CL-4B column chromatography as previously reported [7]. Calpain I (1 mg) in 1 ml of 10 mM-phosphate buffer containing 0.15 M-NaCl and 5 mM-EDTA (pH 7.4) was emulsified with 1 ml of Freund's complete adjuvant and the emulsion was injected subcutaneously into multiple sites in a rabbit. Subcutaneous booster injections of calpain I (each 500 μg) in Freund's complete adjuvant were given twice at monthly intervals, and 1 week after the last booster injection, the blood was drawn from the ear vein and antisera were obtained. The IgG fraction was prepared from the antisera by ammonium sulphate fractionation and Protein A-Sepharose CL-4B chromatography.

Determination of calpain I activation

WPS (2.2×10^9 platelets/ml; 225 μl) was incubated with 5 μl of 52 mM- CaCl_2 (final 1 mM concentration) or various concentra-

tions of EGTA and then stirred with 5 μl of various thrombin concentrations for various times in a 260 μl total volume. The reaction was terminated by addition of 260 μl of SDS sample buffer (10 mM-Tris/HCl, pH 8.0, containing 1 mM-EGTA, 1% SDS and 5% 2-mercaptoethanol) and the mixture was boiled in a water bath for 5 min. A 30 μl sample of the boiled mixture was subjected to SDS/PAGE using a 7% polyacrylamide separating gel, and then the proteins were transferred to a nitrocellulose membrane according to the method of Towbin *et al.* [28]. The membrane was incubated with 10 μg of rabbit anti-(human calpain I) IgG/ml in 20 mM-Tris-buffered saline containing 0.05% Tween 20 (pH 7.5) for 1 h. After washing, the membrane was incubated with biotinylated sheep anti-(rabbit IgG) antibody and avidin-biotin-peroxidase complex using the Vectastain ABC Kit, and then the peroxidase activity was measured using Konica Immunostain HRP IS-50B, composed of aromatic amine and naphthol derivatives as substrates. By densitometric analysis (dual-wavelength flying spot scanner CS-9000; Shimadzu Co., Kyoto, Japan) of the immunoblot bands on the nitrocellulose membranes, the amount of calpain I was measured. In the standard assay, a linear relationship was found between the amount of purified calpain I and the intensity of the immunoblot stain up to 200 ng of calpain I.

RESULTS

Characterization of anti-(human calpain I) antibody

An immunodiffusion test was carried out between purified human erythrocyte calpain I, a whole lysate of human erythrocytes, a whole lysate of human platelets and rabbit anti-(human calpain I) IgG (Fig. 1). The IgG gave a single precipitation arc that showed complete immunochemical identity with the purified calpain I antigen and the cellular calpain antigen in erythrocytes or platelets. Purified calpain I was shown on SDS/PAGE to consist of two subunits: one heavily staining at 80 kDa and one lightly staining at 30 kDa (Fig. 2, lane 1), whereas immuno-

Fig. 1. Immunodiffusion of rabbit anti-(human erythrocyte calpain I) IgG

Rabbit anti-(erythrocyte calpain I) IgG (Ab; 15 μg) was placed in the centre well of a 1% agarose plate and allowed to diffuse overnight with various antigens in wells circling the IgG well. PLT, Triton X-100 lysate of 3.6×10^7 human platelets; Cal I, 5 μg of purified erythrocyte calpain I; RBC, a Triton X-100 lysate of 3×10^7 human erythrocytes. After formation of the precipitation arc, the agarose was soaked in phosphate-buffered saline, dried and stained with 0.25% Coomassie Blue R-250 and 1% Amido Black in a solution that consisted of 5 parts methanol, 5 parts water and 1 part acetic acid.

Fig. 2. Immunoblot specificity of anti-calpain antibody

Coomassie Blue staining of purified erythrocyte calpain I that was used as the immunogen to produce the anti-(calpain I) antibody is shown in lane 1. Purified calpain I (2 µg) was applied to a 7% SDS/PAGE gel after boiling with SDS sample buffer containing 2.6% 2-mercaptoethanol. Immunoblotting with rabbit anti-(human calpain I) IgG of purified calpain I (100 ng, lane 2) or of a whole platelet lysate (2×10^7 platelets, lane 3) is shown on the right. Antigen detection was carried out as described in the Materials and methods section using 10 µg of rabbit anti-(human calpain I) IgG/ml.

blotting of the enzyme using the IgG gave a single band of 80 kDa (Fig. 2, lane 2). The immunoblot of the lysate of unstimulated platelets also exhibited a single band of 80 kDa (Fig. 2, lane 3). These results indicate that this IgG monospecifically recognizes the large subunit of calpain I in human platelets. A minimum of 5 ng of purified calpain I was detected in the immunoblot. A correlation between the amount of purified calpain I and the intensity of the immunoblot stain measured by densitometry was linear up to 200 ng of calpain I per lane (results not shown). The content of calpain I antigen measured by immunoblots from 10 platelet lysates was 539 ± 98 ng/ 10^8 platelets (mean $\pm$ S.D.).

Chelation of external Ca^{2+} by EGTA

After incubation of WPS for 1 min with various concentrations of EGTA, fura-2 was added to the suspension and the fluorescence was measured to determine the chelating effect of EGTA on the external Ca^{2+} concentration. About 3 µM- Ca^{2+} was observed in the external solution of the platelets (2×10^8 platelets/ml), and the fluorescence decreased with increasing EGTA concentrations, completely disappearing after addition of 50 µM-EGTA to the suspension (results not shown). The $[\text{Ca}^{2+}]_i$ was measured in platelets loaded with fura-2-AM, and an elevation occurred within 5 s of stimulation with 0.05, 0.1 or 0.5 NIH units of thrombin/ml (Fig. 3). The $[\text{Ca}^{2+}]_i$ measured in the presence of 1 mM- CaCl_2 exhibited a biphasic response after stimulation. The initial transient elevation of $[\text{Ca}^{2+}]_i$ peaked at 15 s regardless of the amount of thrombin, although the extent of the initial elevation increased in a dose-dependent manner. A sustained secondary elevation of $[\text{Ca}^{2+}]_i$ was observed following the initial elevation. A similar initial transient elevation was observed after stimulation of platelets which were suspended in the absence of 1 mM- CaCl_2 , but the extent of the secondary elevation was decreased. Addition of 50 µM-EGTA into the WPS lowered the initial elevation by about 30% compared with that observed in the presence of 1 mM- CaCl_2 , and the secondary elevation was

Fig. 3. Release of Ca^{2+} into the cytosol of platelets stimulated with thrombin under various conditions

Fura-2-AM-loaded WPS (2×10^8 /ml, 500 µl) in modified Tyrode-Hepes buffer (pH 7.4) was incubated with 10 µl of 52 mM- CaCl_2 or 2.6 mM-EGTA for 2 min at 37 °C. The platelet suspension was stirred at 37 °C in a Ca^{2+} analyser and the $[\text{Ca}^{2+}]_i$ was immediately measured in the fluorimeter after addition of 0.05 (a), 0.1 (b) or 0.5 (c) unit of thrombin/ml in a total volume of 520 µl. The data shown are representative of three experiments.

abolished. These results indicate that a concentration of 50 µM-EGTA is necessary to chelate the external Ca^{2+} in the WPS.

Time course of calpain I activation and elevation of $[\text{Ca}^{2+}]_i$

Activation of calpain I in platelets stimulated with thrombin was measured by immunoblot detection of the 78 kDa and 76 kDa fragments using the monospecific polyclonal antibody to calpain I. A representative immunoblot is shown in Fig. 4 for calpain I in platelets stimulated with 0.05 unit of thrombin/ml. Both fragments appeared within 10 s of stimulation, regardless of the presence or absence of external Ca^{2+} . The intensity of the 80 kDa band decreased in parallel with the appearance of both the 78 kDa and 76 kDa fragments. This result indicates that calpain I in platelets does not require a Ca^{2+} influx from the external medium for activation. The activation ratio of calpain I was measured at various times after stimulation with thrombin (Fig. 5), and was calculated as amount of the 78 kDa and 76 kDa fragments as a percentage of total subunit (80 kDa + 78 kDa + 76 kDa) forms of calpain I. When the platelets suspended in buffer containing 1 mM- CaCl_2 or 50 µM-EGTA were stimulated with 0.05, 0.1 or 0.5 unit of thrombin/ml, a maximal 60% activation of calpain I was achieved at 15 s after stimulation (with the exception of platelets stimulated with 0.05 unit of thrombin/ml in the presence of 50 µM-EGTA). The activation ratio at 15 s of these platelets was limited to about

Fig. 4. Immunoblot detection of calpain I in platelets stimulated with 0.05 unit of thrombin/ml

Washed human platelet suspensions (2.2×10^9 platelets/ml; 225 μ l) were incubated in a tube in the aggregometer with 5 μ l of 52 mM- CaCl_2 (a) or 2.6 mM-EGTA (b) for 2 min at 37 $^\circ\text{C}$ and then stirred with thrombin (0.05 unit/ml) in a total volume of 260 μ l for various times. The reactions were terminated by addition of SDS sample buffer and the mixtures were boiled in a water bath for 5 min. The boiled samples were subjected to SDS/PAGE and the proteins were transferred to nitrocellulose membranes. Detection of calpain I molecules was carried out as described in the Materials and methods section using rabbit anti-(human calpain I) IgG (10 $\mu\text{g}/\text{ml}$).

30% (half-maximal activation). However, the ratio gradually increased up to a maximum of 60% during the following 45 s. The calpain I activation by 15 s occurs within a time frame compatible with that required to achieve the peak in the initial transient elevation of $[\text{Ca}^{2+}]_i$. The initial elevation in $[\text{Ca}^{2+}]_i$ observed at 15 s after stimulation with 0.1 or 0.5 unit of thrombin/ml was more than 250 and 600 nM- Ca^{2+} respectively. The peak $[\text{Ca}^{2+}]_i$ in the platelets stimulated with 0.05 unit of thrombin/ml in 50 μM -EGTA was about 180 nM (initial increase). These results indicate that calpain I in platelets is activated to a maximum level by Ca^{2+} released from internal stores into the cytosol, but the presence of a low $[\text{Ca}^{2+}]_i$ after the initial elevation on stimulation increases the time required to achieve the maximum activation of the enzyme.

Effect of EGTA on calpain I activation and elevation of $[\text{Ca}^{2+}]_i$

The influence of various concentrations of EGTA on the activation ratio of calpain I and the extent of the initial increase in $[\text{Ca}^{2+}]_i$ in stimulated platelets was measured at 15 s after addition of thrombin (Fig. 6). The extent of the initial transient elevation in $[\text{Ca}^{2+}]_i$ was decreased in a dose-dependent manner with EGTA in platelets stimulated with 0.05, 0.1 or 0.5 unit of thrombin/ml. An inflection point in the decrease was observed at 50 μM -EGTA. The $[\text{Ca}^{2+}]_i$ was further gradually decreased by increasing EGTA concentrations to more than 50 μM . These results indicate that a high concentration of EGTA not only chelates external Ca^{2+} , but also affects the extent of the initial elevation in $[\text{Ca}^{2+}]_i$. Although a 55% activation of calpain I was observed in the platelets which were stimulated with 0.05 unit of thrombin/ml in the absence of EGTA, the activation ratio declined in a dose-dependent manner with EGTA to 25% activation by 50 μM -EGTA. Addition of more than 50 μM -EGTA induced a gradual further decrease in the activation ratio. Calpain I was 60% activated in platelets stimulated with 0.1 unit of thrombin/ml in the absence of EGTA, and activation was reduced by only 10% by 50 μM -EGTA. When platelets were stimulated with 0.5 unit of thrombin/ml, no change in activation ratio was observed even in the presence of 200 μM -EGTA. These facts suggest that calpain I activation is regulated by the extent of the initial elevation in $[\text{Ca}^{2+}]_i$.

The data for the activation of calpain I and the initial transient increase in $[\text{Ca}^{2+}]_i$ shown in Fig. 6 were expressed by a saturation curve and Hill plot (Fig. 7). No activation was observed below

Fig. 5. Time course of calpain I activation and increase in $[\text{Ca}^{2+}]_i$ in platelets stimulated with thrombin

To measure calpain I activation ($\bullet$), the stimulation of platelets with thrombin was terminated by addition of SDS sample buffer at various times and the samples were immediately boiled in a water bath for 5 min. The boiled samples were subjected to SDS/PAGE and the proteins were transferred to a nitrocellulose membrane for immunoblot detection as described in the Materials and methods section. The activation ratio of calpain I was expressed as the amount of the 78 + 76 kDa forms as a percentage of the total (80 + 78 + 76 kDa) forms, calculated from densitometric scans of the immunoblots. $[\text{Ca}^{2+}]_i$ of platelets ($\circ$) was measured by the method described in the legend to Fig. 3. The results are expressed as means $\pm$ S.D. of 4 experiments.

80 nM- $[\text{Ca}^{2+}]_i$. A maximum 60% activation of calpain I was observed at $[\text{Ca}^{2+}]_i$ concentrations of more than 300 nM. A line drawn by the least-squares method in the Hill plot crossed the y-axis at -2.2 , and the $[\text{Ca}^{2+}]_i$ required for half-maximal activation of calpain I was calculated to be 160 nM.

DISCUSSION

It is known that $[\text{Ca}^{2+}]_i$ in platelets increases biphasically after stimulation with thrombin in the presence of external Ca^{2+} . The $[\text{Ca}^{2+}]_i$ in the initial and secondary elevations observed in the present experiments were very close to the results reported by other investigators [26,29]. It has been suggested that the initial transient elevation is predominantly dependent upon Ca^{2+} released from internal stores into the cytosol and partially upon Ca^{2+} influx, whereas the secondary sustained elevation is due solely to Ca^{2+} influx [29,30]. The current results support the

Fig. 6. Effect of EGTA on calpain I activation and the increase in $[Ca^{2+}]_i$ in platelets stimulated with thrombin

The WPS was incubated with various EGTA concentrations for 2 min at 37 °C. The platelets were stirred with 0.05 (a), 0.1 (b) or 0.5 (c) unit of thrombin/ml in a total volume of 260 μl in an aggregometer. The stimulation of platelets was terminated at 15 s by addition of SDS sample buffer. Immunoblot detection and the calculation of calpain I activation (●) were carried out as described in the legend to Fig. 5. The $[Ca^{2+}]_i$ of platelets (○) was measured as described in Fig. 3, and was expressed as the peak concentration of the initial transient elevation measured at 15 s after stimulation. The results are expressed as means $\pm$ S.D. of four experiments.

above suggestion, in that the second increase disappeared on addition of 50 μM -EGTA. This concentration was required to chelate both the Ca^{2+} bound to the outer membrane of platelets and the soluble Ca^{2+} in the suspension medium under the present experimental conditions.

It is generally accepted that calpain I composed of the 80 kDa form is a proenzyme which does not have proteinase activity and that the 76 kDa calpain form, which is produced via the intermediate 78 kDa fragment by autoproteolysis of the enzyme after binding of Ca^{2+} , is the active enzyme [8–13,26]. In a previous paper we demonstrated that the 80 kDa form did not exhibit proteinase activity and that activity was observed with the appearance of the 76 kDa form [8]. Therefore the activation of calpain I was confirmed by detection of the 78 kDa and/or 76 kDa fragments by immunoblotting. The results demonstrated that calpain I in platelets was activated by Ca^{2+} released from internal stores into the cytosol, without Ca^{2+} influx, and that the

Fig. 7. Relationship between calpain I activation and $[Ca^{2+}]_i$ in platelets stimulated with thrombin

The values for calpain I activation and $[Ca^{2+}]_i$ in the initial transient elevation as obtained in Fig. 6 were plotted. A Hill plot is shown in the inset.

activation was influenced by the extent of the initial transient elevation in $[Ca^{2+}]_i$ observed just after stimulation. Half-maximal activation occurred at 160 nM- $[Ca^{2+}]_i$ during the initial elevation, and > 300 nM- $[Ca^{2+}]_i$ was required to obtain maximum activation (60 %). The present work is the first study indicating a correlation between $[Ca^{2+}]_i$ and calpain I activation in living cells. When the extent of the initial increase in $[Ca^{2+}]_i$ was small, more time was subsequently required to achieve maximum activation. This could be explained by the proposal that a small amount of calpain I was activated by partial release of Ca^{2+} from the stores, and that the newly generated proteinase activity may gradually have activated adjacent procalsin I. Activation of purified calpain I is accomplished by 5 μM - Ca^{2+} in the presence of plasma membranes [10,15], whereas it requires about 20 μM - Ca^{2+} in the absence of membranes *in vitro* [8]. An endogenous activator that increases the affinity of calpain I for Ca^{2+} was discovered in human neutrophils, and the maximal calpain I activity in the presence of the activator was obtained with less than micromolar Ca^{2+} concentrations [31]. Therefore the activation of calpain I in activated platelets may be accomplished at a $[Ca^{2+}]_i$ near 300 nM through an interaction with the plasma membrane and the activator. Platelets appear to show pseudopods at a $[Ca^{2+}]_i$ of around 160 nM and deform into irregular forms at about 300 nM- $[Ca^{2+}]_i$ [32]. These Ca^{2+} concentrations are compatible with those required for half-maximal and maximal activation of calpain I respectively. The release of ATP from the platelets stimulated with thrombin was found after maximum activation of calpain I (after 15 s of stimulation) (H. Ishii, Y. Suzuki, M. Kuboki, S. Morie & M. Kazama, unpublished work). If calpain I plays a role in platelet responses after the stimulation, the activated enzyme may participate in the early responses before the release reaction.

Because the hydrolysis of supposed calpain substrates in platelets [33] and the appearance of the 78 and 76 kDa forms of calpain [9,26] were inhibited by addition of a high concentration of EGTA or EDTA (> 1 mM) to the platelet suspension, it has been suggested that Ca^{2+} influx is required for calpain activation in platelets. The present results demonstrated that the extent of the initial elevation in $[Ca^{2+}]_i$ was influenced by a high con-

centration of EGTA. An EGTA-releasable pool of Ca^{2+} is present in platelets [32], and the treatment of platelets with a high concentration of EGTA induces a decrease in $[\text{Ca}^{2+}]_i$ [34]. Therefore if platelet responses, including increases in $[\text{Ca}^{2+}]_i$ and calpain activation, are measured in the presence of EGTA, the minimum concentration of EGTA required to chelate the external Ca^{2+} should be used, as in the present work. Calpain I activation, i.e. the appearance of the 78 and 76 kDa forms, was easily observed in stirred platelet suspensions (but not non-stirred ones), even in the presence of 50 μM -EGTA (results not shown). Physical stimulation of platelets may accelerate the activation of calpain I even without a Ca^{2+} influx. It was recently reported that calpain II is externalized and expressed on the surface of platelet membranes after thrombin stimulation [35], and contributes to the generation of procoagulant activity in the presence of factor Va and factor Xa [36]. The present results do not exclude the possibility that calpain II activation requires Ca^{2+} influx, and different functions for calpains I and II in platelet responses are implied.

One of the initial responses of platelets to stimulation is their transformation from discs into irregular shapes, with centralization of subcellular organelles, followed by secretion of the organelles into the external milieu [37,38]. The changes in shape that platelets undergo in response to activation result from modifications to components of the membrane-bound filamentous network. Contraction of non-muscle cells is regulated by activation of myosin light chain kinase which phosphorylates myosin light chains [39]. Myosin light chain kinase is proteolysed by platelet calpain I, and the partially cleaved kinase phosphorylates the myosin light chain independent of Ca^{2+} and calmodulin [33,40–42]. Calpain I of platelets, in concert with its activator, is sufficiently activated on the plasma membrane by Ca^{2+} released to nanomolar concentrations (above 300 nM) in the cytosol after stimulation by thrombin, even in the absence of a Ca^{2+} influx from the external solution. This activated calpain I may participate in the initial responses of platelets through regulation of the filamentous network in the cytoskeleton.

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