Calpain I remains intact and intracellular during platelet activation

Immunochemical measurements with monoclonal and polyclonal antibodies

John A. SAMIS, Gizela ZBORIL and John S. ELCE*

Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

As a step towards understanding the physiological function of calpain (Ca^{2+} -activated neutral proteinase, EC 3.4.22.17) in blood platelets, and in view of some suggestions that calpain is transferred to the platelet external surface during platelet activation, the enzyme was studied with immunochemical methods in resting and thrombin-activated cells. (1) A mouse IgG₁ monoclonal antibody was prepared which binds strongly only to the denatured large subunit of human calpain I, and weakly to that of human calpain II. A polyclonal antibody raised against rat calpain II was available which, apart from binding strongly to rat calpain II, binds to the large subunits of human calpain I and II about equally. (2) With these antibodies, it was found that calpain could be detected in fixed platelets in suspension only after permeabilization with 0.1% saponin, and could not be detected on the exterior surface of resting or of activated platelets, or in the supernatant media of these platelets. It was concluded that calpain is not significantly externalized during platelet activation. (3) Immunoblotting showed that conversion of the larger calpain I subunit from 80 kDa into 76–78 kDa occurred only when thrombin-activated platelets were stirred to permit aggregation, and did not occur during unstirred thrombin activation. Although an action of calpain in the 80 kDa form on possible platelet substrates such as cytoskeletal proteins cannot be excluded, calpain is certainly not present as the 76–78 kDa form, which is assumed to be its active form, until aggregation is initiated.

INTRODUCTION

When platelets are stimulated *in vitro* by physiological agents such as thrombin, they change shape, extend pseudopodia, and secrete the contents of their intracellular granules; when activated with thrombin, they do not aggregate unless they are stirred to promote contact with each other. The response of the cell involves translocation of Ca^{2+} into the cytoplasm, and interactions of Ca^{2+} with phospholipids in the cell, followed by a wide range of subsequent effects [1].

Some of these effects may involve calpain [2], but the role of calpain in platelet activation and aggregation is not fully understood [3]. Platelets contain both calpain I (requiring 5–50 μM Ca^{2+}) and II (requiring 0.5–1 mM- Ca^{2+}) [4–8], and several platelet cytoskeletal proteins are sensitive to calpain hydrolysis, notably actin-binding protein, α -actinin and P235 [5,6,9–14]. Cytoplasmic [Ca^{2+}] rises during platelet activation to 2–3 μM [15–17], which may already be sufficient to permit calpain I activity, and, in addition, the Ca^{2+} requirements of the calpains are decreased, both by autolysis and by the presence of various lipids, to Ca^{2+} concentrations which are physiologically more probable [18–21].

On this basis, a role for calpain in platelet shape change was proposed [22], but more recent evidence implies that calpain may not be involved in the earliest steps of platelet activation. Hydrolysis of actin-binding protein, presumably by calpain, occurred only after the early steps of platelet aggregation by thrombin, and did

not occur if aggregation was prevented by not stirring [9,10]. Other work confirmed this, and suggested that the unknown real substrates of calpain were related to fibrinogen-receptor expression and 5-hydroxytryptamine release [23]. Other proposed substrates, implying other roles for the enzyme, include fibrinogen, kininogen and von Willebrand's factor, which undergo Ca^{2+} -dependent hydrolysis in platelet lysates [24–26], and glycoproteins 1b and V, which are hydrolysed when calpain is added to washed platelets [27]. Glycoprotein 1b, which is a transmembrane protein, is also cleaved by endogenous calpain in platelets treated with A23187 and 5 mM- Ca^{2+} [28].

Since calpain is presumed to be intracellular [2], its real substrates must presumably also be intracellular. It has, however, been suggested that calpain might have brief access to some secreted proteins at the time of α -granule secretion [29], and two reports had given hints that calpain could even have a fully extracellular function: a Ca^{2+} -dependent hydrolysis of casein was detected on the external membrane of activated but not of resting human platelets [30], and clotting Factor Va bound to the surface of bovine platelets was cleaved under conditions favouring calpain activity [31]. For an extracellular function, calpain must become available as a result either of platelet lysis or of externalization during normal platelet activation.

The localization of calpain in platelets, and its molecular mass, have therefore been studied at different stages of platelet activation, by using a monoclonal

Abbreviations used: BSA, bovine serum albumin; e.l.i.s.a., enzyme-linked immunosorbent assay; Mc-anti-hI, mouse monoclonal antibody to human calpain I; Pc-anti-rII, rabbit polyclonal antibody to rat calpain II; PBS, 0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.4.

* To whom correspondence and reprint requests should be addressed.

antibody specific for human calpain I and a polyclonal antibody which binds to both human calpains I and II. A preliminary account of some of this work has appeared [32].

MATERIALS AND METHODS

Calpain

Human calpain I was purified from red blood cells [33]. The cells were washed twice in PBS, and lysed twice in 5 mM-EDTA/1 mM-EGTA/10 mM-2-mercaptoethanol/0.3 mM-phenylmethanesulphonyl fluoride/20 mM-Tris/HCl, pH 7.5. All subsequent buffers contained 5 mM-EDTA, 10 mM-2-mercaptoethanol and 20 mM-Tris/HCl, pH 7.5, unless otherwise stated. Calpain I was recovered from the red-blood-cell lysate by absorption on to DEAE cellulose, elution with 0.15 M-NaCl, precipitation with $(\text{NH}_4)_2\text{SO}_4$, and chromatography successively on columns of Sepharose 6B in 0.1 M-NaCl, on phenyl-Sepharose loaded in 0.3 M-NaCl and finally eluted with 20% (v/v) glycerol/1 mM-EDTA/1 mM-Tris/HCl, pH 7.5 [34,35], and on DEAE-Sephacel with a gradient of NaCl, the enzyme being eluted between 0.08 and 0.12 M-NaCl, which is characteristic of calpain I. It was precipitated from the column eluate with 10 vol. of ethanol at -20°C in the presence of sodium acetate and 20 mM-dithiothreitol. The precipitate was redissolved in 8 M-urea/0.1% SDS at a protein concentration of 1 mg/ml, and used for immunization of mice.

Calpains I and II from rat muscle and calpain II from human lung were obtained by extraction of tissues in 0.1 M- NaHCO_3 , pH 8, absorption of the extract on to DEAE-cellulose, elution with a salt gradient, concentration with $(\text{NH}_4)_2\text{SO}_4$, and then chromatography successively on columns of Sepharose 6B, phenyl-Sepharose and DEAE-Sephacel; all buffers contained 10 mM-2-mercaptoethanol and 5 mM-EDTA. The products were stored in the presence of 40% glycerol at -20°C , and retained their activity for 1–2 years.

For calpain assay, all incubations contained 0.2 ml of casein (10 mg/ml), 5 mM-EDTA or 5 mM- CaCl_2 , and enzyme sample in a total volume of 0.4 ml of 50 mM-Tris/HCl, pH 7.8; the incubations were carried out for 20 min at 25°C , and were stopped by addition of 0.3 ml of ice-cold 10% trichloroacetic acid. The unit of activity for both calpains I and II was defined as a Ca^{2+} -dependent increase in 20 min of 0.1 A_{280} unit in the acid supernatant. The specific activity of purified rat calpain II in these units is approx. 1000 units/mg [36].

Monoclonal-antibody production

Six female CB6F1/J mice, 6–8 weeks old, each received the following intraperitoneal injections of human calpain I: 25 μg in emulsion in Freund's complete adjuvant on day zero; 25 μg in incomplete adjuvant on days 25 and 61; 50 μg without adjuvant on days 77 and 83; and a final dose of 50 μg 4 days before the fusions, which were performed over the period of days 90–110.

The fusions (two from each mouse spleen) were performed with 4×10^7 spleen lymphocytes from the immunized mouse and 2×10^7 Sp2/0 cells, by established procedures [37,38]. The products of each fusion were distributed initially into two 24-well plates in HAT medium, and positive cell lines were cloned twice by limiting dilution down to 0.25 cell/well.

The hybridoma supernatants were tested by e.l.i.s.a. on microtitre plates coated either with human calpain I or with rat calpain II, and selected supernatants were tested further by immunoblotting and platelet immunofluorescence. Cell lines were selected for maximum reaction with human calpain I and minimum reaction with rat calpain II. Isotyping of the secreted immunoglobulins was performed with the Boehringer-Mannheim subtyping kit.

Ascites fluids were raised in female CB6F1/J mice primed with 0.5 ml of Pristane intraperitoneally 7–10 days before injection of 10^7 cells, and the antibodies were purified by means of the Bio-Rad 'MAPS II' kit, which is based on staphylococcal Protein A.

E.l.i.s.a. on purified calpain

Microtitre plates were coated with 0.1 ml of purified calpain solutions per well, at a concentration of approx. 1 unit/ml in 0.1 M- NaHCO_3 /5 mM-EDTA, pH 9.5, overnight at 4°C . The plates were blocked with 3% BSA/5% normal goat serum in PBS, and then incubated with primary antibodies and alkaline-phosphatase-conjugated second antibodies diluted in 0.5% BSA/0.15 M-NaCl/5 mM-EDTA/50 mM-Tris/HCl/0.05% Nonidet P40/5% normal goat serum, pH 7.4, with extensive washing at each step. The substrate for colour development was 1 mg of *p*-nitrophenyl phosphate/ml in 0.5 mM- MgCl_2 /0.1 M-Tris/HCl, pH 9.5.

E.l.i.s.a. to detect calpain

Solutions thought to contain calpain were serially diluted with 0.1 M- NaHCO_3 /5 mM-EDTA, pH 9.5, in some cases with addition of known amounts of standard calpain, in the wells of microtitre plates and left overnight at 4°C to permit any calpain which was present to coat the wells. The plates were then incubated with fixed amounts of primary and secondary antibodies. In the absence of major interference with the coating step by excess foreign protein or detergent, this assay detects calpain at concentrations ≥ 20 units/ml.

Platelet preparation

Human platelets were isolated and washed by a modification of the method of Mustard *et al.* [39]. Whole human blood (10–40 ml) was collected slowly from a forearm vein through a gauge-20 needle into a plastic syringe, and gently mixed with 0.1 vol. of 3.8% (w/v) trisodium citrate in a plastic tube. The blood was centrifuged at 250 *g* for 15 min at room temperature, and the platelet-rich plasma was removed with a plastic transfer pipette to within 3–5 mm of the interface. After adjustment of the pH to 6.5 with 1 M-citric acid, the mixture was centrifuged at 500 *g* for 15 min at room temperature. The supernatant plasma was gently decanted and discarded, and the platelet pellet derived from 10 ml of blood was gently resuspended at 37°C in 1 ml of 136 mM-NaCl/2.7 mM-KCl/0.42 mM- NaH_2PO_4 /12 mM- NaHCO_3 /2 mM- MgCl_2 /2 mM- CaCl_2 /BSA (2.8 mg/ml)/5.56 mM-glucose/apyrase (500 units/ml; Sigma)/22 mM-trisodium citrate, pH 6.5. The resuspended platelets were transferred to a clean plastic tube, made up to 4 ml with the same buffer, and incubated for 20–30 min at 37°C . Resting platelets were obtained after two more washes in this buffer at 37°C , with centrifugation at 500 *g* for 15 min at room temperature, and BSA was omitted from the final resuspension buffer. The platelets were

fixed by addition of an equal volume of freshly prepared 4% (w/v) *p*-formaldehyde in PBS at room temperature, and incubation for 1 h [40]; they were judged to be resting from their discoid shape and absence of pseudopodia when viewed in the electron microscope (J. A. Samis & J. S. Elce, unpublished work). From 10 ml of whole blood, a yield of 4 ml containing 1×10^8 – 5×10^8 platelets/ml was obtained.

Platelet activation

When intended for activation, the cells were finally resuspended at 37 °C in 136 mM-NaCl/2.7 mM-KCl/0.42 mM-NaH₂PO₄/12 mM-NaHCO₃/2 mM-MgCl₂/2 mM-CaCl₂/5.56 mM-glucose/20 mM-Hepes, pH 7.4. These platelets (1×10^8 – 5×10^8 /ml) were activated by addition of bovine thrombin (Sigma) to a final concentration of 1 N.I.H. unit/ml, and mixing by two very gentle inversions of the tube. Activation was allowed to occur for 10 min at 37 °C without stirring, and was stopped by addition of an equal volume of 4% *p*-formaldehyde in PBS, and fixation was allowed to proceed for 1 h at room temperature. The platelets were judged to be activated from their loss of discoid shape and the presence of pseudopodia when viewed in the electron microscope (J. A. Samis & J. S. Elce, unpublished work).

Platelet aggregation

The activated platelets described above had been treated with thrombin without stirring; aggregation was initiated by addition of thrombin to resting platelets stirred with a magnetic stirrer; the aggregation was then clearly visible after about 2 min, and reaction was stopped by heating with an equal volume of gel sample buffer (see below). Aggregation was also monitored in parallel preparations in an aggregometer, generating a characteristic trace; secondary aggregation was visible 1–2 min after thrombin addition, and aggregation was essentially complete after 4–5 min.

Suspension e.l.i.s.a. for platelet calpain

This assay was performed at room temperature. Platelet washing was carried out by centrifugation of fixed platelets at 2000 *g* for 5 min, resuspension, and incubation for 15 min. Each sample contained the fixed platelets derived from 5 ml of blood resuspended at every step in 1 ml of 10 mM-lysine/0.25% BSA/5% normal goat serum in PBS, pH 7.4, either without (unpermeabilized) or with (permeabilized) 0.1% saponin [40]. All antibodies, diluted in the appropriate buffer, were centrifuged for 15 min at approx. 100 000 *g* in a Beckman Airfuge immediately before use to remove aggregated material.

After fixation, the resting or activated platelets were washed three times in PBS. The platelets were incubated for 1 h in buffer, then overnight with the first antibody [diluted 1:100 (monoclonal) or 1:50 (polyclonal) in buffer], washed three times in buffer, and incubated for 4 h with alkaline-phosphatase-conjugated second antibodies, diluted 1:200 in buffer. After three further washings in buffer, and one washing in 0.1 M-Tris/0.5 mM-MgCl₂, pH 9.5, the platelets were finally incubated in 1 ml of 1 mg of *p*-nitrophenyl phosphate/ml in the Tris/MgCl₂ buffer. The *A*₄₁₅ of the supernatants was read 1 h later after centrifugation of the cells (2000 *g* for 3 min). The cells remaining at the end of the

procedure were counted in a haemocytometer, and the absorption values were not corrected for cell numbers, since the recovery of cells was always 70–75%. Contamination with other cell types was less than 0.6%.

Aggregated platelets were not tested by this e.l.i.s.a., since the access of antibodies into an aggregate is uncertain, and only a small proportion of the total membrane surface may be accessible.

SDS/polyacrylamide gels and immunoblotting

When intended for SDS/polyacrylamide-gel analysis, portions of platelet suspensions (unfixed) were mixed with an equal volume of SDS gel sample buffer [41] preheated to 95 °C, and the mixtures were immediately heated at 95 °C for 5 min; after cooling, solid urea (80 mg/0.1 ml of solubilized platelet mixture) was added in some cases, but did not affect the results. SDS/polyacrylamide-gel electrophoresis and immunoblotting were carried out by standard procedures [36,41,42], usually with alkaline-phosphatase-linked second antibodies. For quantification, ¹²⁵I-labelled second antibodies were used, and the immunoblots were then dried and autoradiographed; the autoradiographs were scanned on a densitometer, and the radioactive areas of the blots were cut out for measurement of the bound ¹²⁵I in a γ -radiation counter [43,44].

RESULTS

Twelve fusions were performed with spleen cells from six mice. From three of the mice, a total of 22 wells in the 24-well plates appeared initially to be positive for calpain, and ultimately four different cell lines were selected, after two cloning steps, which appeared to be those most specific for human calpain I. The antibodies produced were all IgG₁ (kappa) and were directed against distinct epitopes on human calpain I, but only one was used in this work. Its laboratory name is 'B27D8 sc E11', but it is referred to here simply as 'Mc-anti-hI'.

A polyclonal antibody has been raised previously in rabbits inoculated with rat calpain II, and was purified by affinity chromatography on rat calpain II covalently linked by a disulphide bond to thiopropyl-Sepharose [36]; it is referred to here as 'Pc-anti-rII'.

Mc-anti-hI. This antibody was selected by means of e.l.i.s.a., by platelet immunofluorescence, and by its binding on immunoblots to the expected 80 kDa band in purified calpains, a band identical with that which binds to Pc-anti-rII. The monoclonal antibody did not bind active human calpain I, so that immunoprecipitation (with the aid of staphylococcal Protein A and rabbit anti-mouse IgG) did not work; similarly, the purified antibody when coupled either to CNBr-Sepharose, or to aminoethyl-Sepharose (with the aid of carbodi-imide), failed to bind active human calpain I. To demonstrate the specificity of Mc-anti-hI for human calpain I, samples were therefore taken from fractions of a DEAE-Sephacel column, run on SDS/polyacrylamide gels, and tested by quantitative immunoblotting. Fig. 1 shows that the amount of Mc-anti-hI bound (to the expected 80 kDa band of highly purified human calpain I) was closely parallel to the calpain activity in the column fractions.

Pc-anti-rII. The crude rabbit anti-rat calpain II antiserum had previously been shown to give a single immunodiffusion line and a single rocket against rat

Fig. 1. Correlation of Mc-anti-hI binding to human calpain I and calpain activity

Human calpain I (100 units) purified from red blood cells was run on a DEAE-Sephacel column (0.9 cm $\times$ 8 cm) with a gradient of NaCl. The eluted fractions (2.1 ml) were assayed for calpain activity, and samples of the fractions were run on SDS/polyacrylamide gels, blotted, and exposed to Mc-anti-hI, followed by 125 I-labelled goat anti-mouse IgG, autoradiography and densitometry of the autoradiograph. $\square$, Calpain activity; $\triangle$, weight of the densitometer peaks.

calpain II, and to bind to the 80 kDa band of rat calpain II on immunoblots [36]. After affinity purification on thiopropyl-Sepharose-rat calpain II, the resultant Pc-anti-rII bound to a single 80 kDa band in blots of human calpain I and II (see below), and showed a close correlation of binding on quantitative immunoblots with enzyme activity in column fractions, of both human and rat calpain II (results not shown), in the same manner as shown above for Mc-anti-hI.

Relative affinities. The relative affinities of these two antibodies for different calpains were compared in e.l.i.s.a. (Fig. 2) and on immunoblots (Fig. 3).

Fig. 2(a) shows that Mc-anti-hI bound very strongly to human calpain I in e.l.i.s.a. and very little to other calpains; in contrast (Fig. 2b), Pc-anti-rII (although binding most strongly to rat calpain II) bound about equally to human calpains I and II.

When calpains were coated on micro-titre plates and then treated with 2% formaldehyde in PBS, the maximum binding of both antibodies was decreased by about 25%; this observation is relevant to the measurements on formaldehyde-fixed platelets described below.

The results of immunoblots developed with the aid of alkaline phosphatase-linked second antibodies are shown in Fig. 3. (The same relative sensitivities were observed with 125 I-labelled second antibodies, but the radioactivity bound by the less-preferred antigens was too low to give neat plots.) Fig. 3(a) shows that Mc-anti-hI can detect down to about 16 munits of human calpain I and 75 munits of human calpain II on a blot, and in addition that it detected a single 80 kDa band in 4×10^6 resting human platelets. Pc-anti-rII (Fig. 3b) could just detect 65 munits of human calpain I and 75 munits of human calpain II, and detected nothing in 4×10^6 platelets.

Fig. 2. Binding specificities of anti-calpain antibodies compared by e.l.i.s.a.

Wells on two microtitre plates were coated with approximately equal amounts of purified calpains: human I ($\square$); human II ($\blacksquare$); rat I ($\triangle$); rat II ($\circ$). One plate (a) was screened with 5-fold serial dilutions of Mc-anti-hI, and the other plate (b) similarly with Pc-anti-rII. Primary antibody was omitted from control wells. The two plates were carried through the e.l.i.s.a. in parallel, and final absorptions were read at 405 nm on a Titer Tek Multiscan after the same time interval.

Quantification of calpain in human platelets

A rough estimate of the calpain content of platelets could be made from Fig. 3(a), but Fig. 4 shows the results of a quantitative immunoblot using Mc-anti-hI and 125 I-labelled goat anti-mouse IgG. The amount of 125 I-labelled anti-mouse IgG bound to the calpain which is present in the gel tracks of solubilized platelets, as indicated by an arrow in Fig. 4, led to a value of 37.5 munits of calpain I/ 8×10^6 platelets, or about 0.47 unit of calpain I/ 10^8 platelets. Immunoblot measurements also showed that the total amount of calpain I per cell did not change during activation and aggregation (result not shown).

Since Pc-anti-rII plus 125 I-labelled goat anti-rabbit IgG did not detect anything in 8×10^6 platelets, and bearing in mind the relative sensitivities of the antibodies shown in Fig. 3, it is possible to say that the amount of human calpain II present is less than about 70 munits/ 8×10^6 platelets, or less than about 0.9 unit/ 10^8 platelets.

Fig. 3. Immunoblot of human calpains I and II and of human platelet extract

Human calpains and solubilized resting human platelets were run on SDS/10% polyacrylamide gels, blotted on to nitrocellulose, and exposed (a) to Mc-anti-hI, or (b) to Pc-anti-rII, followed by alkaline-phosphatase-linked second antibodies and colour development. The tracks on both gels contained: (1)–(6) 8.1, 16.2, 32.5, 65, 98 and 130 munits of human calpain I respectively; (7) 4×10^6 platelets; (8)–(13) 9.4, 18.8, 37.5, 75, 150 and 200 munits of human calpain II respectively.

Release of calpain into supernatant during platelet activation

The supernatants of resting and activated, unfixed, platelets, derived from suspensions containing 2×10^8 platelets/ml, were assayed by e.l.i.s.a. to detect a possible release of calpain into the extracellular medium during activation. Portions of the supernatants were adjusted to 5 mM-EDTA, and standard calpain was added to some samples; these solutions, and solutions of standard calpain alone, were mixed with 0.1 M- NaHCO_3 /5 mM-EDTA, pH 9.5, and used to coat microtitre plates, which were then screened with Mc-anti-hI. It was found that the platelet supernatant did not interfere with the binding of standard calpain to the wells, and that no calpain I was detectable in the supernatants. From the standard curves, it could be calculated that more than 2% of the total platelet calpain I would have been detected by this assay.

Platelet suspension e.l.i.s.a.

The values in Table 1 indicated that calpain could be detected in fixed platelets only after permeabilization, whether they were resting or thrombin-activated. The results given are derived from platelets from a single sample of blood, but the experiment was repeated several times with different blood donors, with fully consistent

Fig. 4. Quantification of calpain I in platelets by immunoblotting

Human calpain I (10–100 munits) and solubilized resting human platelets (8×10^6 cells) were run on SDS/polyacrylamide gel, blotted, and exposed to Mc-anti-hI and ^{125}I -labelled goat anti-mouse IgG, followed by autoradiography and determination of the radioactivity bound to the indicated areas of nitrocellulose by γ -radiation counting. The diagram shows the observed radioactivity (c.p.m.) as a function of munits of human calpain I (Δ), and the arrow indicates radioactivity (c.p.m.) observed for 8×10^6 platelets (---). The line was plotted by linear least-squares approximation.

results. It was also consistently observed, as shown in Table 1, that the A_{415} derived from permeabilized activated platelets was roughly twice that from permeabilized resting platelets, although the total cell calpain content, as measured on a blot, did not change during activation and aggregation.

Changes in the 80 kDa subunit of calpain I during platelet activation and aggregation

The immunoblot shown in Fig. 5 indicates that no change in molecular mass of the large subunit of calpain I occurred in 10 min of thrombin activation (and not even in 30 min; results not shown); on stirring the platelets with thrombin to promote aggregation, a rapid removal of the 80 kDa subunit occurred, and most of it was replaced by a doublet of approx. 76 and 78 kDa, which was just detectable at 1 min, and predominated at 4 min. The doublet is not distinct in the photograph, but was clearly resolved on some blots. A further degradation of calpain also occurred in the aggregating platelets, as shown by the immunoreactive bands of much lower molecular mass which are visible in Fig. 5 in the platelets aggregated for 4 min.

DISCUSSION

Specificity of the antibodies

Much evidence had suggested a close relationship between calpains I and II, and DNA sequencing has now clearly shown that the 80 kDa subunits of the calpains are coded for by very similar but distinct genetic elements [45]. As a consequence of this high degree of similarity, all antisera so far described have shown some cross-reactivity between calpains I and II, and between calpains in different species [46].

Table 1. E.l.i.s.a. of calpain in fixed resting and activated platelets in suspension

The results are given as A_{415} , and each value is the average of duplicate assays

Cells	Monoclonal antibodies			Polyclonal antibodies		
	Positive*	Control†	Δ	Positive‡	Control§	Δ
Resting unpermeabilized	0.095	0.077	0.018	0.060	0.023	0.037
Resting permeabilized	0.369	0.100	0.269	0.287	0.107	0.180
Activated unpermeabilized	0.071	0.085	-0.014	0.066	0.034	0.032
Activated permeabilized	0.703	0.102	0.600	0.444	0.064	0.380

* Mc-anti-hI.
† Irrelevant mouse IgG₁ monoclonal antibody.
‡ Pc-anti-rII.
§ Irrelevant rabbit IgG polyclonal antibody.

Fig. 5. Conversion of calpain I from 80 kDa into 76–78 kDa

Samples of platelet suspensions were mixed with an equal volume of preheated gel sample buffer and heated at 95 °C for 5 min. Portions were run on SDS/polyacrylamide gels, blotted, and probed with Mc-anti-hI and alkaline-phosphatase-linked goat anti-mouse IgG. The tracks contained: (1) standard human calpain I; (2) resting platelets; (3) platelets activated with thrombin for 1 min; (4) platelets activated with thrombin for 10 min; (5) platelets aggregated by stirring with thrombin for 4 min. The platelet samples were each derived from 4×10^6 cells.

The hybridoma-selection procedures had indicated that Mc-anti-hI bound strongly to calpain I and minimally to calpain II. However, the antibody did not immunoprecipitate active calpain I, so that its specificity for human calpain I had to be confirmed in other ways. It was shown that Mc-anti-hI bound on immunoblots to a single 80 kDa band which corresponded to the protein

band in highly purified calpain I and II, and that this 80 kDa band in calpain II is identical with that which also binds Pc-anti-rII (Figs. 3a and 3b); and it was shown that the amount of Mc-anti-hI bound on an immunoblot was closely correlated with the activity of highly purified calpain I in the fractions from an ion-exchange column (Fig. 1).

The monoclonal antibody distinguished between the two forms of human calpain, at least in terms of sensitivity. On blots, where the antigens are thoroughly denatured, the sensitivity of Mc-anti-hI for calpain I was about 5 times greater than for calpain II (Fig. 3a); in e.l.i.s.a., where the extent of denaturation of calpains adsorbed to the plastic surface is not known, Mc-anti-hI bound vastly better to calpain I than to calpain II (Fig. 2a).

It was concluded that Mc-anti-hI is specific for the denatured form of the 80 kDa subunit of calpain, with a marked preference for calpain I.

The polyclonal antibody does not distinguish significantly between calpains I and II, and we have not yet obtained a monoclonal antibody highly sensitive and specific for calpain II, in spite of much effort.

Quantification of calpain I

The immunoblots in Fig. 3, and the quantification in Fig. 4, established that human platelets contain about 0.5 unit of calpain I/ 10^8 cells. Calpain II could not be detected at all in the cell extracts on the blots, although standard calpain II was readily detected. Both calpains are known to be present in human platelets, although the reports differ about the relative amounts of the two forms [4–8]. Our results permit the conclusion only that calpain II must be present at less than about 0.9 unit/ 10^8 cells, and this is not inconsistent with the report that calpain I is present in human platelets in larger amounts than calpain II [7].

Quantitative comparisons cannot be made between the data of Table 1 and those of Figs. 3 and 4, since the state of the calpain differs. In the fixed platelets (Table 1), the extent of aldehyde fixation of the intracellular calpain, and its accessibility to antibodies, are not precisely known, nor is a standard calpain in the same state readily available. In contrast, the calpain bands on the nitrocellulose blots, in both standard and unknown samples, are fully exposed to the antibodies.

Externalization of calpain during platelet activation

Apart from the possible translocation of calpain to the exterior platelet membrane, it was conceivable that calpain might be secreted into the cell-suspension buffer in a soluble form. In fact, no calpain could be detected by e.l.i.s.a. in the medium, although we cannot exclude the possibility that small amounts of calpain, if released into the external medium which contained Ca^{2+} , might be extensively degraded by autolysis. In this case, calpain would not seem likely to have an important extracellular function.

In the platelet-suspension assay (Table 1), each value represented about 5×10^8 cells, 100 times more than in the blots of Figs. 3 and 4, so that the amounts both of calpain I and presumably also of calpain II were sufficient to be readily detected by either antibody. For this reason, it is not possible to determine in these assays the relative contributions of calpains I and II to the final absorption values. Calpain on the outside of cells should be readily accessible to antibodies, and therefore easily detected, since antibody binding is not seriously impaired by formaldehyde treatment. Traces of calpain could, however, be obscured by the control absorption values, but there are no significant changes in the control values in going from resting to activated cells. The values in Table 1 therefore indicate that no significant amount of calpain (I or II) can be detected on the external surface of fixed platelets, before or after thrombin activation.

At present we have no explanation of why the platelet-suspension e.l.i.s.a. values for activated permeabilized cells were always about twice those for resting permeabilized cells, although the control antibody values were unchanged. New calpain synthesis is obviously not involved in the rapid events of platelet activation, and the immunoblots had shown that both the calpain content, and its molecular mass, did not change during platelet activation. One possibility is that a change in the environment or distribution of calpain [47], or in its binding to cytoskeletal proteins, inhibitors or other platelet components, could accompany platelet activation, and permit greater access of the antibodies to calpain in the formaldehyde-fixed saponin-permeabilized cells. This question can be investigated by means of immunocytochemistry.

Autolysis of calpain I

The values of $[\text{Ca}^{2+}]$ reported to be necessary for calpain activity vary widely, but are usually higher than seems physiologically likely, even for calpain I and more obviously for calpain II. For this reason, activation of calpain by phospholipids, and/or by (auto)lysis to forms of lower molecular mass which have a lower Ca^{2+} requirement, have been proposed as regulation mechanisms [19–21]. It is very difficult to exclude the possibility that calpain I may be active even in the 80 kDa form during the early phase of platelet activation, towards some at present unidentified substrates. Further, since our antibodies do not bind to the 30 kDa subunit, a possible regulation by changes in this subunit also cannot be excluded [21].

In this work, it has been shown for calpain I that the 80 kDa form of the larger subunit remains intact during thrombin activation of platelets, and that conversion into a 76–78 kDa form occurs rapidly when the platelets are

stirred to promote aggregation. This correlates interestingly with the report that $0.5 \mu\text{M}-\text{Ca}^{2+}$ is sufficient to induce platelet shape-change, but that $2 \mu\text{M}-\text{Ca}^{2+}$ is necessary for aggregation [15]. It agrees also very well with the previous observation that hydrolysis of actin-binding protein and P235 is not seen until aggregation is allowed to occur [3].

The results therefore support the ideas (i) that calpain I is not active until it is converted into a 76–78 kDa form, and (ii) that calpain is responsible for hydrolysis of actin-binding protein and P235 during platelet aggregation.

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