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# Investigation of the role of calpain as a stimulus–response mediator in human platelets using new synthetic inhibitors

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A series of peptidyl diazomethanes and monofluoromethane with structures specific for calpain have been synthesized and tested for their ability to inhibit calpain activity *in vivo*, using human platelets as a model system. Calpain activity *in vivo* was determined by observing proteolysis of actin-binding protein and talin, two known substrates of calpain. Very potent inhibitors, which emerged from this study, were used to investigate the role of calpain in some platelet response processes. Our results show that calpain-mediated proteolysis in platelets is not an obligatory event leading to change of cell shape, adhesion to glass and spreading, aggregation and 5-hydroxytryptamine release. Two of the inhibitors were iodinated with $^{125}I$ and used to radiolabel the enzyme *in vivo*. To our knowledge, this work also represents the first report describing the affinity labelling of calpain in human platelets using irreversible radioactive inhibitors.

## INTRODUCTION

Calcium-activated neutral proteinases, also known as calpains, are cytosolic or membrane-bound cysteine proteinases which depend on $Ca^{2+}$ for activity (Murachi *et al.*, 1981; Hatanaka *et al.*, 1984). Two forms are known, and their genes have been sequenced (Murachi, 1983; Emori *et al.*, 1986; Miyake *et al.*, 1986; Imajoh *et al.*, 1988). Cytoskeletal proteins are the major protein substrates hydrolysed *in vitro* by calpain (Beckerle *et al.*, 1986; Perides *et al.*, 1987; Fox *et al.*, 1987; Herman *et al.*, 1987; Billger *et al.*, 1988). However, the extensive biochemical knowledge of the structure and distribution of the enzyme has not been matched by knowledge of its physiological role.

Blood platelets are highly reactive and undergo structural changes when activated. Upon stimulation with agents such as thrombin, collagen or the $Ca^{2+}$ ionophore A23187, platelets change shape, secrete the contents of at least two types of secretory granules and aggregate (Rink & Hallam, 1984; Huang & Detwiler, 1986). A dramatic transformation of the cytoskeleton accompanies certain platelet responses. With the exception of phospholipase C stimulation, $Ca^{2+}$ ionophores are able to elicit the same cellular and molecular responses as do physiological agonists (Feinstein *et al.*, 1981; Rittenhouse & Horne, 1984). Platelet activation by many agonists is associated with an immediate rise in the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (Rink & Hallam, 1984; Ware *et al.*, 1986). $Ca^{2+}$-independent pathways of platelet responses have also been proposed (Rink *et al.*, 1982). A $Ca^{2+}$-activated proteinase has been implicated in the reorganization of the cytoskeleton, possibly related to other platelet responses (Verhallen *et al.*, 1987). The role of calpain as an activator of protein kinase C and phosphatidylinositol-specific phospholipase C in platelets has also been suggested (Ishii *et al.*, 1990).

Attempts to use inhibitors of calpain to deduce its role in cellular responses have made use chiefly of leupeptin, which is, however, a reversible inhibitor of very low selectivity. The results thus obtained are necessarily ambiguous. We recently started a project aimed at developing specific, irreversible and cell-permeant calpain inhibitors as an extension of our *in vitro* studies leading to reagents capable of selective inactivation of endopeptidases (Crawford *et al.*, 1988; Kirschke *et al.*, 1988) and exopeptidases (Angliker *et al.*, 1989) of the papain family. New synthetic inactivators of calpain, including radioactive and fluorescent forms, have been obtained. Initial results with platelets indicate a blocking of certain cytoskeletal changes on platelet activation and a more selective action than that of leupeptin.

We report here our results on the use of the radioactive forms of Cbz-Leu-Leu-Tyr-$CHN_2$ and Cbz-Leu-Leu-Tyr-$CH_2F$ (Cbz = benzyloxycarbonyl), two of the novel calpain inhibitors, and an analysis of the labelling pattern of intracellular proteins in platelets in an attempt to correlate loss of platelet responses (such as shape change, secretion, adhesion to glass and spreading, and aggregation) with the inactivation of calpain.

## MATERIALS AND METHODS

The following materials were purchased from the sources indicated: Iodo-beads, Pierce Chemical Co.; fura-2/AM and $Ca^{2+}$ ionophore 4-bromo-A23187, Sigma Chemical Co.; leupeptin, Bachem Feinchemikalien, Bubendorf, Switzerland; ADP, Fluka AG, Buchs, Switzerland; 5-hydroxy[G-$^3$H]tryptamine creatinine sulphate and $Na^{125}I$, Amersham; dimethyl-BAPTA/AM, Molecular Probes. Human $\alpha$-thrombin was kindly provided by Dr. Jan Hoffstenge, Friedrich Miescher-Institut, Basel, Switzerland. Cbz-Leu-Leu-Tyr-$CHN_2$, Cbz-Tyr(I)-Ala-$CHN_2$, Boc-Val-Lys(Z)-Leu-Tyr-$CHN_2$ and Cbz-Phe-Ala-$CHN_2$ were synthesized as described previously (Crawford *et al.*, 1988). Synthesis of the other peptidyl diazomethanes and monofluoromethane (H. Angliker, J. Anagli & E. Shaw, unpublished work) studied in this work will be described later, and details are available from the authors on request.

### Iodination of inhibitor

Cbz-Leu-Leu-Tyr-$CHN_2$ and Cbz-Leu-Leu-Tyr-$CH_2F$ were iodinated using the Iodo-bead method (Markwell, 1982). One Iodo-bead was added to a micro-reaction vessel containing 10 $\mu$l of 50 mM-sodium phosphate buffer, pH 7.5, 10 $\mu$l of $Na^{125}I$

Abbreviations used: ABP, actin-binding protein; Ac, acetyl; Boc, t-butyloxycarbonyl; Cbz or Z, benzyloxycarbonyl; PAB, *p*-aminobenzoyl; Tyr(I), iodotyrosine; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; PRP, platelet-rich plasma.

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(1 mCi) and 25 $\mu$l of inhibitor (1 mM in 50 % ethanol) at 0 °C for 10 min. Then 455 $\mu$l of buffer was added and the reaction was stopped by removing the Iodo-bead.

### Isolation of platelets

Freshly drawn venous blood mixed with 3.8 % (w/v) trisodium citrate (9:1, v/v) was centrifuged at 200 *g* for 15 min to obtain platelet-rich plasma (PRP). This and subsequent isolation steps were done at room temperature. The PRP was further centrifuged with EDTA (final concentration 5 mM) at 2500 *g* for 15 min. The sedimented platelets were washed three times in a solution containing 68.44 mM-NaCl, 2.7 mM-KCl, 0.5 mM-$MgCl_2$,$6H_2O$, 0.24 mM-$NaH_2PO_4$,$H_2O$, 12 mM-$NaHCO_3$, 5.55 mM-glucose and 1 mM-EDTA, pH 7.4 (buffer A), resuspended in buffer A [$(4–5) \times 10^8$ platelets/ml] and incubated at 37 °C.

### Treatment of platelets with inhibitors

Portions (1 ml) of platelet suspension were incubated with various concentrations of the synthetic proteinase inhibitors at 37 °C for 1 h. Platelets were then activated in buffer A containing inhibitor or washed and resuspended in inhibitor-free buffer before activation.

For shape change and aggregation experiments, the inhibitors were added to PRP and incubated at 37 °C for 1 h. Stock solutions of the inhibitors of 10 mM in dimethyl sulphoxide were used, and the final dimethyl sulphoxide concentrations were less than 1 %.

### Activation of platelets

Platelets in buffer A containing 5 mM-$CaCl_2$ were activated with either 1 $\mu$M of the $Ca^{2+}$ ionophore 4-bromo-A23187 or 0.1 unit of thrombin/ml at 37 °C for 30 min. Shape change of platelets in PRP was induced with 0.2 $\mu$M-ADP. To initiate aggregation, 1 mM-$CaCl_2$ was added to PRP followed 30 s later by 40 $\mu$M-ADP.

### Measurement of changes in $[Ca^{2+}]_i$

A portion of PRP was incubated with 1 $\mu$M-fura-2/AM at 37 °C for 30 min. The fura-2-loaded platelets were washed and 600 $\mu$l portions were incubated in the presence or absence of inhibitors (10–100 $\mu$M), as described above. Changes in $[Ca^{2+}]_i$ were determined by recording the fura-2 fluorescence excitation spectra from 300 to 400 nm (the emission was collected at 510 nm), before and after platelet stimulation with thrombin or ionophore, at 37 °C in a Perkin–Elmer 610S fluorimeter by the method of Grynkiewicz *et al.* (1985). Control experiments with platelets not loaded with fura-2 were performed, and corrections made for autofluorescence.

### Measurement of shape change and aggregation

Platelet shape change in PRP was determined in an Elvi 840 aggregometer by measuring the maximal light absorption as described previously (Laubscher & Pletscher, 1979). For this, the platelet number in the PRP was adjusted to $10^8$ platelets/ml with Tyrode's solution without $Ca^{2+}$. ADP was added to PRP, incubated at 37 °C with constant stirring at 1000 rev./min, to induce shape change response. Aggregation was measured according to Born & Cross (1963).

### Videomicroscopy

A glass coverslip was glued over a hole in the bottom of a Petri dish, and 50 $\mu$l of a platelet suspension was added to the dish filled with Tyrode's solution containing 2 mM-$CaCl_2$. Settling and spreading of the platelets was observed with an inverted microscope (Zeiss Axiovert 35) equipped with a Newvicon camera (Hamamatsu C2400-07). A Plan-Neofluar 100× objective lens was used, and the differential interference contrast images were digitized and processed by an Imagel/AT image processing system (Universal Imaging, West Chester, PA, U.S.A.). At intervals of 20 s, 128 video frames were summed, an out-of-focus background was subtracted, and the resulting images were autoenhanced and recorded on an optical disc recorder (Panasonic, TQ-2028F).

### Secretion studies with 5-hydroxy[G-$^3$H]tryptamine

PRP was incubated with 10 $\mu$Ci of 5-hydroxy[G-$^3$H]tryptamine/ml at room temperature for 30 min, and the platelets were washed and treated with inhibitors as described above. After activation with thrombin, the platelets were fixed with an equal volume of 3.7 % (v/v) formaldehyde. Controls were treated under the same conditions. The fixed platelets were centrifuged at 2500 *g* for 10 min and the radioactivity in the supernatant was determined by liquid-scintillation counting (Beckman LS 3801).

### Labelling of proteins with radioactive inhibitor

Portions of platelet suspension (1 ml) were preincubated at 37 °C for 30 min, with or without the non-radioactive form of the inhibitor of cathepsins B and L, Cbz-Tyr(I)-Ala-$CHN_2$ (100 $\mu$M) (Mason *et al.*, 1989). Then Cbz-Leu-Leu-Tyr($^{125}$I)-$CHN_2$ or Cbz-Leu-Leu-Tyr($^{125}$I)-$CH_2F$ (0.1–0.5 $\mu$M) was added to the cell suspensions which were subsequently incubated for 1 h. The inhibitor-treated platelets were activated with $Ca^{2+}$ ionophore as described above. In some labelling experiments, platelets were loaded with an intracellular $Ca^{2+}$ chelator (15 $\mu$M-dimethyl-BAPTA/AM) for 15 min at 37 °C, incubated with proteinase inhibitor(s) in the presence of EDTA and then challenged with ionophore (1 $\mu$M-4-bromo-A23187). Samples were washed with buffer A and then with 1 ml of acetone to remove excess inhibitor.

### Gel electrophoresis and autoradioagraphy

SDS/PAGE was carried out according to Laemmli (1970). Autoradiography of $^{125}$I-labelled proteins was performed with Kodak X-OMAT film exposed at −70 °C.

## RESULTS

### Inhibition of $Ca^{2+}$-dependent proteolysis

Previous studies have shown that platelet calpain activity *in vivo* is manifested by the degradation of 260, 235 and 200 kDa proteins and the appearance of fragments of 190, 135, 93 and 48 kDa (see Phillips & Jakabova, 1977; White, 1980; Fox *et al.*, 1983; present paper, Fig. 1*a*). We therefore evaluated our synthetic inhibitors for potency and cell-penetrability by examining their ability to prevent $Ca^{2+}$-dependent proteolysis of the high-molecular-mass proteins cited above. Intact platelets were treated with inhibitors (at 50 $\mu$M final concentration) and activated with a $Ca^{2+}$ ionophore as described in the Materials and methods section. Cbz-Leu-Leu-Tyr(I)-$CHN_2$, Cbz-Leu-Leu-Tyr-$CHN_2$ and Cbz-Leu-Leu-Tyr-$CH_2F$, in an increasing order of inhibitory activity, were the most powerful in preventing proteolysis. Boc-Val-Lys(Z)-Leu-Tyr-$CHN_2$, Cbz-Leu-Leu-homoPhe-$CHN_2$, PAB-Leu-Leu-Val-$CHN_2$, Cbz-Tyr-Leu-Leu-Val-$CHN_2$ and Cbz-Tyr(I)-Leu-Leu-Ala-$CHN_2$ partially inhibited proteolysis. Cbz-Tyr(I)-Ala-$CHN_2$, Cbz-Phe-Ala-$CHN_2$ and Ac-Leu-Leu-Arg-H (leupeptin) could not protect the high-molecular-mass proteins examined from proteolytic degradation (Figs. 1*a* and 1*b*). *In vitro* studies have revealed Cbz-Tyr(I)-Ala-

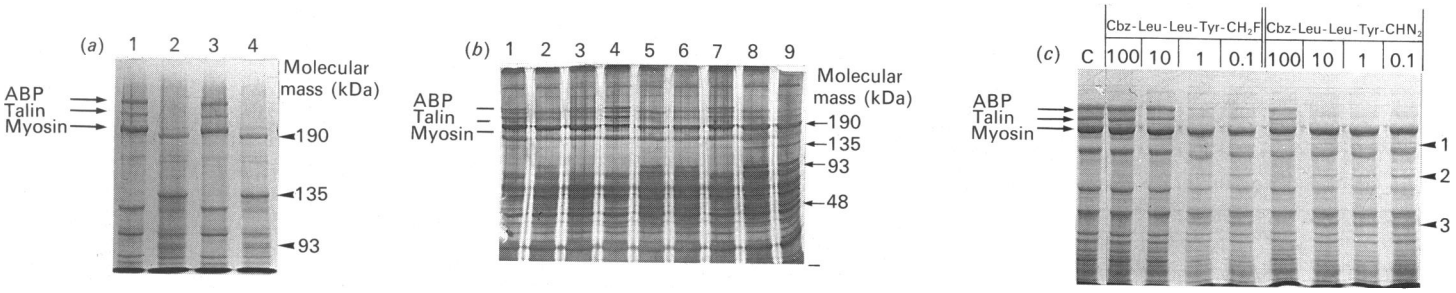


**Fig. 1. Evaluation of inhibitors for calpain inhibitory activity in intact human platelets**

SDS/7.5%-polyacrylamide-gel electrophoretograms of platelets treated with (*a*, *b*) 50 $\mu$M-inhibitor or (*c*) various concentrations of inhibitor for 1 h at 37 °C, followed by activation with $Ca^{2+}$ ionophore for 30 min. (*a*) Lane 1, 50 $\mu$M-Cbz-Leu-Leu-Tyr-$CHN_2$; lane 2, 50 $\mu$M-Cbz-Phe-Ala-$CHN_2$; lanes 3 and 4, no inhibitors (no $Ca^{2+}$ in lane 3). (*b*) Lane 1, Cbz-Leu-Leu-Tyr-$CHN_2$; lane 2, Cbz-Leu-Leu-homoPhe-$CHN_2$; lane 3, Boc-Val-Lys(Z)-Leu-Tyr-$CHN_2$; lane 4, Cbz-Leu-Leu-Tyr-$CH_2F$; lane 5, Cbz-Tyr-Leu-Leu-Val-$CHN_2$; lane 6, Cbz-Tyr(I)-Leu-Leu-Ala-$CHN_2$; lane 7, Cbz-Leu-Leu-Tyr(I)-$CHN_2$; lane 8, PAB-Leu-Leu-Val-$CHN_2$; lane 9, Ac-Leu-Leu-Arg-H (leupeptin). (*c*) Values indicate concentrations of inhibitors ($\mu$M); C, no treatment with inhibitor or with ionophore. In all parts, substrates are indicated on the left and fragments on the right.

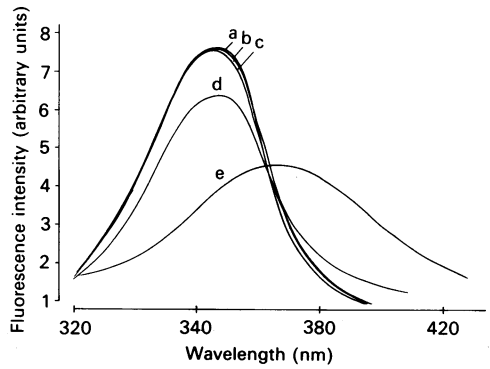


**Fig. 2. Fura-2 fluorescence spectra of resting and $Ca^{2+}$-ionophore-stimulated platelets**

Platelets were loaded with fura-2, incubated with or without inhibitor and the fura-2 fluorescence spectra were recorded before and after stimulation with $Ca^{2+}$ ionophore, as described in the Materials and methods section. Spectra a, b, c, d and e, fura-2-loaded platelets; spectra a, b and c, stimulation with $Ca^{2+}$ ionophore; spectrum a, stimulation in the presence of 100 $\mu$M-Cbz-Leu-Leu-Tyr-$CHN_2$; spectrum b, stimulation in the absence of inhibitor; spectrum c, stimulation in the presence of 100 $\mu$M-Cbz-Leu-Leu-Tyr-$CH_2F$; spectrum d, pretreatment with 100 $\mu$M-Cbz-Leu-Leu-Tyr-$CH_2F$ followed by the addition of $CaCl_2$ only.

$CHN_2$ and Cbz-Phe-Ala-$CHN_2$ to be very powerful inactivators of cathepsins B and L but unreactive with calpain (Crawford *et al.*, 1988). Their intracellular action has also been demonstrated (Shaw & Dean, 1980; Delaisse *et al.*, 1980; Grinde, 1983; von Figura *et al.*, 1986; Mason *et al.*, 1989). These results confirm the involvement of calpain but not cathepsins B and L in the degradation of the proteins examined. Cbz-Leu-Leu-Tyr-$CHN_2$ and Cbz-Leu-Leu-Tyr-$CH_2F$ emerged as the most powerful calpain inhibitors, the latter being effective at an extracellular concentration as low as 10 $\mu$M (Fig. 1*c*).

### Intracellular $Ca^{2+}$ concentrations

The fura-2 fluorescence excitation spectra shifted to shorter wavelengths and the amplitudes of the peaks increased as $[Ca^{2+}]_i$ increased (Fig. 2). $[Ca^{2+}]_i$ was estimated using ratios of fluorescence intensities at 340 and 380 nm, and a calibration curve obtained with a 5 mM-$Ca^{2+}$/EGTA buffer and 2 $\mu$M-fura-2 (free acid). The $[Ca^{2+}]_i$ was raised from a basal level of 90 nM to about 3 $\mu$M after stimulation with 4-bromo-A23187. Monitoring of $[Ca^{2+}]_i$ showed that the inhibition of protein degradation was not due to an inhibition of $Ca^{2+}$ entry into platelets. The rise of $[Ca^{2+}]_i$ after platelet stimulation was not affected by the presence of either Cbz-Leu-Leu-Tyr-$CHN_2$ or Cbz-Leu-Leu-Tyr-$CH_2F$.

**Table 1. Effect of calpain inhibitors on ADP-induced shape change and aggregation of human platelets**

Portions of PRP of 250 $\mu$l ($4 \times 10^8$ platelets/ml) and 500 $\mu$l ($2 \times 10^8$ platelets/ml), for aggregation and shape change responses respectively, were pretreated with various concentrations of the proteinase inhibitors and the responses were induced with ADP as described in the Materials and methods section. Controls (no inhibitor) contained 1% dimethyl sulphoxide. The aggregometer was calibrated to 100% light transmittance with platelet-poor plasma and 0% light transmittance with PRP for the aggregation experiments. For shape change measurements, the recorder was set to the baseline before platelet stimulation and the minimal transmittance (maximal absorbance) was recorded for each shape change response. The light absorption is expressed as deflection on the recorder scale in mV. N.D., not determined.

| Inhibitor concn. ($\mu$M) | *n* | Shape change (light absorption, mV) | Aggregation (%) |
|---|---|---|---|
| Cbz-Leu-Leu-Tyr-$CHN_2$ | | | |
| 0.01 | 3 | N.D. | 36±1 |
| 0.10 | 3 | N.D. | 41±2 |
| 1.0 | 3 | 73±2 | 32±1 |
| 10 | 3 | 78±5 | 36±2 |
| 100 | 4 | 69±3 | 49±6 |
| Cbz-Leu-Leu-Tyr-$CH_2F$ | | | |
| 1.0 | 3 | 60±1 | N.D. |
| 10 | 3 | 62±1 | N.D. |
| 100 | 3 | 62±2 | N.D. |
| No inhibitor | 4 | 63±3 | 35±2 |

### Change of cell shape and aggregation

Having obtained very potent calpain inhibitors suitable for use in biological systems, we proceeded to observe their effects on a number of platelet responses. ADP (0.2 $\mu$M) was added to PRP to induce shape change, which was manifested by a decrease in light transmission. This was followed by a gradual increase in light transmission when platelets were stimulated (with 40 $\mu$M-ADP in the presence of $Ca^{2+}$) to aggregate and thus clear the way for light to be transmitted through the platelet suspension. Shape change

and aggregation were induced separately under different conditions, as described in the Materials and methods section. Final inhibitor concentrations up to 100 $\mu$M did not decrease the degree of ADP-induced shape change and aggregation (Table 1). Proteolysis was, however, completely blocked with 10 $\mu$M-Cbz-Leu-Leu-Tyr-$CH_2F$ (Fig. 1*c*).

### Adhesion and spreading on glass

Platelets attaching and spreading on glass surfaces extend filopodia and veils which fill the spaces between them (J. Hagmann & M. M. Burger, unpublished work). Treatment of platelets with the calpain inhibitor did not affect either of these processes (Fig. 3). We also did not observe an effect on the rapidity with which platelets were spreading.

### 5-Hydroxytryptamine release

The results summarized in Table 2 show that Cbz-Leu-Leu-Tyr-$CHN_2$ did not inhibit the thrombin-activated release of 5-hydroxy[G-$^3$H]tryptamine from preloaded platelets. However, leupeptin, a less specific proteinase inhibitor, had some inhibitory effect on thrombin-activated secretion. This effect is probably due to inhibition of thrombin, a serine proteinase, by leupeptin.

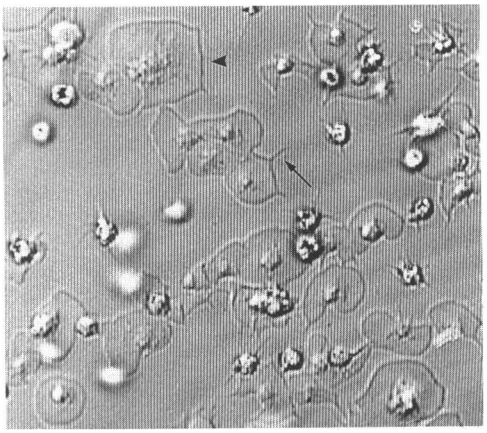

**Fig. 3. Videoenhanced image of platelets pretreated with Cbz-Leu-Leu-Tyr-$CHN_2$**

The platelets shown were recorded after spreading on the coverslip for 15 min. Filopodia (arrow) and veils (arrowhead) can be seen. Control platelets looked identical and are not shown.

**Table 2. 5-Hydroxy[G-$^3$H]tryptamine release from platelets**

Platelets were preloaded with 5-hydroxy[G-$^3$H]tryptamine and treated with inhibitors. Thrombin-mediated secretion was determined as described in the Materials and methods section.

| 5-Hydroxy[G-$^3$H]tryptamine-loaded platelets in buffer A | $10^{-5}$ × 5-Hydroxy[G-$^3$H]-tryptamine released into incubation medium (c.p.m.) |
|---|---|
| Resting platelets | 0.00026 |
| Resting platelets (in the presence of 3 mM-$CaCl_2$) | 0.86 |
| Thrombin-stimulated platelets (in the presence of 3 mM-$CaCl_2$) | 5.3 |
| + 50 $\mu$M-Cbz-Leu-Leu-Tyr-$CHN_2$ | 4.7 |
| + 50 $\mu$M-leupeptin | 1.6 |

### Protein labelling experiments

In order to identify the proteinase(s) which are inactivated, platelets were treated with radioactive forms of the inhibitors and then analysed by SDS/PAGE and autoradiography. Cbz-Leu-Leu-Tyr($^{125}$I)-$CHN_2$ labelled three major proteins of 78, 40 and 23 kDa and three minor proteins of 80, 55 and 30 kDa, whereas Cbz-Leu-Leu-Tyr($^{125}$I)-$CH_2F$ labelled a major protein of 80 kDa and four minor bands of 78, 55, 40 and 25 kDa (Fig. 4). The intensities of the major bands obtained by labelling with the fluoromethyl ketone were about three times those of the major diazomethyl ketone-labelled bands. Our calpain inhibitors do not have an absolute specificity for the calpains; they also inactivated cathepsin L *in vitro*. However, by using them subsequent to a treatment with Cbz-Tyr(I)-Ala-$CHN_2$, an inhibitor which inactivates cathepsins B and L but not calpain, labelling of cathepsin L can be avoided. In platelets pretreated with Cbz-Tyr(I)-Ala-$CHN_2$, the low-molecular-mass proteins of 30 and 23 kDa which correspond to the active forms of cathepsin L were

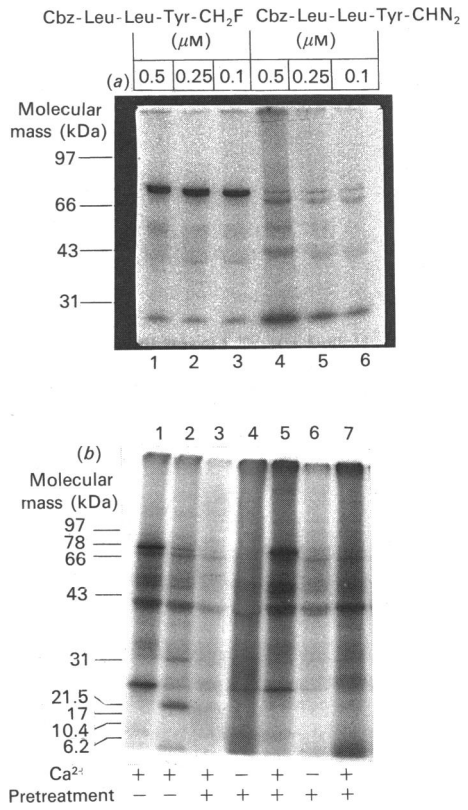


**Fig. 4. Affinity labelling of calpain with radioactive inhibitors**

(*a*) Washed platelets were radiolabelled with various concentrations of Cbz-Leu-Leu-Tyr-$CH_2F$ (lanes 1, 2 and 3) or Cbz-Leu-Leu-Tyr-$CHN_2$ (lanes 4, 5 and 6). Proteins were resolved with SDS/7.5%-polyacrylamide gel and autoradiographed for 12 h. Weakly labelled bands are not shown. (*b*) Washed platelets were incubated in the presence (lanes 3, 4, 5, 6 and 7) or absence (lanes 1 and 2) of 100 $\mu$M-Cbz-Tyr(I)-Ala-$CHN_2$ (pretreatment) and then radiolabelled with Cbz-Leu-Leu-Tyr($^{125}$I)-$CHN_2$ (lanes 2, 3, 6 and 7) or Cbz-Leu-Leu-Tyr($^{125}$I)-$CH_2F$ (lanes 1, 4 and 5) in the presence of $Ca^{2+}$ (lanes 1, 2, 3, 5 and 7). In some samples (lanes 4 and 6), platelets were treated with radioactive inhibitors in the presence of 15 $\mu$M-dimethyl-BAPTA and 5 mM-EDTA (intracellular and extracellular $Ca^{2+}$ chelators respectively). Proteins were resolved in a SDS/7.5–20% gradient polyacrylamide gel and autoradiographed for 1 week to show both major and minor bands.

not labelled by the radioactive calpain inhibitors. When platelets were deprived of $Ca^{2+}$ (with dimethyl-BAPTA and EDTA), radiolabelling of the 80, 78 and 55 kDa proteins was not possible and the intensities of the 40 and 25 kDa bands diminished considerably; labelling was $Ca^{2+}$-dependent.

## DISCUSSION

Substantial changes in the cytoskeletal structure and a rise in the $[Ca^{2+}]_i$ accompany most agonist-mediated responses in platelets. One of the possible targets for an increase in $[Ca^{2+}]_i$ is calpain. Inhibitors found to inactivate calpain *in vitro*, namely peptidyl epoxides and aldehydes, have indeed been used to block the $Ca^{2+}$-dependent proteolysis of ABP and talin in intact platelets (Tsujinaka *et al.*, 1988; McGowan *et al.*, 1989). Moreover, several investigators have correlated a calpain-mediated reorganization of the platelet cytoskeleton with secretion and aggregation, using inhibitors which belong to the classes of epoxides (Toyo-oka *et al.*, 1989) or aldehydes (Okita *et al.*, 1989; Puri *et al.*, 1989).

Even though the peptidyl epoxides are irreversible inhibitors of cysteinyl proteinases, they react more slowly with calpain than with other cysteinyl proteinases (Barrett *et al.*, 1982; Parkes *et al.*, 1985). Leupeptin, which is the peptidyl aldehyde chiefly used in calpain studies, is a powerful inhibitor of serine proteinases, some of which are involved in platelet physiology, as well as being an inhibitor of cysteinyl proteinases (Aoyagi & Umezawa, 1975; Rao *et al.*, 1987). Improved forms of peptidyl aldehydes with structures more specific for calpain have been synthesized and used in attempts to elucidate the enzyme's physiological role (Kajiwara *et al.*, 1987; Tsujinaka *et al.*, 1988). However, the aldehyde inhibitors form only reversible complexes and are also readily oxidized under physiological conditions (Imperiali, 1988; Shaw, 1990). They are, therefore, not suitable for identifying their target proteinases *in vivo*.

Peptidyl diazomethanes and monofluoromethanes are very powerful tools for investigating the physiological roles of cysteine proteinases. The peptidyl diazomethanes react specifically with cysteine proteinases (Green & Shaw, 1981; Shaw, 1990), whereas the monofluoromethanes are more than one order of magnitude less reactive with serine proteinases than with cysteine proteinases (Angliker *et al.*, 1988). Their properties of irreversibility and non-reactivity with free thiol groups (Green & Shaw, 1981; Angliker *et al.*, 1987) give them the added advantage of being excellent probes for studying proteinase activity in cellular environments. By varying the amino acid residues in the peptidyl portions of these reagents, highly selective and irreversible calpain inhibitors have been designed. The inhibitors penetrated to intracellular sites and protected platelet cytoskeletal proteins from $Ca^{2+}$-dependent proteolysis. This anti-proteolytic effect did not, however, prevent ADP-mediated shape change and aggregation, thrombin-mediated secretion of 5-hydroxytryptamine or platelet adhesion to glass and spreading, even at concentrations higher than those required for full protection of the platelet cytoskeleton from $Ca^{2+}$-dependent proteolysis. The results show that calpain-mediated proteolysis may not be an obligatory event leading to any of the platelet responses we examined. Our observations also suggest that the less selective inhibitors blocked certain platelet processes which are calpain-independent.

We used radioactive forms of our inhibitors to label the platelet proteinase(s) which were inactivated. An ideal situation would be one in which a single proteinase, to which the observed proteolytic action can be attributed, is labelled. As mentioned in the Results section, the labelling patterns showed bands corresponding to 80, 78, 55 and 40 kDa when cathepsin L was blocked with Cbz-Tyr(I)-Ala-$CHN_2$ before radiolabelling with a calpain inhibitor. Labelling of the 80, 78, 55, 40 and 25 kDa proteins was $Ca^{2+}$-dependent. This observation was not unexpected, since calpains autolyse to produce active fragments of 78 and 55 kDa within 5 min of activation (Crawford *et al.*, 1987). An active-site-containing peptide of 37 kDa was also found after a 5-min activation. The 80 kDa subunit has been shown to be active before autolysis (Cong *et al.*, 1989). Calpain inhibitors would be expected to label all forms of the enzyme which contain a functional active site. Cbz-Leu-Leu-Tyr($^{125}$I)-$CH_2F$ labelled a major protein of 80 kDa and minor proteins of 78, 55, 40 and 25 kDa, and Cbz-Leu-Leu-Tyr($^{125}$I)-$CHN_2$ reacted with proteins which had molecular masses of 78, 55 and 40 kDa (see Fig. 4). The different intensities of radiolabelling obtained with the fluoromethyl ketone and the diazomethyl ketone reflect their differences in reactivity towards the target enzyme and/or in cell-penetrability. Interestingly, the fluoromethyl ketone was incorporated into calpain forms which exist during the very early stage of autolysis, whereas the diazomethyl ketone labelled mostly the later forms. The pattern of inhibitor labelling will depend on the rate of autolysis, the rate of reactivity of the radiolabel with the active centre of calpain and the state of the enzyme in a particular cellular environment.

The morphological and metabolic responses of platelets to various types of activation are coupled through a complex network of intracellular messengers. The response reactions may involve multiple parallel mechanisms and co-operativity among individual regulatory events. The involvement of calpain in these processes is still not clear. Our results confirm a recent report in which no correlation was found between calpain I activation and aggregation in human platelets (Elce *et al.*, 1989). To our knowledge, the present work represents the first intracellular labelling of calpain using more selective irreversible inhibitors.

The new inhibitors will be very useful in examining other cell biochemical processes, such as protein phosphorylation (Kajiwara *et al.*, 1987; Ishii *et al.*, 1990), long-term memory potentiation (Lynch & Baudry, 1987), myocardial ischaemia (Tsuchida *et al.*, 1986), mitosis (Schollmeyer, 1988), clot retraction (Fox, 1986) and procoagulant activity (Verhallen *et al.*, 1988), in which calpain is thought to be involved.

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