Affinity labelling of the Ca^{2+} -activated neutral proteinase (calpain) in intact human platelets

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Two irreversible calpain inhibitors, benzyloxycarbonyl (Cbz)-Leu-Leu-Tyr- CH_2F and Cbz-Leu-Leu-Tyr- CHN_2 , were shown earlier [Anagli, Hagmann and Shaw (1991) *Biochem. J.* **274**, 497–502] to penetrate intact platelets and to inactivate calpain. This permitted an evaluation of certain functions attributed to this proteinase. For example, in platelets pretreated with these inhibitors, talin and actin-binding protein were protected from subsequent degradation when the Ca^{2+} level was raised. On the other hand, additional properties of stimulated platelets attributed to calpain remained unaffected by this treatment, and such hypotheses may be dismissed. Radioiodinated inhibitors permitted confirmation of the labelling of calpain by the procedures used. Although Cbz-Leu-Leu-Tyr- CHN_2 is more effective *in vitro* than the corresponding fluoromethyl ketone, we now show that the latter penetrates more readily. These two

inhibitors, and two additional ones, t-butyloxycarbonyl-Val-Lys(Cbz)-Leu-Tyr- CHN_2 and Cbz-Leu-Tyr- CH_2F , have been radioiodinated to permit a comparison of their intracellular labelling patterns in activated platelets. Calpain is the major target of all four inhibitors. Although they are closely related peptide structures, variations with respect to the labelling of additional proteins were observed. These were minor in the case of the peptidyl diazomethyl ketones, but were major in the case of the fluoromethyl ketones. However, in contrast to calpain, this labelling was neither time-dependent nor Ca^{2+} -dependent. Radiolabelling and cellular fractionation studies were used to localize active calpain during platelet activation. Calpain appears to be activated in the cytosol and translocated to the membrane or cytoskeletal sites.

INTRODUCTION

The calcium-activated neutral proteinases, commonly known as calpains, are intracellular non-lysosomal thiol proteinases which are widely distributed in eukaryotic cells (Murachi et al., 1981; Johnson, 1990). Two isoforms of calpain, calpain I and calpain II, which require micromolar- and millimolar-order concentrations of Ca^{2+} ions respectively for activity *in vitro*, are known (Mellgren, 1980; Murachi, 1983). The true function of the Ca^{2+} -dependent proteolytic system is not clear, in spite of many suggestions. Physiological roles in which calpains have been implicated include cell division (Schollmeyer, 1988), signal transduction (Pontremoli & Melloni, 1986; Hirai et al., 1991; Kuroda et al., 1991), platelet procoagulant activity (Fox et al., 1990, 1991) and long-term memory potentiation (Lynch and Baudry, 1987). The enzymes might also play a significant part in pathological conditions such as degradation of myofibrillar or cytoskeletal proteins in degenerative diseases of muscle and nerve (Pontremoli and Melloni, 1986; Banik et al., 1987; Spalla et al., 1987; Arai et al., 1990; Siman, 1990; Johnson et al., 1991; Shimohama et al., 1991) and hypertension (Pontremoli et al., 1988b,c).

The autoproteolytic modification of the Ca^{2+} -activated neutral proteinases *in vitro* has been carefully studied in a number of laboratories, and it is now generally accepted that Ca^{2+} -induced limited autoproteolysis plays a central role in the activation and regulation of the enzyme (DeMartino and Blumenthal, 1986; Cottin et al., 1991; Zimmerman and Schlaepfer, 1991). Even though these *in vitro* studies examined systematically and in detail the structural and catalytic modifications of calpain during autoproteolysis, they give little clue as to where and when the

proteinase(s) is active in the cell. The activity of calpain *in vivo* seems to be under tight and complex regulation (Mellgren, 1987; Pontremoli et al., 1988a; Melloni and Pontremoli, 1991). This may result from the direct and co-ordinated interaction of inhibitory and stimulatory molecules with calpain, modulated by changes in the intracellular Ca^{2+} concentration. Alteration of the susceptibility of physiological substrates to proteolysis, and the intracellular location of the substrate(s) and enzyme at a given time, are potential factors which can indirectly control calpain action in cells. Cell-permeant, irreversible and highly selective inhibitors which serve as affinity labels for the identification and subcellular localization of active forms of the proteinase are required to clarify the uncertainty about proposed roles for the calpains in various physiological functions, and to shed more light on the mechanism(s) of activation of the enzyme.

Our previous studies on the role of calpain in platelet activation (Anagli et al., 1991) revealed the excellent potential of peptidyl diazomethanes and monofluoromethanes as probes for examining the function and mode of regulation of the enzyme in intact cells. In that work the activity of the inhibitors was deduced from their ability to protect actin-binding protein and talin, two known substrates of calpain (White, 1980; Fox et al., 1983), from Ca^{2+} -dependent proteolysis. This antiproteolytic 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 the Ca^{2+} -dependent proteolysis.

In the present study, the four most potent inhibitors which emerged from our earlier work (Anagli et al., 1991; Angliker et al., 1992) were chosen for further examination *in vivo*. The results

Abbreviations used: Boc, t-butyloxycarbonyl; Cbz, benzyloxycarbonyl; BAPTA/AM, [bis-(o-aminophenoxy)ethane-*NNN'*-tetra-acetic acid], tetrasodium salt; PVDF, poly(vinylidene difluoride); NEM, *N*-ethylmaleimide; NH-Mec, 4-methyl-7-coumarylamide; PMSF, phenylmethanesulphonyl fluoride; Suc, succinyl; Tyr(¹²⁵I), radio-iodinated tyrosine.

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demonstrate the effect of variations in the peptide structure or functional groups on the cell-penetrability of the inhibitors and on their selectivity in a complex biological environment. Time-dependent affinity labelling and subcellular localization of the active enzyme during platelet activation was carried out in an attempt to obtain results which could lead to a better understanding of the mechanism of activation of the enzyme *in vivo*.

MATERIALS AND METHODS

The following materials were purchased from the sources indicated: Iodobeads, Pierce Chemical Co.; AG 50W-X8 and AG 1-X8 ion-exchange resins, Bio-Rad Laboratories Ltd.; 4-bromo-A23187, Sigma Chemical Co.; Na¹²⁵I and ECL Western blotting detection system, Amersham; dimethyl-BAPTA/AM, Molecular Probes; succinyl (Suc)-Leu-Tyr-4-methyl-7-coumarylamide (NH-Mec), Bachem (Bubendorf, Switzerland); horseradish peroxidase-conjugated rabbit anti-goat second antibody, Dako-patts; benzyloxycarbonyl (Cbz)-Leu-Nle-H (Cbz-Leu-Nle-aldehyde, calpeptin), Novabiochem (Läufelfingen, Switzerland). Cbz-Leu-Leu-Tyr-CHN₂ and t-butyloxycarbonyl (Boc)-Val-Lys(ϵ -Z)-Leu-Tyr-CHN₂ were synthesized as described previously (Crawford et al., 1988). Cbz-Leu-Leu-Tyr-CH₂F and Cbz-Leu-Tyr-CH₂F were synthesized according to Angliker et al. (1992). Human platelets were isolated as described previously (Anagli et al., 1991) from fresh venous blood obtained from the blood bank of the Cantonal Hospital, Basel, Switzerland. The polyclonal anti-(platelet calpain II) antiserum was generously provided by Dr. Alvin H. Schmaier, The University of Michigan Medical Center, Ann Arbor, MI, U.S.A.

Iodination of inhibitor

Inhibitors were iodinated with ¹²⁵I as described previously (Anagli et al., 1991) with some modifications. Subsequent to treatment with Na¹²⁵I in the presence of an Iodobead, 500 μ l of inhibitor solution (50 μ M) was added to a 2 ml mixed bed of AG 50W-X8 and AG 1-X8 ion-exchange resins and excess iodide was removed from the reaction mixture by batch procedure. Specific radioactivities of the ¹²⁵I-labelled inhibitors were estimated by γ -radiation counting (Beckman Gamma 5500B). The radioactive inhibitors were used immediately to label platelet proteinases.

Evaluation of cell permeability of calpain inhibitors

Portions (1.5 ml) of washed platelet suspension in buffer A (Anagli et al., 1991) (2×10^9 platelets/ml) containing 1 mM EDTA and 15 μ M dimethyl-BAPTA/AM were treated with various concentrations of Cbz-Leu-Leu-Tyr-CHN₂, Cbz-Leu-Leu-Tyr-CH₂F or Cbz-Leu-Nle-H at 37 °C for 1 h. Platelets were washed three times and sonicated in 0.5 ml of the same buffer. The platelet lysate was incubated with 5 mM CaCl₂ for 3 min, and the residual calpain activity was measured at 37 °C by monitoring the Ca²⁺-dependent hydrolysis of Suc-Leu-Tyr-NH-Mec (Crawford et al., 1988). In a second set of experiments, platelets were first lysed by sonication before treating with various concentrations of the inhibitors. Residual calpain activity in the lysate was determined as in the first set of experiments.

The inhibitors were radiolabelled with ¹²⁵I as described above. Portions (5 ml) of platelet suspension (1.5×10^9 platelets/ml) were incubated with 2 μ M ¹²⁵I-labelled inhibitor [Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F or Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂] of the indi-

cated specific radioactivity (Table 1) at 37 °C for 30 min. Platelets in buffer A containing 5 mM CaCl₂ were activated with 1 μ M of the Ca²⁺ ionophore 4-bromo-A23187 for 2 min. Then 10 mM EDTA was added and the platelets were pelleted. The radiolabelled platelets were washed three times in buffer A containing 10 mM EDTA and the amount of inhibitor retained per cell was estimated by γ -radiation counting (Beckman Gamma 5500B).

Assay of calpain activity in platelet lysate

Calpain activity was determined fluorimetrically by adding 50 μ l of platelet lysate to 1 ml of 50 mM Tris/acetate buffer, pH 7.5, containing 5 mM CaCl₂, 2.5 mM dithiothreitol and 0.1 % Triton X-100 at 37 °C, using Suc-Leu-Tyr-NH-Mec (2.5×10^{-4} M) as substrate. The appearance of the aminomethylcoumarin product was recorded continuously with a Perkin-Elmer 650-10S spectrofluorimeter, with excitation at 370 nm and emission at 460 nm. Ca²⁺-independent Suc-Leu-Tyr-NH-Mec-hydrolytic activity was measured by replacing CaCl₂ with 10 mM EDTA in the substrate solution. Activity due to calpain was obtained after subtracting Ca²⁺-independent activity from total Suc-Leu-Tyr-NH-Mec-hydrolytic activity in the presence of Ca²⁺.

Labelling of proteins with radioactive inhibitor

Portions of platelet suspension (1 ml) were incubated with radioactive inhibitor (6 μ M diazomethyl ketone or 2 μ M monofluoromethyl ketone) and then activated with Ca²⁺ ionophore as described previously (Anagli et al., 1991). Activation was stopped by adding 200 μ l of a solution of 100 mM EDTA, 0.1 % 2-mercaptoethanol, 5 mM *N*-ethylmaleimide (NEM), 5 mM phenylmethanesulphonyl fluoride (PMSF), 10 μ M-Cbz-Leu-Leu-Tyr-CH₂F and 5 % Triton X-100 (quenching solution) to the platelet suspension with vigorous mixing. Platelet proteins were precipitated with a methanol/chloroform/water mixture according to Wessel & Flügge (1984).

For time-dependent labelling of calpain in intact cells, platelets (2.5 ml) were loaded with Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F, and 250 μ l portions of platelet suspension [$(3-5) \times 10^8$ platelets/ml] were activated with Ca²⁺ ionophore for different periods of time.

Subcellular localization of affinity-labelled proteinases

Proteinases in intact human platelets were affinity-labelled with Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F as described above. At different time points during activation, aliquots of 250 μ l of the platelet suspension were removed from the incubation mixture, added to an equal volume of a solution containing 20 mM Tris/HCl, 1 mM PMSF, 1 mM NEM and 20 mM EDTA, pH 7.5 (lysis buffer), and frozen immediately in a solid CO₂/methanol mixture to stop activation. Platelet lysates were prepared by sonication on ice (Labsonic 2000; B. Braun; low position; 10×5 s cycles) and centrifuged at 100 000 *g* for 1 h at 4 °C to separate soluble (cytosol) and particulate (membrane and cytoskeleton) fractions. The cytosolic proteins were precipitated with a methanol/chloroform/water mixture as described above. Protein samples were solubilized in SDS sample buffer and electrophoresed.

Electrophoretic and immunochemical analyses

SDS/PAGE was carried out according to Laemmli (1970). Gels were stained with Coomassie Blue R-250, dried and exposed to X-ray film to detect radiolabelled proteins. Immunoblots were performed according to the technique of Towbin et al. (1979),

except that proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes for 40 min at 4 °C at 15 V in a Bio-Rad trans-blot semi-dry electrophoretic transfer cell using a running buffer of 25 mM Tris/HCl, 0.1% SDS and 192 mM glycine, pH 8.3, containing 20% methanol. Non-specific protein adsorption on the PVDF membrane was blocked with 5% non-fat dried milk in phosphate-buffered saline containing 1% Triton X-100 and 0.5% Tween 20. A polyclonal anti-(platelet calpain II) antiserum raised in goat was used as the primary antibody, and horseradish peroxidase-conjugated rabbit anti-goat antibody was used as second antibody. Labelled proteins were detected with the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham RPN 2106).

RESULTS

Cell penetrability of calpain inhibitors

Affinity labelling of human platelet proteinases *in vivo* using radioactive forms of the two inhibitors revealed a more intense labelling of calpain by the fluoromethyl ketone-derivatized peptide after platelet stimulation with the Ca^{2+} ionophore 4-bromo-A23187 (Figure 1). This result suggests a higher reactivity of Cbz-Leu-Leu-Tyr- CH_2F with calpain and/or a better cell penetrability than Cbz-Leu-Leu-Tyr- CHN_2 . In order to distinguish between these possibilities, the inhibitors were compared, together with the reversible inhibitor Cbz-Leu-Nle-H (Tsujinaka et al., 1988, 1990), for their ability to cross the plasma membrane. The inhibitors were either incubated with intact human platelets prior to lysis or added directly to the lysed platelet homogenates. The residual hydrolytic activity, using Suc-Leu-Tyr-NH-Mec as substrate, included a Ca^{2+} -independent component of about 42%. As shown in Figure 2(a), the extracellular concentrations of inhibitor needed for half-maximal inhibition of calpain activity were 9 μM Cbz-Leu-Leu-Tyr- CH_2F , 17 μM Cbz-Leu-Leu-Tyr- CHN_2 and 30 μM Cbz-Leu-Nle-H. Cbz-Leu-Leu-Tyr- CH_2F partly inhibited the Ca^{2+} -independent activity at extracellular concentrations above 30 μM . When the same number of cells (3×10^9) were lysed and then treated with the inhibitors, Cbz-Leu-Leu-Tyr- CH_2F and Cbz-Leu-Nle-H both inhibited 50% of the calpain activity at 0.12 μM . Cbz-Leu-Leu-Tyr- CHN_2 was the most potent inhibitor *in vitro*; it inactivated 50% of the calpain activity at a concentration of 0.07 μM .

The intracellular inhibitor concentration achieved by incubating whole platelets with inhibitors can be estimated from the results of the cell penetrability studies. The available platelet water space has been estimated at 50–55% of the cell volume, i.e. at 2–3 fl (Hughes and Crawford, 1989). The total intracellular water space of 3×10^9 platelets is about 9 μl . The concentration of inhibitor loaded in 3×10^9 platelets is therefore diluted 55-fold when the platelets are lysed in 0.5 ml of buffer prior to activation by Ca^{2+} . As shown in Figure 2(b), 0.07 μM Cbz-Leu-Leu-Tyr- CHN_2 is required to half-maximally inhibit calpain activity in the lysate. This is equivalent to an intracellular concentration of 3.85 μM . Since an extracellular concentration of 17 μM Cbz-Leu-Leu-Tyr- CHN_2 was required to pretreat the intact platelets in order to obtain half-maximal inhibition in the lysate, the intracellular inhibitor concentration was 23% of the extracellular concentration, as estimated under our experimental conditions. Calculations performed for the other two inhibitors give intracellular concentrations of 73% and 22% for Cbz-Leu-Leu-Tyr- CH_2F and Cbz-Leu-Nle-H respectively. These results suggest that the fluoromethyl ketone derivative penetrates the platelet plasma membrane better than does the diazomethyl ketone

Figure 1 Comparison of Cbz-Leu-Leu-Tyr(^{125}I)- CH_2F and Cbz-Leu-Leu-Tyr(^{125}I)- CHN_2 for their ability to affinity label calpain in intact platelets

Platelets were incubated with various concentrations of Cbz-Leu-Leu-Tyr(^{125}I)- CH_2F (lanes 1, 2 and 3) or Cbz-Leu-Leu-Tyr(^{125}I)- CHN_2 (lanes 4, 5 and 6) as described in the Materials and methods section. Proteins were separated on a SDS/7.5%-polyacrylamide gel and autoradiographed for 12 h. The arrow indicates the position of calpain on the gel (76–78 kDa).

Figure 2 Membrane penetrability of the calpain inhibitors Cbz-Leu-Leu-Tyr- CH_2F , Cbz-Leu-Leu-Tyr- CHN_2 and Cbz-Leu-Nle-H

(a) Intact human platelets were incubated with various concentrations of Cbz-Leu-Leu-Tyr- CHN_2 (∇), Cbz-Leu-Leu-Tyr- CH_2F ($\bullet$) and Cbz-Leu-Nle-H ($\circ$) before lysis by sonication and measurement of residual hydrolytic activity using the substrate Suc-Leu-Tyr-NH-Mec as described in the Materials and methods section. $\square$ indicates calcium-independent activity. (b) As for (a), except that the platelets were lysed by sonication before incubating with the inhibitors.

Table 1 Estimation of the amount of radioactive inhibitor in platelets[I]_i is the intracellular concentration of inhibitor.

Inhibitor	Specific radioactivity of inhibitor (mCi/mol)	Total radioactivity retained (mCi/cell)	Amount of inhibitor per cell (mol)	[I] _i (μM)
Cbz-LLY(¹²⁵ I)-CHN ₂	1.6 × 10 ⁹	1.65 × 10 ⁻¹²	1 × 10 ⁻²¹	0.3
Cbz-LLY(¹²⁵ I)-CH ₂ F	1.2 × 10 ⁹	3.6 × 10 ⁻¹²	3 × 10 ⁻²¹	1.0

Figure 3 Effect of structural variation on selectivity of inhibitors

Platelets were incubated with radioactive inhibitor (5 μM diazomethyl ketone or 2 μM monofluoromethyl ketone) for 1 h and then activated with Ca²⁺ ionophore for 30 min as described in the Materials and methods section. Lane 1, Coomassie Blue staining. Lanes 2–5 show autoradiography: 2, Boc-Val-Lys(ε-Z)-Leu-Tyr(¹²⁵I)-CHN₂; 3, Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂; 4, Cbz-Leu-Tyr(¹²⁵I)-CH₂F; 5, Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F. The dried gel was exposed to an X-ray film at -70 °C for 1 week.

derivative of the same tripeptide. The order of cell penetrability is: Cbz-Leu-Leu-Tyr-CH₂F > Cbz-Leu-Leu-Tyr-CHN₂ ≈ Cbz-Leu-Nle-H.

The diazomethane and monofluoromethane derivatives of Cbz-Leu-Leu-Tyr were radioiodinated with ¹²⁵I and incubated with platelet suspensions as described in the Materials and methods section. As shown in Table 1, the amount of Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F retained in the washed platelets was about three times the amount of the diazomethyl ketone derivative of the same peptide structure. These results provide further confirmation of the higher cell penetrability of the fluoromethyl ketone derivative.

Effect of structural variation on selectivity of inhibitors

In order to examine the effect of variations in the peptide structure or in the functional group of the inhibitors on selectivity in a complex biological environment, four calpain inhibitors were iodinated with ¹²⁵I and used to label proteinases in intact platelets (Figure 3). Two peptidyl diazomethanes [Boc-Val-Lys(ε-Z)-Leu-Tyr(¹²⁵I)-CHN₂ and Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂] and two monofluoromethanes [Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F and

Figure 4 Time-dependent radiolabelling of calpain during platelet activation

(a) Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F-loaded platelets were activated for different periods of time (0, 0.25, 0.5, 1, 2, 5 and 15 min) with the Ca²⁺ ionophore 4-bromo-A23187. Whole cell proteins were solubilized in sample buffer, electrophoresed and analysed by autoradiography as described in the Materials and methods section. (b) Time course for radiolabelling of active calpain during platelet activation.

Cbz-Leu-Tyr(¹²⁵I)-CH₂F] were compared for the effects of changes in the peptide structure on selectivity. In the group of monofluoromethyl ketones, the tripeptide (Figure 3, lane 5) with an additional Leu in P₃ labels fewer proteins than the dipeptide (lane 4), even though it is more reactive towards calpain and cathepsin L *in vitro* (Anglikier et al., 1992). The tetrapeptidyl diazomethane, Boc-Val-Lys(ε-Z)-Leu-Tyr(¹²⁵I)-CHN₂ (lane 2), was compared with the most potent inhibitor *in vitro*, i.e. Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂ (lane 3). Both Boc-Val-Lys(ε-Z)-Leu-Tyr(¹²⁵I)-CHN₂ and Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂ labelled calpain (76–78 kDa). In addition, Boc-Val-Lys(ε-Z)-Leu-Tyr(¹²⁵I)-CHN₂ labelled two minor proteins of about 47 and 35 kDa, whereas Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂ labelled two minor proteins of about 25 and 33 kDa. Comparing reagents with the same peptide structure but different functional groups, Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F labelled five major proteins and two minor proteins (lane 5), whereas Cbz-Leu-Leu-Tyr(¹²⁵I)-CHN₂ labelled two major proteins and two minor proteins (lane 3). The diazomethyl ketones are more selective, labelling fewer platelet proteins. The labelling patterns not only confirm the higher

Figure 5 Immunodetection of calpain during platelet activation

(a) Platelets were treated with $2 \mu\text{M}$ Cbz-Leu-Leu-Tyr- CH_2F and activated with Ca^{2+} ionophore as described in the Materials and methods section. At different time points during activation, $100 \mu\text{l}$ portions of whole cell suspension were solubilized in sample buffer, electrophoresed on a SDS/7.5%-polyacrylamide gel and electrotransferred to a PVDF membrane. Calpain was immunodetected as described in the Materials and methods section. (b) Time course of immunodetection of calpain fragments during platelet activation, as described in the Methods and materials section.

selectivity of the diazoketone group relative to the monofluoroketone, but they also indicate how structural variations in the peptidyl (non-covalently binding) portion of the inhibitor can lead to reagents which discriminate between proteinases of the same mechanistic class.

Time-dependent radiolabelling using Cbz-Leu-Leu-Tyr(^{125}I)- CH_2F and immunodetection of calpain during platelet activation

Radiolabelling of a single platelet protein by the calpain inhibitor Cbz-Leu-Leu-Tyr(^{125}I)- CH_2F proceeded in a time-dependent manner (Figure 4a). It should be noted that no labelling of this protein was observed when platelets were deprived of Ca^{2+} (Figure 4a, lane 1). The labelling of additional proteins was not time-dependent. The time course of calpain labelling in activated intact human platelets showed an increase during the first 5 min of platelet activation with 4-bromo-A23187, after which a maximal level was slowly approached (Figure 4b). Half-maximal activation was reached after about 2 min.

Samples from the experiment shown in Figure 4(a) were separated in an SDS/7.5%-polyacrylamide gel, and the proteins were electrotransferred on to a PVDF membrane. A goat polyclonal antibody raised against human platelet calpain II recognized the 76–78 kDa protein which was labelled by Cbz-Leu-Leu-Tyr(^{125}I)- CH_2F . Interestingly, the antibody not only showed a time-dependent labelling of the lower 76–78 kDa protein, but it also recognized a protein band of about 80 kDa (Figure 5a). The calpain inhibitor, on the other hand, did not label the upper band when used at an extracellular concentration of $2 \mu\text{M}$. The generation of the 76–78 kDa fragment paralleled the time course of radiolabelling (Figure 5b). The results presented here confirm our previous observation that the interaction between calpain and our substrate-like inhibitors is Ca^{2+} -dependent. The inhibitor labelled only the autolysed form (76–78 kDa) of calpain when the cells were incubated with inhibitor at a concentration which was not sufficient to completely inactivate the enzyme *in vivo*. The antibody did not react with radioactive bands that did not show time-dependent labelling.

Figure 6 Subcellular distribution of active calpain during platelet activation

(a) Calpain was affinity-labelled in intact human platelets with radioactive Cbz-Leu-Leu-Tyr(^{125}I)- CH_2F as described in the Materials and methods section. At different time points during activation, aliquots were separated into soluble and particulate fractions and analysed by autoradiography. (b) Time course of calpain labelling in the soluble (S) and particulate (P) platelet fractions.

Contrary to our earlier suggestion (Anagli et al., 1991), these bands are probably not calpain fragments.

Subcellular distribution of active calpain during platelet activation

Labelling of calpain by Cbz-Leu-Leu-Tyr(¹²⁵I)-CH₂F was time-dependent in both the particulate (membrane and cytoskeleton) and soluble (cytosol) fractions (Figure 6). Radiolabelling rapidly increased in the cytosol. This shows that a greater part of the total intracellular active calpain is in the cytosol early during cell activation. The time course of labelling was slower in the particulate fraction. The intensity of labelling in the cytosol reached its maximal level at about 4 min. Subsequently, calpain labelling increased in the particulate fraction and decreased in the cytosolic fraction. These results suggest that calpain activation occurs in the cytosol, and that active calpain is redistributed to membrane and/or cytoskeletal sites.

DISCUSSION

Several investigators have used proteinase inhibitors to study the physiological role of calpain. However, most of these studies have employed peptidyl aldehydes (Kajiwara et al., 1987; Mehdi et al., 1988; Tsujinaka et al., 1988, 1990; Ariyoshi et al., 1991; Mehdi, 1991) which have poor selectivity. Furthermore, use of peptidyl aldehydes does not permit covalent labelling of the target proteinase (Shaw, 1990; Mehdi, 1991). It is therefore difficult to examine the selectivity of such inhibitors in a complex biological environment.

The proteinase inhibitory activity of peptidyl diazomethanes is limited to cysteine proteinases, whereas peptidyl monofluoromethanes are more selective for cysteine proteinases than for serine proteinases (Shaw, 1990). Highly selective inhibitors have been designed for certain cysteine proteinases by making structural changes in the peptidyl (non-covalently binding) portion of the reagents (Crawford et al., 1988; Angliker et al., 1988). For intracellular studies, cell-permeable molecules are needed. Therefore a compromise has to be made between selectivity, reactivity and cell permeability when designing an inhibitor suitable for studying the biological function of a proteinase. We had previously demonstrated the superiority of Cbz-Leu-Leu-Tyr-CH₂F over Cbz-Leu-Leu-Tyr-CHN₂ in protecting cytoskeletal proteins in intact human platelets from Ca²⁺-dependent proteolysis mediated by the Ca²⁺ ionophore 4-bromo-A23187 (Anagli et al., 1991). In the present studies, the effects of structural variations on the inhibitor properties *in vivo* were determined. Cbz-Leu-Leu-Tyr-CHN₂ and Cbz-Leu-Leu-Tyr-CH₂F were compared for their ability to cross the platelet plasma membrane. The results summarized in Figure 2 and Table 1 show that the monofluoroketone (-CH₂F) derivative penetrates the cell about 3-fold more efficiently than the diazoketone (-CHN₂) derivative of the same tripeptide. The reason for this difference is not known. Cbz-Leu-Nle-H (calpeptin), which is as effective as Cbz-Leu-Leu-Tyr-CH₂F in inactivating calpain in a platelet lysate, is less effective in intact platelets. It should be noted that calpeptin has been cited several times in the current literature as a very effective cell-penetrating calpain inhibitor (Tsujinaka et al., 1988; Ariyoshi et al., 1991; Fox et al., 1991). Cbz-Leu-Nle-H is as good as Cbz-Leu-Leu-Tyr-CHN₂ in penetrating the plasma membrane.

In our earlier *in vitro* studies (Crawford et al., 1988), it was shown that calpain inhibitors with hydrophobic residues in their P₂ and P₃ positions [using the terminology of Schechter and Berger (1968)] are very potent inhibitors of cathepsin L, a lysosomal cysteine proteinase. However, inhibitors such as Cbz-

Phe-Ala-CHN₂ and Cbz-Tyr(I)-Ala-CHN₂, which have aromatic residues in the P₂ position, inactivate cathepsins B and L but not calpain (Crawford et al., 1988; Mason et al., 1989). These results were further confirmed by *in vivo* proteinase labelling studies carried out in intact human platelets (Anagli et al., 1991). In platelets treated with Cbz-Tyr(I)-Ala-CHN₂, calpain (76–78 kDa), but not the low-molecular-mass proteins of 30 and 23 kDa which correspond to the active forms of cathepsin L, were labelled by the radioactive calpain inhibitors. It is likely that cysteinyl proteinases other than cathepsin L and calpain, which have a preference for hydrophobic residues in S₂ and S₃, are labelled by the calpain inhibitors, and studies are in progress to identify the radiolabelled proteins detected by autoradiography. In the present study, a higher extracellular concentration of radioactive inhibitor (5 and 2 μ M for diazomethyl and monofluoromethyl ketones respectively) was used (compare labelling in Figures 1 and 3) and autoradiography was carried out at longer exposure times in order to detect proteinases which are weakly labelled by the inhibitors. Certain variations in the peptidyl portions of our inhibitors improved their selectivity considerably (Figure 3). It was possible to detect in a highly selective manner proteins with which our inhibitors reacted in a complex biological environment. Such studies on specificity *in vivo* cannot be done with the other calpain inhibitors, namely peptidyl aldehydes (Sasaki et al., 1990; Wang, 1990; Ariyoshi et al., 1991; Mehdi, 1991), α -diketones and α -keto and trifluoromethyl esters (Angelastro et al., 1990), since they only form reversible complexes which are displaceable by protein substrate in the intracellular environment. Boc-Val-Lys(ϵ -Z)-Leu-Tyr-CHN₂ and Cbz-Leu-Leu-Tyr-CHN₂ are the most selective calpain inhibitors. Because the peptidyl diazomethanes and monofluoromethanes react by irreversible covalent bond formation, the altered cellular constituents can be localized. Thus the investigator may see what other components of the system, in addition to the target proteinase, become labelled. An ideal situation would be one in which a single proteinase, to which the observed proteolytic action can be attributed, is labelled. Caution must be exercised in the interpretation of the results when more than one proteinase is labelled.

The greater part of activated calpain was localized in the cytosol. Only the autolysed active form of calpain was labelled by the inhibitor (Figure 4a). By combining immunodetection and affinity radiolabelling techniques, the intracellular distribution of the active and inactive forms of calpain during platelet activation was determined. According to one of the hypotheses proposed to explain the activation of calpain in the cell (Mellgren, 1987; Suzuki et al., 1988; Melloni and Pontremoli, 1991), procalpain is translocated to the plasma membrane, where its calcium requirement is lowered and the enzyme is activated by autolysis. The autolysed active form of the enzyme would then be released into the cytosol or redistributed to other sites in the cell. The present studies locate active calpain first in the cytosol, and then in the particulate fraction (Figure 6). These results suggest that calpain may be activated in the cytosol and subsequently translocated to the membrane or to cytoskeletal sites.

The mechanism of regulation of calpain activity seems to be very complex. Any model to explain the way the proteinase is activated and regulated in the cell must consider the participation of the endogenous inhibitor (Murachi et al., 1981; Murachi, 1989; Pontremoli et al., 1991), the protein activator (Pontremoli et al., 1988a; Melloni and Pontremoli, 1991; Michetti et al., 1991), and other cell components located in the membrane (Kuboki et al., 1990; Inomata et al., 1990), in the cytoskeleton or on intracellular organelles (Golapakrishna and Barsky, 1986; Mellgren, 1991). The presence of two forms of the enzyme with

different requirements for calcium further complicates the picture. Highly selective affinity labels such as those used in the present studies will help answer certain questions concerning calpain function and regulation in these systems as well. A proposed function should be knocked out in cells whose calpain can be shown to be irreversibly inactivated. The inhibitors should be used at the lowest level that inactivates the proteinase to avoid conflicting results from the labelling of other proteinases. The selectivity of affinity labelling reagents is dependent on their kinetic properties and diminishes with high concentration or long exposure. Of the reagents we have examined, the peptidyl diazomethyl ketones were more selective than the fluoromethyl ketones. We have demonstrated that cytoskeletal elements in platelets can be protected when platelets are preloaded with such reagents prior to activation. Other imputed roles of calpain, such as in the thrombin-promoted secretion of 5-hydroxytryptamine, were not substantiated, since the inactivation of calpain was demonstrated by labelling without interfering with this cellular response.

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