Calpastatin in erythrocytes of young and old individuals

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To gain knowledge about the behaviour of calpastatin (the specific inhibitor of the Ca^{2+} -dependent thiol protease calpain) in the intact cell, we analysed the inhibitor by specific antibodies and determined its activity in erythrocytes from individuals 20–34 years old (young) and 70–93 years old (old). Differences between old and young in the behaviour of erythrocyte calpastatin were observed. Erythrocytes of old individuals had lower amounts of calpastatin and less calpastatin activity than those of young ones. A difference between old and young was also found in the molecular-mass distribution of calpastatin subunits. Increasing the erythrocyte Ca^{2+} induced changes in calpastatin in young individuals, rendering it similar to calpastatin in cells of

old individuals. When calpastatin (isolated from erythrocytes of a young individual) was added to erythrocyte membranes, the initial binding and subsequent association of calpastatin with the membrane were lower in old than in young individuals. We had previously found that calpain binding and activation were enhanced in erythrocyte membranes from old individuals, along with enhanced degradation of band 3 (a major erythrocyte transmembrane anion-transport protein). The overall results indicate an interaction of calpain with calpastatin in the intact cell. Enhanced activation of erythrocyte calpain and degradation of calpastatin occur under conditions of increased cellular Ca^{2+} and in cells of the aged.

INTRODUCTION

Calpastatin is a specific inhibitor of the Ca^{2+} -dependent thiol proteases, μ -calpain and m-calpain. Calpastatin is an intracellular protein and is widely distributed. The amounts of calpastatin in relation to calpain vary with cell type and species [1–3]. Calpain is usually present in an inactive form in the cell cytosol and is activated by limited autolysis in the presence of Ca^{2+} , a process which is enhanced by the translocation of calpain to cell membrane [4,5]. Calpastatin is also usually isolated from soluble cell extracts, but may be associated in part with cell membranes [3,6]. The mechanism by which calpastatin inhibits calpain activity has not been fully elucidated. In cell-free extracts, calpastatin associates with calpain in the presence of Ca^{2+} , and under certain conditions is degraded to low-molecular-mass fragments, some of which may retain the ability to inhibit calpain activity. In addition, calpastatin inhibits the binding of calpain to cell membranes [4,7]. The relevance of the interaction *in vitro* and modifications to the behaviour *in vivo* of the calpain–calpastatin system is not clear. Little information is available on the intracellular location and activities of the protease and inhibitor within the intact cell [3,6,8]. It is not known whether degradation of calpastatin by calpain or by other enzymes occurs *in vivo*.

Calpain causes limited proteolysis of some membrane and cytoskeletal proteins, so that the relative amounts, location and interaction of the protease with the inhibitor may influence degradation of membrane proteins [3,6]. We have described a role for the calpain–calpastatin system in membrane fusion, in particular, erythrocyte fusion induced by the membrane-mobility agent A_2C . In such fusions, degradation of certain membrane proteins by calpain is a necessary prerequisite, and there is a relationship between cellular calpain activity and that of calpastatin that determines the fusibility of the cell membranes [9–11]. The dependence of cell fusibility on the ratio of calpain to calpastatin points to the importance of the interaction of calpain,

calpastatin and Ca^{2+} in certain membrane events and cell function.

The degradation of band 3 (a major erythrocyte transmembrane glycoprotein that mediates chloride–bicarbonate exchange) by μ -calpain is enhanced in erythrocytes of old people [12]. Using anti- μ -calpain antibodies, we have found less calpain in the erythrocytes from old individuals than in cells of young ones. We have shown that when calpain is added to membranes, the Ca^{2+} -induced binding, autolysis and activation of calpain are greater in old than in young individuals [13]. These findings suggest that, in the old individual, erythrocyte calpain is subjected to enhanced activation, resulting in degradation of certain membrane proteins and eventual loss of some native calpain through autolysis.

In the present study we used antibodies for the analysis of calpastatin in the erythrocyte cytosol and membranes and determined the activity of calpastatin in erythrocytes of old and young individuals. Differences between old and young individuals in the behaviour of erythrocyte calpastatin were observed. The results are consistent with the occurrence of enhanced activation of calpain in the erythrocytes of old individuals. They indicate an interaction of calpain with calpastatin in the intact cell and degradation of intracellular calpastatin by calpain under conditions of increased cellular Ca^{2+} and in cells of the aged.

MATERIALS AND METHODS

Erythrocytes, cytosol and membranes

Blood was obtained from healthy humans, 20–34 years old (young) and 70–93 years old (old), and mixed with 1.0 mM EDTA to prevent coagulation. Blood was centrifuged, plasma and buffy coat were removed and the erythrocytes washed three times with 150 mM NaCl. Erythrocytes were haemolysed in 5

vol. of 10 mM Tris/HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol, 1.0 mM EDTA and 1.0 mM EGTA (Tris-DE) and centrifuged at 30000 *g* for 10 min to yield membrane-free haemolysate (cytosol). One portion of the cytosol was heated to 100 °C for 5 min and centrifuged at 30000 *g* for 10 min to obtain supernatant free of precipitated haemoglobin (heated cytosol). Another portion of the cytosol was used for obtaining partially purified calpastatin by chromatography of the cytosol on a DEAE-cellulose (DE-52) column. The cytosol was loaded on the DE-52 column pre-equilibrated with Tris-DE, and the column was washed with Tris-DE containing 50 mM NaCl until the eluate was free of haemoglobin. A haemoglobin-free fraction which contains non-haemoglobin proteins, including calpain and calpastatin, was then eluted with Tris-DE containing 150 mM NaCl [10]. The eluate was dialysed against 0.1 mM EDTA/0.1 mM EGTA/0.1 mM phenylmethanesulphonyl fluoride (PMSF), and then freeze-dried, resuspended in 10 mM Tris/HCl buffer, heated to 100 °C for 3 min to inactivate calpain (calpastatin is not inactivated by heating [1–3,10]), then centrifuged to clarify the DE-52 eluate (DE-cytosol) containing the partially purified calpastatin. Erythrocyte ghosts were obtained by haemolysing cells in 5 mM phosphate buffer, pH 8.0. Membranes were washed with this buffer, and then with 10 mM NaCl to obtain haemoglobin-free ghosts (white ghosts).

Calpastatin purification and preparation of antibodies to calpastatin

Cytosol obtained from erythrocytes of young individuals was used for the isolation and purification of calpastatin. The partially purified DE-52 fraction was further chromatographed to obtain purified calpastatin by established procedures used previously [10]. Calpastatin activity was determined as previously described [10], with 1 unit of calpastatin defined as the amount inhibiting 50 % of casein degradation by one unit of calpain. The chromatographed non-denatured calpastatin (having a molecular mass of about 280 kDa [10]) was used for obtaining polyclonal antibodies. Rabbits were injected intradermally at multiple sites at the neck region, once with calpastatin in complete Freund's adjuvant followed 1 month later by four to five additional booster injections in incomplete Freund's adjuvant (0.1 mg/injection) at 2-week intervals. Blood was obtained from the rabbits 10 days after the last booster injection. Purified antibody fraction was obtained by established methods [3], by ammonium sulphate precipitation of serum, followed by dialysis and chromatography on a DEAE-cellulose column. The whole serum or the purified antibodies were used, with similar results. Preimmunized rabbit serum served as a control. The antiserum or the antibody fraction did not cross-react with calpain, nor with the known membrane protein bands.

Treatment of erythrocytes with *N*-ethylmaleimide (NEM) and with Ca^{2+} and ionophore

Erythrocytes were suspended in a buffer containing 135 mM NaCl and 10 mM Tris/HCl, pH 7.4 (TBS). Erythrocyte suspensions (8 % packed cells) were incubated at 37 °C for 10–60 min without or with 0.5 mM Ca^{2+} and the ionophore A23187 (final concn. 10 μM , by using a stock solution of 5.0 mM in dimethyl sulphoxide), referred to as Ca^{2+} /Iph. In some experiments, portions of erythrocyte suspensions were preincubated at 37 °C for 30 min with 1.0 mM NEM and washed, then incubated with or without Ca^{2+} /Iph. Erythrocytes were then washed with 150 mM NaCl containing 2.0 mM EDTA and DE-cytosol, and erythrocyte white ghosts prepared as described above.

Treatment of erythrocyte white ghosts with calpastatin

Erythrocyte white ghosts were suspended at 4 °C in 10 mM Tris/HCl, pH 7.4, containing 0.2 mM PMSF (Tris/PMSF). The ghost suspensions were mixed with calpastatin (0.5–1.0 unit/ml of 10 % ghost suspension) and 0.5 mM Ca^{2+} and kept at 4 °C for 10 min, then incubated at 30 °C for 5–30 min. Ghost suspensions were then placed at 4 °C and washed in cold Tris/PMSF to remove unbound calpastatin.

Analysis of calpastatin by immunoblotting

Cytosol, heated cytosol and DE-cytosol were used for the analysis of soluble intracellular calpastatin. Erythrocyte white ghosts were used for the analysis of calpastatin in the membranes. Protein concentration in the cytosol was determined by haemoglobin absorption at 540 nm. Protein concentration in heated cytosol and in the DE-cytosol was estimated by absorption at 280 nm. Protein concentration in membranes was determined by the Lowry method [14]. In order to compare the calpastatin content of the membrane to that of the cytosol, the number of cells represented in each was estimated (based on values of 5 μmol of haemoglobin/ 10^{10} cells and 5 mg of protein/ 10^{10} white ghosts).

Cytosol, heated cytosol, DE-cytosol and membrane suspensions were mixed with Laemmli's SDS buffer [15]. Protein analysis was carried out by SDS/PAGE, using 10 % acrylamide in the gels. Gels were fixed and stained with Coomassie Brilliant Blue by established methods used previously [12]. Other gels, run in parallel, were used for immunoblotting. Proteins were transferred to nitrocellulose, treated with the anti-calpastatin antibodies, and then by reaction with secondary antibody, in accordance with established procedures [16]. Alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody was used as a secondary antibody, with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-Nitro Blue Tetrazolium chloride Bio-Rad reagents as substrates.

Degradation of calpastatin by μ -calpain

Calpastatin solution (0.2 unit) was mixed with calpain solution (0.1 unit of calpain, purified from human erythrocytes [10]) in a total volume of 0.25 ml of 10 mM Tris/HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA and 0.5 mM dithiothreitol. Samples were incubated at 30 °C for 15 min, with or without 1.0 mM Ca^{2+} and with or without 0.5 mM NEM. Samples were analysed for calpastatin and for calpain by immunoblotting, using the polyclonal antibodies to calpastatin described above and monoclonal antibody to calpain described previously [13].

Determination of calpastatin activity

Samples of heated cytosol (10–50 μl of heated cytosol solution, estimated to contain 10–50 μg of protein, based on 1.0 A_{280} unit = 1.0 mg of protein) were added to calpain solutions (0.2–0.25 units) in a total volume of 0.25 ml of 10 mM Tris/HCl buffer, containing 0.1 mM EDTA, 0.1 mM EGTA and 0.5 mM dithiothreitol. Samples were incubated for 10 min at room temperature, then activity of calpain was determined by methods previously used, with casein as a substrate [10]. Inhibition of 50 % of calpain activity was determined from the curve of casein degradation by calpain obtained by using increasing volumes of the heated cytosol as the source for calpastatin.

RESULTS

Calpastatin in erythrocyte cytosol and effects of increased intracellular Ca^{2+}

Cytosol and heated cytosol were prepared, electrophoresed and analysed for calpastatin by immunoblotting, as described in the Materials and methods section. In immunoblots of cytosol, calpastatin appeared as a very wide band composed of multiple fractions in the region of about 80–120 kDa. Less calpastatin was found in cytosol of old individuals than in cytosol of young ones, as detected by antibodies (Figure 1, lanes a and b). In heated cytosol, calpastatin appeared mostly as a wide band of about 70 kDa and a minor band of about 110 kDa. As was the case for cytosol, less calpastatin was found in the heated cytosol of the old than in that of the young individuals (Figure 1, lanes c and d). Purified calpastatin [9] appears as a wide band of about 70 kDa, with traces of bands of lower molecular masses (Figure 1, lane e). The protein profiles of the cytosol and the heated cytosol (Coomassie Blue-stained) appeared similar in old and young individuals (results not shown).

Erythrocytes of young and old individuals were incubated with and without Ca^{2+} /Iph; then calpastatin was analysed in DE-cytosol. In the absence of Ca^{2+} , calpastatin in DE-cytosol of young individuals appeared as a wide band of about 70 kDa, with traces of bands of higher molecular masses and no, or only a trace of a, band of 45 kDa. In the old, calpastatin appeared as bands of 70 kDa and 45 kDa (Figure 2, lanes a and b). Ca^{2+} /Iph treatment of erythrocytes from young individuals led to the disappearance of the minor higher-molecular-mass bands, to a diminution in the calpastatin band of 70 kDa and to the appearance of a 45 kDa band (Figure 2, lane c). A diminution in the calpastatin band of 70 kDa was also observed in the Ca^{2+} /Iph-treated cells from the old, along with an enhancement of the band of 45 kDa (Figure 2, lane d). Calpastatin obtained from erythrocytes treated with NEM showed a profile similar to that of control cells (Figure 2, lanes e and f, versus lanes a and b). Pretreatment of erythrocytes with NEM prevented the Ca^{2+} -induced changes, i.e. calpastatin fractions in cells treated with

Figure 2 Effects of NEM and of Ca^{2+} on erythrocyte calpastatin: detection by immunoblotting of calpastatin in DE-cytosol

Portions of erythrocytes of young and old individuals were kept at 4 °C (lanes a and b) or incubated at 37 °C for 30 min with or without NEM, followed by incubation for 10 min with or without Ca^{2+} (lanes c–h). Cytosol was chromatographed on DEAE-cellulose column, and the haemoglobin-free fraction containing calpastatin analysed by immunoblotting. Key: Y, young individual; O, old individual. Lanes a and b, control unincubated cells; lanes c and d, Ca^{2+} /Iph-treated cells; lanes e and f, NEM-treated cells; lanes g and h, NEM-treated and Ca^{2+} /Iph-treated cells; lanes i and j, Coomassie Blue (CB)-stained gels. For details, see the Materials and methods section. Results similar to those shown here were obtained in eight of nine young and in nine of 11 old individuals. In one young individual, the calpastatin appearance was similar to that of most old individuals; in two old individuals, calpastatin appearance was similar to that of the eight young individuals. The kDa values on the right refer to marker proteins.

NEM and Ca^{2+} /Iph (Figure 2, lanes g and h) were similar to those in cells treated with NEM alone (Figure 2, lanes e and f).

The protein profiles (Coomassie Blue-stained) of the DE-cytosol of the old were similar to those of the young individuals (Figure 2, lanes i and j).

Calpastatin in erythrocyte membranes

Very little calpastatin was found in white ghosts from either young or old individuals. Based on the number of erythrocytes used for the calpastatin analysis, we estimated the membrane calpastatin to be less than 1.0% of the total cell calpastatin. In the membranes from young individuals, calpastatin appeared as a band of 70 kDa (Figure 3, lane a). A band of 70 kDa was also observed in the old, but in lower amount than in membranes of the young. In addition, a smear of calpastatin in the range between 70 kDa to about 45 kDa was observed in the membranes of old individuals (Figure 3, lane b). Ca^{2+} /Iph treatment led to an initial increase in membrane calpastatin in both young and old individuals (Figure 3, lanes c and d), followed by diminution in calpastatin upon further incubation (Figure 3, lanes e and f).

Binding of added calpastatin to erythrocyte membranes

Membranes prepared from cells of young ('young membranes') and old individuals ('old membranes') were incubated with calpastatin (purified from erythrocytes of a young individual) in the presence of Ca^{2+} . Calpastatin bound to both types of membranes, with more calpastatin associated with young membranes than with old ones (Figure 4). Initial incubation resulted in an increase in membrane-bound calpastatin (Figure 4, lanes a–c, e–g), followed by diminution in the membrane-associated calpastatin in both young and old membranes upon continued incubation (Figure 4, lanes d and h). More calpastatin remained

Figure 1 Detection by immunoblotting of calpastatin in cytosol and heated cytosol of erythrocytes of young and old individuals

Key: Y, young individual; O, old individual; Std, purified calpastatin. Lanes a and b, cytosol; lanes c and d, heated cytosol; lane e, Std. For details, see the Materials and methods section. Results similar to those shown here were obtained in seven of nine experiments; calpastatin in the old ranged between 35 and 65% of calpastatin in the young individuals, run in parallel, as estimated by densitometry of the immunoblots. In two old individuals, the amounts of calpastatin in the cytosol were similar to those observed in the young individuals. The kDa values on the left refer to marker proteins.

Figure 3 Detection by immunoblotting of calpastatin in erythrocyte membranes

Haemoglobin-free membranes were prepared from erythrocytes of young and old individuals. Key: Y, young individual; O, old individual. Lanes a and b, control unincubated cells; lanes c and d, cells treated with Ca²⁺/lph for 10 min; lanes e and f, cells treated with Ca²⁺/lph for 60 min. For details, see the Materials and methods section. Results similar to those shown here were obtained in four young and four old individuals.

associated with the young membranes than with the old ones (Figure 4, lanes d and h).

Degradation of calpastatin by calpain

Calpastatin was incubated with calpain, with or without Ca²⁺ and with or without NEM. In the presence of calpain and Ca²⁺, calpastatin was degraded. Degradation was inhibited in the presence of NEM (Figure 5, lanes a–c). In parallel, autolysis of calpain occurred in the presence of Ca²⁺ and was inhibited by NEM (Figure 5, lanes d–f).

Activity of calpastatin in cytosol of young and old individuals

Activity of calpastatin was estimated by measuring the extent of inhibition of calpain-induced degradation of casein upon addition of heated cytosol, as described in the Materials and methods section. The inhibition curve of calpain activity obtained by addition of heated cytosol from old individuals was shifted to the right as compared with the curve obtained for heated cytosol

Figure 4 Binding of calpastatin added to erythrocyte membranes

Membranes, prepared from erythrocytes of young and old individuals, were incubated with calpastatin in the presence of Ca²⁺. Samples were removed at time intervals, washed and analysed by immunoblotting. Lanes a–d, young individual; lanes e–h, old individual. For details, see the Materials and methods section. Similar results were obtained in four young and four of six old individuals.

Figure 5 Degradation of calpastatin by calpain

Calpastatin was incubated with calpain, with or without Ca²⁺ and with or without NEM. Samples were immunoblotted with anti-calpastatin (lanes a–c) or anti-calpain (lanes d–f) antibodies. For details, see the Materials and methods section.

Figure 6 Activity of calpastatin in erythrocytes of young and old individuals

Portions of heated cytosol were added to calpain solution, and degradation of casein by the calpain–calpastatin mixtures was determined. ○, Young individual; ●, old individual. For details, see the Materials and methods section. The inhibition curve of calpain activity was shifted to the right in six of nine old individuals, as compared with those of young individuals, run in parallel; calpastatin activity in the six samples ranged between 54% and 74% of that in the young ones, based on the point of half-maximal inhibition. In three experiments, the curves for the old were similar to those for the young individuals.

from young individuals (Figure 6). The activity of calpastatin in old and young individuals was estimated from the inhibition curves, with 1 unit of calpastatin defined as the amount of heated cytosol protein required for half-maximal inhibition of casein degradation by 1 unit of calpain. The calpastatin activity in nine young and nine old individuals was 54 ± 2.53 and 40.6 ± 3.26 units/mg of protein respectively ($P < 0.02$).

DISCUSSION

Calpastatin exhibits an abnormal behaviour on SDS/PAGE, migrating more slowly than expected for the number of amino acid residues in the protein [17]. This may be due to the particular nature of the protein, having a high content of negative amino acids and proline [1,2]. Various molecular masses for the native protein have been reported, ranging from 200 kDa to 400 kDa, with subunits of 17 to 170 kDa. The most commonly reported calpastatin subunits are those of about 45 kDa, 70 kDa, 90 kDa, 110 kDa and 120 kDa, with one or several of these observed in extracts of a variety of cells and tissues [2,5,17–20]. In addition,

Table 1 Calpastatin bands in SDS/PAGE

For all details see the Materials and methods section. Preparation procedures: A, cytosol mixed with SDS, then heated; B, cytosol heated, then mixed with SDS; C, cytosol loaded on DE-52 column, and the eluted calpastatin fraction heated, then mixed with SDS. Bands are estimated from marked to traces (+ + + to $\pm$), as identified by immunoblotting.

Pretreatment of erythrocytes	Preparation procedure	Bands (kDa)					
		Young			Old		
		(80–120)	(70)	(45)	(80–120)	(70)	(45)
None	A	+ + +			+ + / +		
None	B	$\pm$	+ + +	$\pm$	$\pm$	+ + / +	$\pm$
None	C	$\pm$	+ + +	$\pm$		+ +	+
Ca ²⁺ /Iph	C		+	+ +		+	+ +
NEM	C	$\pm$	+ + +	$\pm$		+ +	+
NEM → Ca ²⁺ /Iph	C	$\pm$	+ + +	$\pm$		+ +	+

wide diffuse calpastatin bands are commonly observed in SDS/PAGE by Coomassie Blue staining or by immunoblotting [10,19–22].

The reasons for the heterogeneity observed in the molecular masses and for the appearance of the diffuse calpastatin bands in SDS/PAGE are not known. Multiple forms of calpastatin may exist in different cells, due to alternative splicing and/or to post-translational modifications [2,5,17,19]. However, as shown here, differences in isolation procedures and steps used in the preparation may account in part for the heterogeneity in the molecular masses of calpastatin subunits reported previously. The results, summarized in Table 1, show differences in the electrophoretic migration of calpastatin from the same cells in the same SDS/PAGE system after different preparation procedures. Calpastatin migrated as a very wide, diffuse, band (range 80–120 kDa) when the samples were mixed with SDS buffer, then heated (Figure 1, lanes a and b). In contrast, it migrated mainly as a wide band of about 70 kDa if the haemolysates were heated before the addition of SDS buffer (Figure 1, lanes c and d), or after a partial purification by DE-52 column chromatography (Figure 2). Thus, it is important to specify the method of preparing samples of calpastatin for electrophoresis. Heating a crude biological sample after dissociation by SDS solution may result in larger calpastatin subunits than heating the sample before dissociation by SDS (e.g. subunits of over 100 kDa and of 70 kDa, respectively). Removal of some cytoplasmic proteins (such as the removal of haemolysate proteins by DE-52 column chromatography) before heating seems to preserve calpastatin subunits of 45 kDa present in the cell. The properties of calpastatin responsible for this behaviour remain to be studied, but may be related in part to the unique structure and nature of calpastatin [2,5,10,18–20,23].

In the present study, we have found significant differences in the nature of calpastatin derived from old individuals and that derived from young individuals. The differences do not depend on the isolation procedures, as summarized in Table 1. Less calpastatin was found in the cytosol from old than from young individuals (preparation procedures A and B, Table 1). An additional band of 45 kDa was found in samples from old individuals (preparation procedure C). Pretreatment of the cells with Ca²⁺ and the ionophore A23187 leads to enhanced formation of the 45 kDa calpastatin band in both young and old individuals. This suggests that intracellular calpain is responsible for a partial degradation of the calpastatin to a 45 kDa calpastatin subunit. Pretreatment of the cells with NEM (which inhibits calpain activity) inhibited the Ca²⁺-induced increase in the 45 kDa band

from young individuals; the 45 kDa band of old individuals remained (Table 1). Therefore, the 45 kDa calpastatin subunit was already present in the cells of old individuals. An altered calpastatin is thus present within the erythrocytes of old individuals.

Calpastatin does not bind Ca²⁺, but associates with calpain in the presence of Ca²⁺ [4,6,8]. Fragmentation of calpastatin by calpain in the presence of Ca²⁺ occurs in isolated systems *in vitro* [24–26]. Calpain is autolysed under these conditions *in vitro* and, as shown in the present work, both the autolysis of calpain and the degradation of calpastatin are inhibited by NEM. As has been recently pointed out, information on the degradation of calpastatin in the intact cell is lacking [2]. The results described here indicate that calpastatin may associate with calpain in the presence of Ca²⁺ and be degraded by the protease in the intact cell, in a manner similar to degradation *in vitro*. The behaviour of calpastatin in cells of old individuals is similar to the behaviour of calpastatin induced by Ca²⁺ in cells of young individuals. The results therefore suggest that erythrocyte calpastatin in the old individual is subjected to partial degradation, resulting in decreased activity and in diminution in the major band and appearance of bands of lower molecular masses, both in the cytosol and in the membrane. Calpain is probably responsible for the diminution in calpastatin in cells of the aged. However, since calpastatin is very sensitive to degradation by various enzymes [5], degradation of calpastatin in the erythrocytes of old individuals by enzymes other than or in addition to calpain is not excluded.

The alterations in calpastatin in the old individuals may be one of a general suite of age-associated processes. Calpastatin activity has been found to be diminished in certain age-related pathological conditions, e.g. in erythrocytes in untreated hypertension [23,27], and in brain cortical tissues in association with degenerative changes [28], along with enhanced calpain activity [28–30].

The activation of calpain by Ca²⁺ is enhanced by the association of calpain with the cell membrane [4,5]. Activated calpain causes a limited degradation of certain membrane and cytoskeletal proteins and enzymes. The calpain-induced limited proteolysis may regulate activities of membrane-associated enzymes and modulate the behaviour of membrane structural proteins [5,11,12,31]. Therefore, association of calpastatin with cell membrane may be important in preventing calpain binding and/or activation at the membrane [5,7,21].

Calpastatin has been shown to bind to cellular membranes via binding of the N-terminal region of calpastatin to acidic phospho-

lipids [21]. It has been suggested that the lack of binding of erythrocyte calpastatin to the membrane is due to a missing N-terminal domain [21]. That some calpastatin can bind to the erythrocyte membrane within the cell is evident from the increase in membrane-associated endogenous cellular calpastatin following increased intracellular Ca^{2+} . Our results also show that added calpastatin binds to erythrocyte membranes of both young individuals ('young membranes') and old individuals ('old membranes'), but that more of the added calpastatin is associated with young membranes than with the old ones. Since calpastatin from the same source (isolated from erythrocytes of a young individual) was added to young and old membranes, membrane properties seem to influence its interaction with calpastatin. The altered behaviour of the old membranes suggests age-associated changes in the membrane, leading to diminished binding and/or enhanced degradation of bound calpastatin.

In conclusion, our results show significant differences in erythrocyte calpastatin behaviour and activity between young and old individuals and indicate an altered association of the calpain-calpastatin system within the erythrocytes and with the erythrocyte membranes from old individuals. The changes observed may be due to alterations in both intracellular and membrane components, changes which may result in enhanced degradation of certain membrane proteins [12].

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