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Calpain (Ca^{2+}-dependent thiol protease) in erythrocytes of young and old individuals

(aging/band 3 protein/calpain autolysis/membrane proteolysis)

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ABSTRACT **Limited proteolysis by calpain (Ca^{2+}-activated protease; EC 3.4.22.17) is believed to regulate the function of membrane enzymes and modify the behavior of membrane structural proteins. Calpain is activated by autolysis. The degradation of band 3 protein by μ-calpain is known to be enhanced in erythrocyte membranes from human individuals >70 years old (old) as compared with that from individuals 20–30 years old (young). In the present study, monoclonal antibody to μ-calpain was used to study the behavior of calpain in erythrocytes of young and old individuals. Less calpain was found in erythrocyte cytosol and membranes from old than in those from young. Increasing the erythrocyte Ca^{2+} induced translocation of calpain to the cell membrane and autolysis of the enzyme. Alkylation of erythrocyte thiols also promoted translocation of calpain to the membrane, especially in the presence of Ca^{2+}. When calpain was added to erythrocyte membranes, initial binding was greater and subsequent autolysis faster in old than in young individuals, possibly arising from alterations in cell membranes of old individuals. The enhanced calpain autolysis was accompanied by enhanced degradation of band 3 protein in the old. The results suggest that calpain in old individuals is translocated to the cell membrane and is activated by autolysis, resulting in degradation of certain membrane proteins and loss of calpain. Enhanced calpain-induced membrane proteolysis may play a role in abnormal cell destruction (e.g., shortening the life span of erythrocytes in the aged, neuronal degeneration, etc). The erythrocyte membrane provides a convenient model for the study of age-associated alterations in cell membranes and in calpain behavior.**

Calpain is a neutral Ca^{2+}-dependent thiol protease (EC 3.4.22.17), present in a wide variety of vertebrate cells. Two major isozymes are known, μ-calpain and m-calpain, requiring μM and mM Ca^{2+} for activation, respectively (1–5). The isozyme types and amounts of the protease vary among cells and among species. Calpain is found mainly in the cell cytosol and is usually present in an inactive form. It has been suggested that, in the presence of Ca^{2+}, calpain is activated by limited autolysis, a process that is enhanced by the translocation of calpain to the cell membrane (4–10). Activated calpain causes a limited degradation of certain membrane and cytoskeletal proteins and enzymes. The calpain-induced limited proteolysis may regulate enzyme activities and modulate the behavior of membrane structural proteins (3–5, 11–13).

The mammalian erythrocyte contains the μ-calpain isozyme (2). We have shown previously that calpain plays a role in membrane fusion: specifically, that degradation of certain membrane proteins by calpain is a prerequisite for membrane fusion induced by the membrane mobility agent A_2C (14–16). In addition to calpain-induced changes associated with membrane fusion, we have also discovered that the degradation of band 3 protein by μ-calpain is enhanced in erythrocyte membranes of old human individuals (>70 years old) as compared with that in erythrocyte membranes of young ones (20–30 years old) (17). The observed age-related enhanced sensitivity to calpain may be due to changes in band 3 protein, changes in other membrane components, and/or altered association with calpain.

In the present study we used monoclonal antibody to μ-calpain for the identification of calpain in the cytosol and membranes of erythrocytes from young and old individuals. Age-related differences in the behavior of erythrocyte calpain were found. Our results suggest that age-related changes in the cell membrane may lead to an enhanced binding of calpain to the cell membrane, followed by calpain activation, degradation of certain membrane proteins, and loss of the enzyme. Calpain-induced membrane proteolysis may play a role in the shortening of erythrocyte life span in the aged (18).

MATERIALS AND METHODS

Erythrocytes, Cytosol, Membranes, and Band 3 Protein Fragments. Blood was obtained from healthy humans, 20–30 years old (young) and 70–93 years old (old) and prevented from coagulation by the addition of 5 mM EDTA. Blood was centrifuged, plasma and buffy coat were removed, and the erythrocytes were washed three times with 150 mM NaCl. Erythrocytes were hemolyzed in 10 vol of 10 mM Tris·HCl buffer, pH 7.4 (Tris), containing 0.5 mM dithiothreitol (DTT), 1.0 mM EDTA, and 1.0 mM EGTA (Tris-DE) and centrifuged at 30,000 × *g* for 15 min to yield membrane-free hemolysate (cytosol). Partially purified, hemoglobin-free cytosol (Hb-free cytosol) was obtained by loading the cytosol on a DEAE-cellulose column (preequilibrated with Tris-DE) and washing with Tris-DE containing 50 mM NaCl until the eluate was free of hemoglobin. A fraction containing non-hemoglobin proteins, including calpain, was then eluted with Tris-DE containing 150 mM NaCl (15, 16). The eluate was dialyzed against 1.0 mM EDTA/1.0 mM EGTA/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/0.5 mM DTT, then lyophilized and resuspended in Tris. Erythrocyte ghosts were obtained by hemolyzing cells in 5 mM sodium phosphate buffer, pH 8.0 (5p8). Membranes were washed with 5p8, then further washed with 10 mM NaCl to obtain hemoglobin-free ghosts (white ghosts). Membranes stripped of peripheral proteins (membrane vesicles), band 3 chymotrypsin fragment of 60 kDa (CH-60), and trypsin fragment of 55 kDa (TR-55)

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; Inp, ionophore.

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were prepared by established methods, as described previously (17).

Calpain Purification and Preparation of Antibodies to Calpain. Cytosol obtained from young individuals was used for the isolation and purification of calpain according to published procedures (2, 16), with units of calpain activity defined as described previously (16, 17). Monoclonal antibodies specific to human erythrocyte μ-calpain were generated according to established procedures (19). BALB/c mice were initially injected twice (intradermally and subcutaneously, at a 3-week interval) with 50 μg of calpain in complete Freund's adjuvant. Four or five additional booster injections (25–50 μg in incomplete Freund's adjuvant) were administered over a period of several months, with titer of serum antibodies determined by ELISA. Mice displaying the highest titer of anti-calpain antibodies were then injected intraperitoneally with 50 μg of calpain, spleens were removed 3–4 days later, and spleen cells were fused with NSO myeloma cells (19). Subsequent procedures (screening and cloning of hybridoma cells, clone propagation as ascites, and isolation and identification of the antibodies) were carried out by established procedures (19). Ascites fluid or purified antibodies were used. The monoclonal antibody used here is an IgG antibody (CmAb 3) which recognizes the 80-kDa large subunit of μ-calpain (1) (Fig. 1). The antibody did not react with purified m-calpain or with any other erythrocyte nonhemoglobin proteins.

Treatment of Erythrocytes with *N*-Ethylmaleimide (NEM) and with Ca^{2+} and Ionophore (Inp). 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 with or without 1.0 mM NEM at 37°C for 30 min, then washed and resuspended in TBS. Control and NEM-treated cells were further incubated with or without $CaCl_2$ (final concentration 0.5 mM) and the Inp A23187 (final concentration 10 μM, from a stock solution of 5.0 mM in dimethyl sulfoxide), referred to as Ca^{2+}/Inp. Erythrocytes were incubated at 37°C for 30 min, then washed with 150 mM NaCl. Hb-free cytosol, membrane vesicles, and band 3 protein fragments were then prepared as described above.

Treatment of Erythrocyte White Ghosts and Membrane Vesicles with Calpain. Erythrocyte white ghosts were suspended in 10 mM Tris·HCl buffer, pH 7.4, containing 0.1 mM PMSF (Tris-PMSF). The ghost suspensions were mixed with calpain (0.5–1.0 unit/ml of 10% ghost suspension) and 0.5 mM DTT and kept at 4°C for 10 min, then 0.5 mM $CaCl_2$ was added and suspensions were incubated at 30°C for 0.5–5 min. Ghost suspensions were then washed twice in Tris-PMSF to remove unbound calpain. Membrane vesicles were suspended at 4°C in Tris-PMSF, mixed with calpain (0.2–0.5 unit/ml of membrane vesicle suspension containing 0.5 mg of protein) and 0.5 mM DTT, 0.5 mM $CaCl_2$ was added, and suspensions were incubated at 30°C for 5–10 min, then washed with Tris-PMSF.

Analysis of Calpain by Immunoblotting. Cytosol, Hb-free cytosol, white ghosts, and membrane vesicles were used for the analysis of calpain. Protein content in the cytosol was estimated by measuring hemoglobin concentration. Protein content in Hb-free cytosol and in the membranes was determined by the method of Lowry (20). To compare the calpain content of the membrane to that of the cytosol, the number of cells represented in each was estimated (based on values of 5 mmol of hemoglobin per 10^{10} cells and 5 mg of protein per 10^{10} white ghosts).

Samples were mixed with Laemmli buffer (21). Protein analysis was carried out by SDS/PAGE under reducing conditions, using 10% acrylamide in the gels. Gels were fixed and stained with Coomassie brilliant blue according to established methods (17). Other gels, run in parallel, were used for immunoblotting. Proteins were transferred to nitrocellulose membranes and allowed to react with the monoclonal antibody, followed by reaction with secondary antibody, according to established procedures (22). Alkaline phosphatase-conjugated rabbit antibody to mouse IgG served as a secondary antibody; 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) and *p*-nitroblue tetrazolium chloride (NBT) Bio-Rad reagents were used as substrate and color developer, respectively.

RESULTS

Calpain in Erythrocytes from Young and Old Individuals. Cytosol and Hb-free cytosol were used for the analysis of soluble intracellular calpain. White ghosts and membrane vesicles were used for the analysis of membrane calpain. Most of the erythrocyte calpain was found in the cytosol in both young and old individuals, with very little noted in the white ghosts from either young or old (Fig. 1). On the basis of the number of erythrocytes used for the calpain analysis, we estimated the membrane calpain to be about 1% of the total cell calpain.

Erythrocyte cytosol obtained from old individuals contained significantly less calpain than that from young individuals (Fig. 1). The membranes of old individuals also contained less calpain than the erythrocyte membranes from young individuals (Fig. 1). Similar results were obtained in seven of nine old people studied. In the remaining two old individuals the amount of calpain was similar to that observed in young individuals.

To allow for the detection of possible minor calpain fractions, more concentrated samples (i.e., Hb-free cytosol and membrane vesicles) were analyzed by electrophoresis and immunoblotting. The Hb-free cytosol of erythrocytes from old individuals contained less calpain than that of the young, as found in the whole cytosol, and no other calpain fractions were observed. The results indicated that the calpain from the old behaved similarly to that of the young during the partial purification and that the hemolysates did not contain components that may modulate or interfere with the antibody reaction. Membrane vesicles of old individuals also contained less calpain than those of young ones, as found in the whole membranes (data not shown). These results indicate that native calpain was lost from the cytosol and from the cell membranes in old individuals. Since the specific monoclonal antibody used may not recognize certain calpain degradation products, degraded calpain fractions of low molecular masses may be present in the cytosol or cell membranes.

Increased Intracellular Ca^{2+} and Thiol Alkylation: Effects on the Behavior of Erythrocyte Calpain. Erythrocytes of young and old individuals were incubated with or without

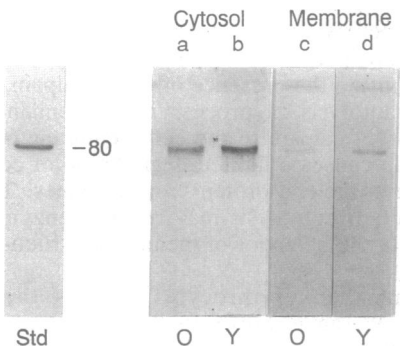

FIG. 1. Detection by immunoblotting of calpain in cytosol and membranes of erythrocytes from old and young individuals. Std, purified calpain; O, old individual; Y, young individual. Molecular mass is given in kDa.

NEM, followed by incubation with or without Ca^{2+}/Inp, then analyzed for calpain in Hb-free cytosol and membrane vesicles. Results obtained for erythrocytes of a young individual are shown in Fig. 2. One calpain band of 80 kDa was observed in Hb-free cytosol obtained from control cells (Fig. 2, lane a). Two calpain bands (the original one of 80 kDa and one of about 76 kDa) were observed in the Hb-free cytosol prepared from Ca^{2+}/Inp-treated erythrocytes (Fig. 2, lane b). Only the original calpain band of 80 kDa was observed in cells incubated with Ca^{2+} alone or with the ionophore alone (not shown). In cells treated with NEM, one calpain band (80 kDa) was observed (Fig. 2, lane c). When NEM-alkylated erythrocytes were treated with Ca^{2+}/Inp, little or no calpain was detected in the cytosol (Fig. 2, lane d). The changes in the cytosol were accompanied by changes in membrane calpain. Very little calpain was detected in control membranes (Fig. 2, lane e). A significant increase in calpain was observed in membrane vesicles of Ca^{2+}/Inp-treated cells as compared with the control (Fig. 2, lane f). A slight increase in the amount of calpain was noted in membranes of NEM-alkylated cells (Fig. 2, lane g). A very large amount of calpain was observed in membranes of cells treated with both NEM and Ca^{2+}/Inp (Fig. 2, lane h). Similar results were obtained for cell membranes of old individuals (data not shown). Thus, the changes observed in the cytosol of the alkylated and Ca^{2+}/Inp-treated erythrocytes were mirrored by the changes observed in the membranes.

Association of Cellular Calpain with Band 3 Protein Fragments. We have previously found that band 3 protein is degraded by calpain and that the band 3 CH-60 fragment is the sensitive domain, whereas the TR-55 fragment is not sensitive to calpain (17). It was therefore of interest to find out whether the endogenous calpain binds to the band 3 fragments, especially under conditions of enhanced calpain translocation to the membrane. Erythrocytes of young and old individuals were incubated with or without NEM, followed by further incubation with or without Ca^{2+}/Inp. Band 3 fragments were prepared as described (17). Results obtained in old erythrocytes are shown in Fig. 3. In control untreated cells, calpain was found to be associated with the CH-60 fragment but not with the TR-55 fragment (Fig. 3, lanes a and e). Enhanced association of calpain was found in CH-60 isolated from cells treated with Ca^{2+}/Inp, with two bands of calpain noted (Fig. 2, lane b). A slight increase in calpain bound to CH-60 fragment was observed after NEM treatment (Fig. 3, lane c) and a very large increase was noted after both NEM and Ca^{2+} (Fig. 3, lane d). In contrast, no calpain was associated with the TR-55 fragment after Ca^{2+}/Inp or NEM, with very little present even in alkylated cells treated with Ca^{2+}/Inp (Fig. 3, lanes *f–h*). Thus, under conditions of enhanced translocation from the cytosol, calpain binds preferentially to the band 3 sensitive domain. Very little enzyme is bound to the fragment which is not degraded by calpain, even under conditions of translocation of most of the cellular enzyme to the membrane. Similar results were obtained for band 3 protein fragments prepared from erythrocytes of young individuals (data not shown).

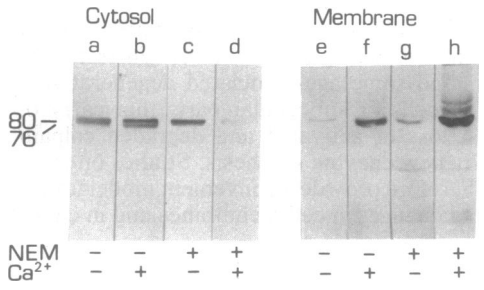

FIG. 2. Effects of Ca^{2+} and of thiol alkylation on erythrocyte calpain. Erythrocytes from a young individual were incubated with or without NEM, then washed and further incubated with or without Ca^{2+}/Inp. Hb-free cytosol and membrane vesicles were prepared and analyzed by immunoblotting. Lanes a–d, immunoblots of calpain in Hb-free cytosol; lanes e–h, immunoblots of calpain in membrane vesicles.

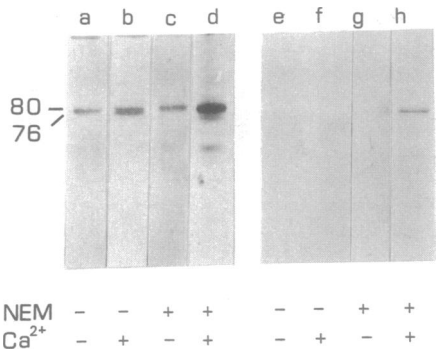

FIG. 3. Detection by immunoblotting of cellular calpain associated with band 3 protein fragments. Erythrocytes from an old individual were incubated with or without NEM, then washed and incubated with or without Ca^{2+}/Inp. Band 3 protein fragments were prepared and analyzed for the presence of calpain by immunoblotting. Lanes a–d, immunoblots of calpain associated with band 3 chymotrypsin-generated fragment of 60 kDa (CH-60); lanes e–h, immunoblots of calpain associated with band 3 trypsin-generated fragment of 55 kDa (TR-55).

Interaction of Added Calpain with Erythrocyte Membranes and Band 3 Protein from Old and Young Individuals. Calpain, prepared from erythrocytes of a young individual, was added to white ghosts prepared from cells of old individuals ("old" membranes) and of young ones ("young" membranes). Very little calpain bound to any of the membrane preparations when added in the absence of Ca^{2+} (data not shown). In the presence of Ca^{2+}, more calpain was initially bound to "old" membranes than to "young" ones (Fig. 4, lanes a and d). Upon a short incubation, calpain added to "old" membranes was degraded to a significant extent (Fig. 4, lanes b and c), whereas calpain added to "young" membranes changed little (Fig. 4, lane e). When calpain and Ca^{2+} were added to membrane vesicles containing band 3 protein, the enhanced degradation of band 3 in the old (Fig. 5, lanes a and b) was paralleled by enhanced degradation of calpain (Fig. 5, lane f), whereas little degradation of band 3 and of added calpain was noted in membrane vesicles isolated from young individuals (Fig. 5, lanes c, d, and g). These results were obtained in 7 of 10 old people studied. In the other 3, the behavior of band 3

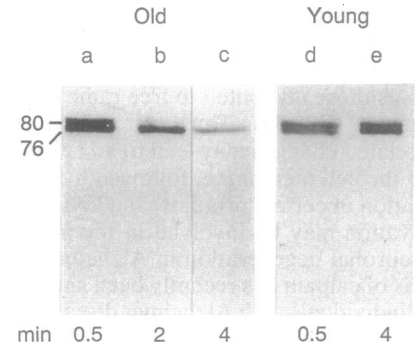

FIG. 4. Binding and autolysis of calpain added to erythrocyte membranes. Membranes, isolated from erythrocytes of old and young individuals, were incubated in the presence of calpain (0.5 unit/10% membrane suspension) and 0.5 mM Ca^{2+} at 30°C, then washed, solubilized, and analyzed by immunoblotting. Lanes a–c, old individual; lanes d and e, young individual. Incubation times are given at the bottoms of the lanes.

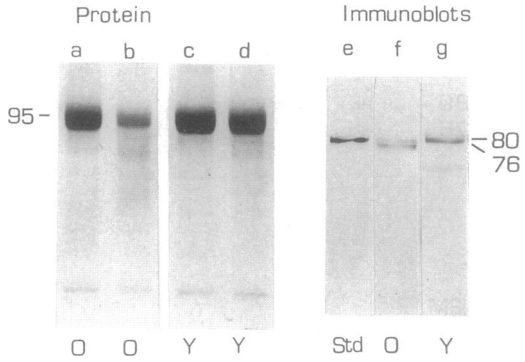

FIG. 5. Band 3 degradation and calpain autolysis in old and young individuals. Erythrocyte membrane vesicles, obtained from old and young individuals, were incubated with or without calpain (0.5 unit/ml of membrane vesicle suspension containing 0.5 mg of protein) at 30°C for 6 min, then solubilized and analyzed. Lanes a–d, SDS/PAGE patterns of proteins (Coomassie blue staining); lanes e–g, immunoblots of calpain; lanes a and c, control vesicles; lanes b, d, f, and g, calpain-treated vesicles. Std, purified calpain; O, old individual; Y, young individual.

protein and of the added calpain was similar to that observed in the young.

DISCUSSION

An increase in cellular Ca^{2+} promotes the activation of calpain by autolysis, followed by degradation of substrates and loss of calpain, as found in neural tissues (23) and in erythrocytes (24, 25). In the mature erythrocyte, which is unable to synthesize proteins, any activation and autolysis of calpain would result in an irreversible loss of calpain from the cell. The results presented here are consistent with the idea that calpain in the erythrocytes from old individuals is subjected to activation and autolysis, leading to a diminution in cellular calpain. Of interest are our findings that thiol alkylation results in enhanced translocation of cytosolic calpain to the membrane. With both alkylation of erythrocyte thiols and increased cellular Ca^{2+}, most of the erythrocyte calpain is translocated to the membrane. The results indicate that thiol depletion alters the membrane proteins and/or the enzyme in such a way that it binds more easily to the membrane, especially in the presence of Ca^{2+}. Though complete cellular and enzyme alkylation would result in enzyme inactivation, a partial diminution in thiols would, in the presence of Ca^{2+}, allow for a loss of cytosolic calpain, enhanced binding of calpain to the membrane, and activation of the enzyme by intermolecular calpain autolysis (7, 10). An increase in cellular Ca^{2+} and diminution in thiols may occur in certain tissues as a result of age-associated processes (11, 26–29), such as those attributed to free radical reactions and oxidative damage (30, 31). Thus, our results suggest that certain age-related changes may lead to an enhanced binding of calpain to the cell membrane, followed by calpain activation, degradation of certain proteins, and loss of the enzyme. Calpain activation may be involved in the abnormal proteolysis and neuronal degeneration in Alzheimer disease (32–34). Autolysis of calpain has recently been shown to occur in the brains of individuals with Alzheimer disease (34). Such an autolysis indicates an increase in intracellular calcium in Alzheimer disease (34). Similarly, though no data are available on Ca^{2+} levels in erythrocytes of old individuals, our results suggest that an increase in Ca^{2+} may occur in these cells.

Band 3 degradation is induced by increasing erythrocyte Ca^{2+} (35). Band 3 degradation has been proposed to be responsible for the appearance of senescence antigen to serve as a signal for the removal of aged cells (36). The enhanced degradation of band 3 protein by calpain in the old individual may provide a senescence signal and play a role in the shortened erythrocyte life span observed in aged animals and humans (18, 37). Proteins related to band 3 protein are found in other cell membranes (38, 39), so age-related alterations in membranes of different cells may be similar to the changes observed in the erythrocytes from old individuals. Other membrane proteins may also show differential sensitivity to calpain. We have recently found that spectrin from erythrocytes of old individuals is more susceptible to degradation by calpain than is spectrin from young ones (T.G., N.S.-B., Y. Zipser, and N.S.K., unpublished data). It should be emphasized that subtle changes (e.g., limited membrane proteolysis by activated calpain) might be sufficient to cause a significant change in membrane behavior (36).

The calpain translocated from the cytosol to the membrane may bind to substrates or to other membrane components, proteins or phospholipids (9, 40). Phospholipids play a role in the activation process (10, 40). We have previously found that the difference between young and old people in the susceptibility of band 3 protein to calpain is also observed in membrane vesicles stripped of peripheral proteins and in the band 3 CH-60 fragment, which contains the N-terminal cytoplasmic domain. The transmembrane TR-55 fragment is not degraded by calpain in either young or old individuals (17). The isolated soluble N-terminal cytoplasmic 43-kDa fragment is degraded to a similar extent in young and old individuals (17). In the present study we found that calpain was bound to the band 3 fragment containing the susceptible domains (CH-60) but not to the nondegradable fragment (TR-55). This selective binding was also observed when essentially all of the cellular enzyme was translocated from the cytosol to the membrane under conditions which inhibited its activity. The results therefore suggest that the enzyme binds to the sensitive domains of substrates. However, since all the preparations contained other components and especially lipids, the binding of the enzyme may involve membrane components other than the substrate.

The fact that calpain from the same source (calpain purified from erythrocytes obtained from young individual) underwent a greater autolysis when added to "old" than when added to "young" membranes indicates age-associated alterations in membrane components and/or organization. These may include alterations in proteins and/or lipids due to oxidative damage (40–42) or to other post-translational modifications, though primary changes in protein polypeptides are not excluded. Changes at the level of the cell membrane do not, of course, exclude changes in calpain itself with aging. It should also be noted that changes may occur in the specific calpain inhibitor calpastatin, which is present in many types of cells (2, 5, 6).

Elucidation of the changes responsible for the altered interaction of cellular calpain with the substrates may help us to understand some age-associated degenerative processes (32–34). In cells or subcellular parts that carry on protein synthesis, loss of activated and degraded calpain may be masked by new enzyme synthesis. Studies on mature erythrocytes therefore provide a convenient model for the study of age-related changes in cell membranes and in calpain behavior.

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