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The possible self-down-regulation of calpain triggered by cell membranes

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Abstract

In order to confirm whether the binding sites for μ-calpain on the inner surface of erythrocyte membranes are substrate proteins themselves, we examined the binding properties of μ-calpain to μ-calpain-pretreated inside-out membranes. When native μ-calpain was incubated with μ-calpain-pretreated membranes, however, newly added calpain was degraded rapidly in a time- and Ca^{2+}-dependent manner. Although the degradation of μ-calpain was not inhibited by various proteinase inhibitors, it was strongly inhibited by digestible substrates for calpain that possess the ability to inhibit the binding of μ-calpain to erythrocyte membranes. On the other hand, when μ-calpain inactivated by *N*-ethylmaleimide was incubated with μ-calpain-pretreated membranes, no degradation was observed. These results indicate that the degradation of μ-calpain occurs on the surface of μ-calpain-modified membranes and that it depends on the autoproteolytic activity of μ-calpain itself. It seems likely that the autoproteolytic activity of μ-calpain is accelerated markedly by some component(s) exposed on the surface of membranes during the pretreatment with μ-calpain. The possibility is thus proposed that cell membranes possess the ability to down-regulate calpain to protect cell membranes from overdegradation by excessively bound calpain. The active factor(s) in the membranes that can accelerate the autoproteolytic degradation of μ-calpain could be almost completely removed from μ-calpain-modified membranes by treatment with Triton X-100.

Keywords: Calpain; Autoproteolysis; Cell membrane; Down regulation

1. Introduction

Calcium-activated neutral proteinase (calpain; EC 3.4.22.17) is an intracellular cysteine proteinase distributed in various tissues and cells throughout the animal kingdom. Two forms of calpain have been identified, namely μ-calpain which is activated at μM concentrations of calcium ions and m-calpain for which mM concentrations are required. An endogenous specific inhibitor, called calpastatin, also exists in various tissues and cells. Although the exact physiological function of these enzymes is not yet established, the calpain/calpastatin system has been suggested to play important roles in cellular functions in response to mobilized calcium ions [1–5].

From many experiments in vitro, the mechanism underlying the activation of calpain has been visualized. The activation process appears to include calpain autoproteolysis in the presence of calcium ions during which the N-terminal portion of the large subunit, which inhibits proteinase activity, is removed [6–8]. This autoproteolysis reaction is accompanied by an increase in the calcium sensitivity of calpain. In the case of μ-calpain, the molecular mass of the large subunit decreases from the original 79 kDa to 76 kDa via a 77 kDa intermediate [7]. Calpain binds to cytoplasmic membranes in a Ca^{2+}-dependent manner and its autoproteolytic activation is facilitated greatly by this binding [9–11]. We have also demonstrated that intracellular μ-calpain in erythrocytes is activated upon increases in intracellular Ca^{2+} levels in the same manner as that established in vitro and that the cytoplasmic

Abbreviations: kDa, kilodaltons; PAGE, polyacrylamide gel electrophoresis; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; E-64c, L-*trans*-epoxysuccinylleucylamido(3-methyl)butane; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; IgG, immunoglobulin G; NEM, *N*-ethylmaleimide.

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cell membrane is also involved in the activation of μ-calpain in vivo as well as in vitro [12]. Although little is known about the precise binding sites for μ-calpain on the membrane, our previous data strongly suggest that these are composed of substrate proteins rather than membrane phospholipids [11,17]. Therefore, we examined the binding of μ-calpain to μ-calpain-pretreated erythrocyte membranes to confirm that the binding sites are substrate proteins that can be removed by pretreatment. In the course of this experiment, however, we noticed that μ-calpain newly added to μ-calpain-pretreated membranes for binding assay is always degraded rapidly. We considered that this phenomenon might be related to the down-regulation of calpain in which excess calpain molecules bound to membranes are to be removed. In this paper, we report in detail on the interaction between μ-calpain and μ-calpain-pretreated membranes, in order to clarify the mechanism for the degradation of μ-calpain.

2. Materials and methods

2.1. Materials

μ-Calpain and calpastatin were purified from rabbit skeletal muscle and rabbit cardiac muscle, respectively, as described [13,14]. A monoclonal antibody specific for μ-calpain ($1A_8A_2$) that recognizes only the large subunit of μ-calpain was produced by immunizing female BALB/c mice with purified μ-calpain from rabbit skeletal muscle and screening hybridoma cells that secreted μ-calpain specific antibodies, as described previously for the isolation of m-calpain specific antibodies [15]. Chemicals were purchased as follow: casein Hammersten from Merck; phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), EGTA, TLCK, and human hemoglobin from Sigma; leupeptin, E-64c and pepstatin A from Peptide Research Foundation; prestained molecular weight markers for immunoblotting from Bio-Rad; nitrocellulose membranes from Schleicher and Schuell; peroxidase-conjugated anti-mouse IgG (Fab)′ from Medical and Biological Labs; Triton X-100 from Wako Chemicals. Other chemicals were obtained from Nacalai Tesque.

2.2. Preparation of inside-out membrane vesicles

Hemoglobin-free erythrocyte membranes (unsealed white ghosts) were prepared from fresh heparinized human peripheral blood and the inside-out vesicles were prepared from unsealed white ghosts according to the method of Steck and Kant [16]. The purity of inside-out vesicles was determined from the ratio of acetylcholinesterase activities in the presence and absence of 0.01% Triton X-100 and was estimated to be 85–90% [11].

2.3. Preparation of μ-calpain-treated erythrocyte membranes

100 μg as protein of inside-out vesicles or unsealed white ghosts were incubated with 1 μg of purified μ-calpain at 25° C for 15 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol in Ca^{2+}-EGTA buffer containing 1.4 μM free Ca^{2+}. The reaction was stopped by the addition of EGTA to a final concentration of 2 mM and the mixture was centrifuged at $35\,000 \times g$ for 10 min. The pelleted modified membranes were washed once with 20 mM Tris-HCl (pH 7.5) containing 2 mM EGTA and three times with 20 mM Tris-HCl (pH 7.5), and resuspended in the same buffer to the original volume.

2.4. Treatment of μ-calpain-treated erythrocyte membranes with Triton X-100

In order to remove the major integral proteins and glycerophospholipids, μ-calpain-pretreated erythrocyte membranes were further treated with 0.5% (v/v) Triton X-100 as described before [17]. Triton X-100-treated membranes were prepared from the μ-calpain-pretreated white ghosts instead of the μ-calpain-pretreated inside out vesicles, since Triton shells from μ-calpain-pretreated inside out vesicles were not recovered as pellets by centrifugation, probably due to destruction of lining protein network. Triton shells were resuspended to the original volume.

2.5. Detection of membrane-associated proteinase activity in μ-calpain-treated membranes

To detect proteinase activity at neutral pH, 41 μg of μ-calpain-pretreated inside-out vesicles were incubated with 30 μg of alkali-denatured casein at 30° C for 180 min in 20 mM Tris-HCl, 250 μM $CaCl_2$, 10 mM 2-mercaptoethanol, pH 7.5. After incubation, the reaction mixture was cooled immediately on ice and centrifuged at $386\,000 \times g$ for 10 min. The soluble fraction was heated in the sample buffer for SDS-PAGE at 100° C for 5 min. The proteinase activity was detected by electrophoresis on an SDS-polyacrylamide gel. For the detection of acidic proteinase activity, 41 μg of μ-calpain-pretreated inside-out vesicles were incubated with 30 μg of acid-denatured hemoglobin at 30° C for 180 min in 100 mM acetate, 250 mM $CaCl_2$, 10 mM 2-mercaptoethanol, pH 4.0. The other procedures were the same as for the assay of the neutral proteinase activity.

2.6. Preparation of inactivated μ-calpain

Inactivated μ-calpain was prepared by modification with *N*-ethylmaleimide according to the method described previously [18].

2.7. Preparation of phospholipids from erythrocyte membranes

The phospholipid fraction was prepared according to the method of Saito et al. [19] and stored under N_2 at $-20°$ C. The amount of phospholipid was determined by inorganic phosphorous analysis [20].

2.8. Preparation of phospholipid vesicles

Phospholipids or phospholipid mixtures isolated from erythrocyte membranes were dried on the bottom of assay tubes under a stream of nitrogen and sonicated in buffer prior to assay.

2.9. Determination of free calcium ions

Ca^{2+}-EGTA buffer was prepared by mixing 1 mM EGTA and appropriate amounts of $CaCl_2$ solution in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol. Free Ca^{2+} concentrations were calculated using a dissociation constant of $26.8 \cdot 10^{-6}$ M [21].

2.10. Protein determination

Protein concentration was determined by Lowry's method [22] using bovine serum albumin as the standard protein.

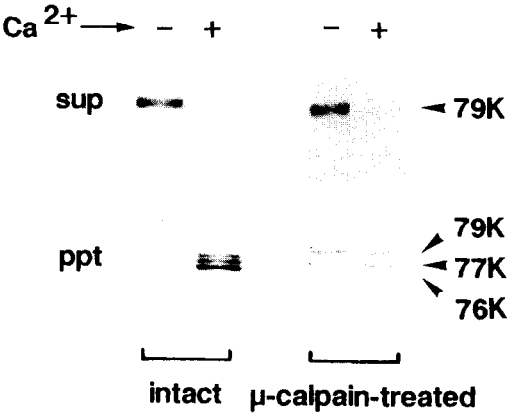

Fig. 1. Binding properties of μ-calpain to intact and μ-calpain-pretreated inside-out vesicles. 1 μg of native μ-calpain was incubated with 66 μg as protein of intact or μ-calpain-pretreated inside-out vesicles at 25° C for 2 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing 1 mM EGTA or Ca^{2+}-EGTA buffer (1.4 μM free Ca^{2+}). The reaction was stopped by the addition of leupeptin to a final concentration of 1 mM and the mixture was immediately cooled on ice. The reaction mixture was centrifuged at 35000 × g for 10 min to separate the soluble fraction from the membrane fraction. The membrane fraction was washed three times, heated in the sample buffer for SDS-PAGE at 100° C for 5 min, and electrophoresed on SDS-polyacrylamide gels. The proteins in the soluble fraction were precipitated in 10% trichloroacetic acid and treated for electrophoresis as the membrane fraction. After electrophoresis, the proteins in the gels were transferred onto a nitrocellulose membrane and immunostained with monoclonal antibody ($1A_8A_2$) against the large subunit of μ-calpain. sup, soluble fraction; ppt, membrane fraction.

3. Results

3.1. Degradation of μ-calpain in the presence of μ-calpain-pretreated erythrocyte membranes

We have previously reported that the major binding site for μ-calpain on the inner surface of erythrocyte membranes are proteins rather than membrane phospholipids [11,17]. In the present study, we examined the binding of μ-calpain to μ-calpain-pretreated membranes, in order to confirm that the binding site is a substrate protein for μ-calpain itself. If the binding site is a substrate protein, the amount of μ-calpain binding to the membrane might be markedly decreased by pretreatment of membranes with μ-calpain.

However, when μ-calpain was newly added to the binding assay reaction mixture, it disappeared almost completely not only from the membrane fraction, but also from the soluble fraction (Fig. 1). These results suggest the possibility that the newly added μ-calpain was degraded by exposure to μ-calpain-modified membranes. The time course for the degradation of μ-calpain in the presence of μ-calpain-modified membranes was explored in the presence of 1.4 μM free calcium ions (Fig. 2). When μ-calpain was incubated with intact membranes, the 79 kDa pro-enzyme autoproteolyzed at once in a restricted manner, and

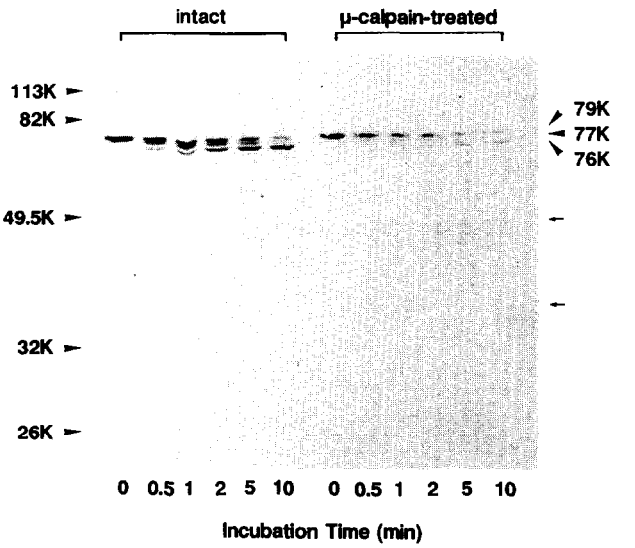

Fig. 2. Time-dependent degradation of μ-calpain in the presence of μ-calpain-pretreated inside-out vesicles. 1 μg of native μ-calpain was incubated with 95 μg as protein of intact or μ-calpain-pretreated inside-out vesicles at 25° C for various periods in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing Ca^{2+}-EGTA buffer (1.4 μM free Ca^{2+}). The reaction was stopped by the addition of the sample buffer for SDS-PAGE. The other procedures were the same as those in Fig. 1. The two faint bands indicated by arrows are the position of the degradation product of μ-calpain.

was converted to the 76 kDa active enzyme via a 77 kDa intermediate as previously reported [11]. In the case of the μ-calpain-modified membranes, however, μ-calpain was degraded rapidly without restricted autoproteolysis. If μ-calpain substrates are removed completely from membranes during the first incubation with μ-calpain, it might induce rapid autoproteolysis of μ-calpain during the second incubation of μ-calpain with the μ-calpain-treated membranes. As shown in Fig. 3, however, considerable amounts of μ-calpain substrates such as spectrin and band 3 remained on the μ-calpain-treated membranes. Therefore, it seems likely that the rapid degradation of μ-calpain in the presence of μ-calpain-treated membranes is induced not by exhaustion of substrate proteins.

3.2. Effect of substrate proteins on the degradation of μ-calpain in the presence of μ-calpain-pretreated membranes

As a first step to analyze the degradation mechanism of μ-calpain, it is important to clarify whether the association of μ-calpain with μ-calpain-pretreated membranes is a prerequisite for degradation. Thus, we examined the effect of substrate proteins on the degradation of μ-calpain since, as we previously reported, digestible substrate proteins for calpain, such as alkali-denatured casein and calpastatin

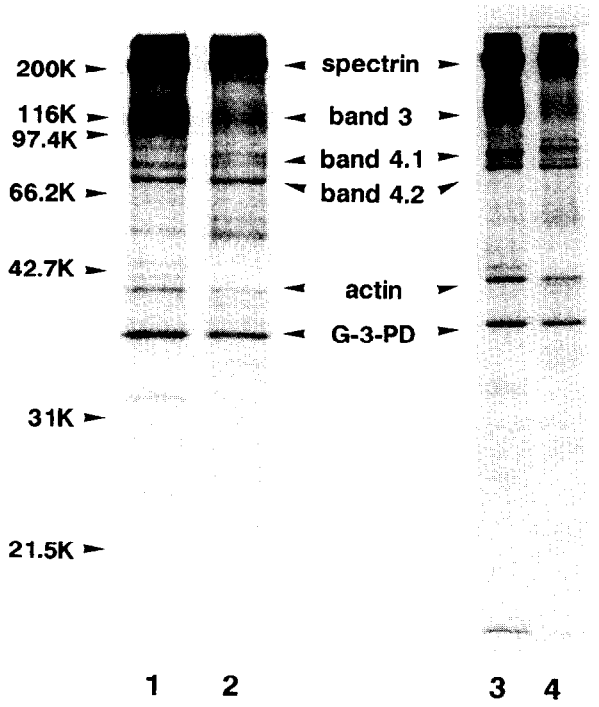

Fig. 3. Electrophoresis pattern of intact and μ-calpain-treated erythrocyte membrane proteins. The samples were electrophoresed on 12.5% SDS-polyacrylamide gels and the proteins were stained with Coomassie brilliant blue. Lanes: 1, intact inside-out vesicles; 2, μ-calpain-treated inside-out vesicles; 3, intact white ghosts; 4, μ-calpain-treated white ghosts. Spectrin, band 3, band 4.1, band 4.2, actin and glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) were identified by their mobilities.

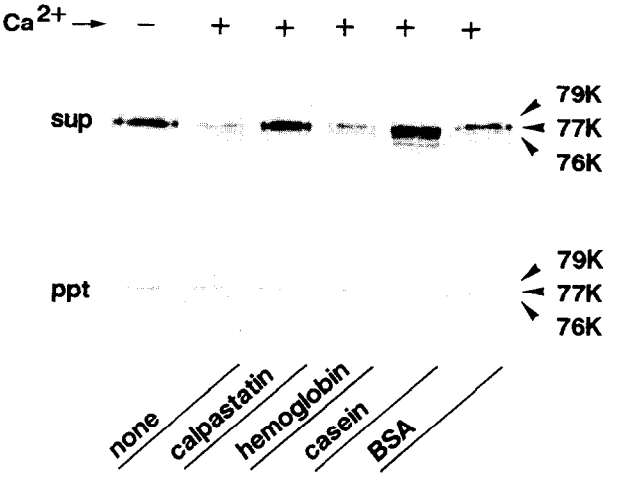

Fig. 4. Effect of substrate proteins on the degradation of μ-calpain by μ-calpain-pretreated inside-out vesicles. 1 μg of native μ-calpain (0.3 unit) was incubated with 95 μg as protein of the μ-calpain-pretreated inside-out vesicles at 25° C for 2 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing Ca^{2+}-EGTA buffer (1.4 μM free Ca^{2+}) in the presence of substrates. The other procedures were the same as those in Fig. 1. none, with Ca^{2+} only; calpastatin, with Ca^{2+} and rabbit cardiac muscle calpastatin (0.7 unit/4 μg/ml); hemoglobin, with Ca^{2+} and nondenatured hemoglobin (0.25 mg/ml); casein, with Ca^{2+} and alkali-denatured casein (0.25 mg/ml); BSA, with Ca^{2+} and nondenatured bovine serum albumin (0.25 mg/ml). sup, soluble fraction; ppt, membrane fraction.

(suicide substrate), can inhibit the binding of μ-calpain to erythrocyte membranes [11]. The characteristics of these substrate proteins could prove very useful answering this question. As undigestible proteins, nondenatured hemoglobin and BSA were chosen. As shown in Fig. 4, the degradation of μ-calpain in the presence of μ-calpain-modified membranes was strongly interrupted by digestible substrates. On the other hand, undigestible substrates had little or no effect on the degradation of μ-calpain. These results strongly suggest that the degradation of μ-calpain is triggered by the association of μ-calpain with μ-calpain-modified membranes.

3.3. Effect of proteinase inhibitors on the degradation of μ-calpain in the presence of μ-calpain-pretreated membranes

There are a few possible reasons for the degradation of μ-calpain by μ-calpain-pretreated membranes. One is that some unidentified membrane-associated proteinase is exposed and/or activated by pretreatment with μ-calpain, and the newly added μ-calpain is a good substrate for this proteinase. In order to explore this possibility, we examined the effects of various proteinase inhibitors on the degradation of μ-calpain. As shown in Fig. 5, however, a proteinase inhibitor cocktail containing leupeptin, E-64c, TLCK, and pepstatin A did not inhibit the degradation of μ-calpain on μ-calpain-pretreated membranes. PMSF and DFP also showed no inhibitory effect (data not shown). In

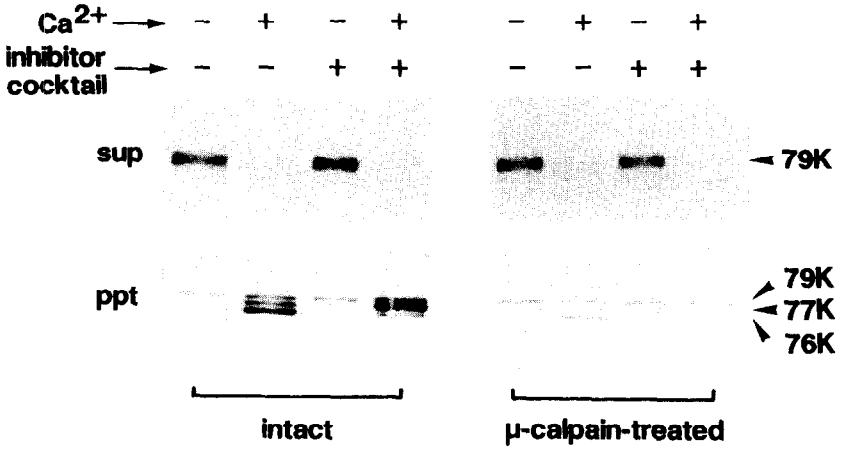

Fig. 5. Effect of proteinase inhibitors on the degradation of μ-calpain on μ-calpain-pretreated inside-out vesicles. 1 μg of native μ-calpain was incubated with 53 μg as protein of intact or μ-calpain-pretreated inside-out vesicles at 25° C for 2 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing 1 mM EGTA or Ca^{2+}-EGTA buffer (1.4 μM free Ca^{2+}) in the presence or absence of proteinase inhibitor cocktail (500 μM leupeptin, 500 μM E-64c, 500 μM TLCK and 50 μM pepstatin A). The other procedures were the same as those in Fig. 1. sup, soluble fraction; ppt, membrane fraction.

separate experiments, we could detect proteinase activities in μ-calpain-pretreated membranes at neutral and acidic pH, using alkali-denatured casein and acid-denatured hemoglobin as substrates, respectively. Both proteinase activities, however, were completely inhibited in the presence of the same inhibitor cocktail (data not shown). Therefore, the possibility that some membrane-associated proteinase is involved in the degradation of μ-calpain can be excluded.

3.4. Degradation of native and NEM-inactivated μ-calpains on μ-calpain-pretreated membranes

It has been shown that calpain is highly susceptible to Ca^{2+}-induced autoproteolysis and that restricted autoproteolysis plays an essential regulatory role in activation as a proteinase. However, extensive autoproteolysis can lead to the loss of activity and ultimate destruction of the enzyme. Therefore, it is likely that the degradation of μ-calpain proceeds autoproteolytically on μ-calpain-modified membranes if some component(s) with an ability to accelerate the autoproteolytic activity of μ-calpain is exposed on membrane surfaces upon treatment with μ-calpain. To verify whether the degradation of μ-calpain depends on the autoproteolytic activity of μ-calpain itself, *N*-ethylmaleimide-inactivated μ-calpain was incubated with μ-calpain-pretreated membranes (Fig. 6). When this inactivated μ-calpain was incubated with active form of μ-calpain (76 kDa), it was degraded and the degradation pattern was the same as in the autoproteolysis of native μ-calpain [18], suggesting no gross conformational change in μ-calpain molecule by the treatment with *N*-ethylmaleimide. Intact μ-calpain was degraded in a Ca^{2+}-dependent manner as described above. On the other hand, no degradation of inactivated μ-calpain was observed. These results clearly demonstrate that the degradation of μ-calpain on μ-calpain-modified membranes occurs autoproteolytically. However, one question now arises. Why could not the proteinase inhibitor cocktail, which contains leupeptin and E-64c, inhibit the autoproteolytic degradation of μ-calpain? Although we have no good explanation for this at the moment, it is possible that the velocity of the autoproteolytic degradation of μ-calpain is much faster than that of the association of μ-calpain with proteinase inhibitors, or, because the autoproteolytic degradation proceeds intramolecularly, substrate-like inhibitors can not interact with the active sites of μ-calpain.

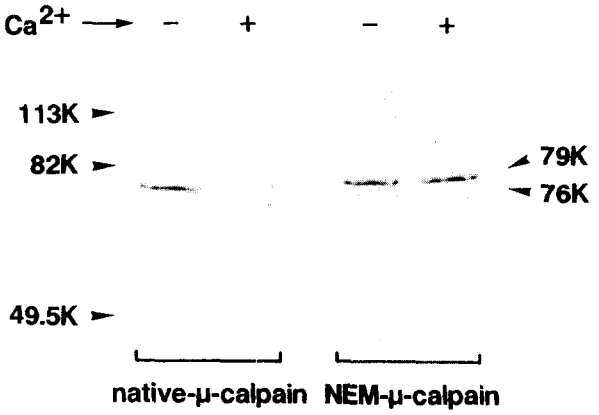

Fig. 6. Degradation of native and NEM-inactivated μ-calpains on μ-calpain-pretreated inside-out vesicles. 1 μg of native or NEM-inactivated μ-calpain was incubated with 96 μg as protein of μ-calpain-pretreated inside-out vesicles at 25° C for 15 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing 1 mM EGTA or Ca^{2+}-EGTA buffer (1.4 μM free Ca^{2+}). The reaction was stopped by the addition of the sample buffer for SDS-PAGE. The other procedures were the same as those in Fig. 1.

3.5. Effect of Triton shells prepared from μ-calpain-pretreated erythrocyte membranes on the autodegradation of μ-calpain

In order to characterize the factor(s) that exists on the surface of μ-calpain-modified membranes and induce the

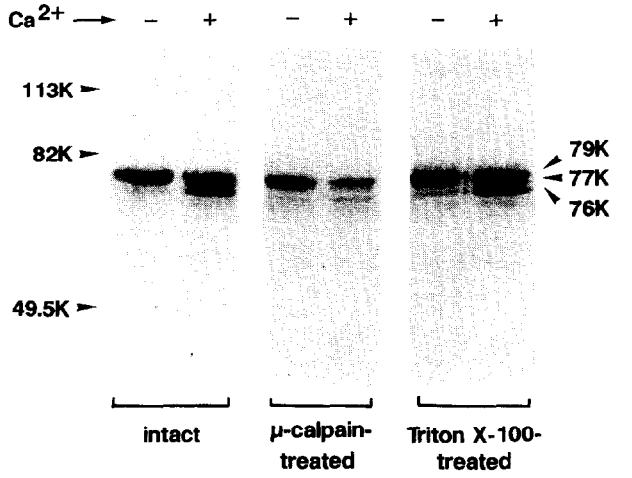

Fig. 7. Effect of Triton shells prepared from μ-calpain-pretreated white ghosts on the autodegradation of μ-calpain. 1 μg of native μ-calpain was incubated with intact white ghosts, μ-calpain-pretreated white ghosts or Triton shells prepared from the μ-calpain-pretreated white ghosts (95 μg, 75 μg, 50 μg as protein, respectively) at 25° C for 5 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing Ca^{2+}-EGTA buffer (1.4 μM free Ca^{2+}). The reaction was stopped by the addition of the sample buffer for SDS-PAGE. The other procedures were the same as those in Fig. 1.

autoproteolytic degradation of μ-calpain, we examined the effect of Triton shells prepared from μ-calpain-modified membranes on the autoproteolytic degradation of μ-calpain. In this experiment, we used unsealed white ghosts instead of inside-out vesicles as a starting material to prepare Triton shells, since it is very difficult to prepare Triton shells from μ-calpain-pretreated inside-out vesicles. No appreciable difference was found between the μ-calpain-treated inside-out vesicles and white ghosts in the composition of remaining membrane proteins (Fig. 3). μ-Calpain, when incubated with intact unsealed white ghosts, was activated autoproteolytically in a restricted manner, while in the case of the μ-calpain-modified white ghosts, it was degraded (Fig. 7). These results are identical to those of intact and the μ-calpain-modified inside-out vesicles. On the other hand, when μ-calpain was incubated with Triton shells, no degradation was observed and the enzyme was autoproteolytically activated in a restricted manner as occurs when intact unsealed white ghosts were used. This result clearly shows that the factor(s) that induces the autoproteolytic degradation of μ-calpain was removed from μ-calpain-modified membranes by treatment with Triton X-100.

3.6. Effect of phospholipids on the autoproteolysis of μ-calpain

We showed previously that most integral proteins and glycerophospholipids are extracted from erythrocyte white ghosts by treatment with Triton X-100 [17]. In this paper, we thus examined the effect of glycerophospholipids on the autoproteolysis of μ-calpain (Fig. 8). In the case of phosphatidylethanolamine, the 79 kDa subunit of μ-calpain was autoproteolyzed to an active 76 kDa form and considerable amounts of active species accumulated. On the other hand, μ-calpain was degraded in the presence of phospha-

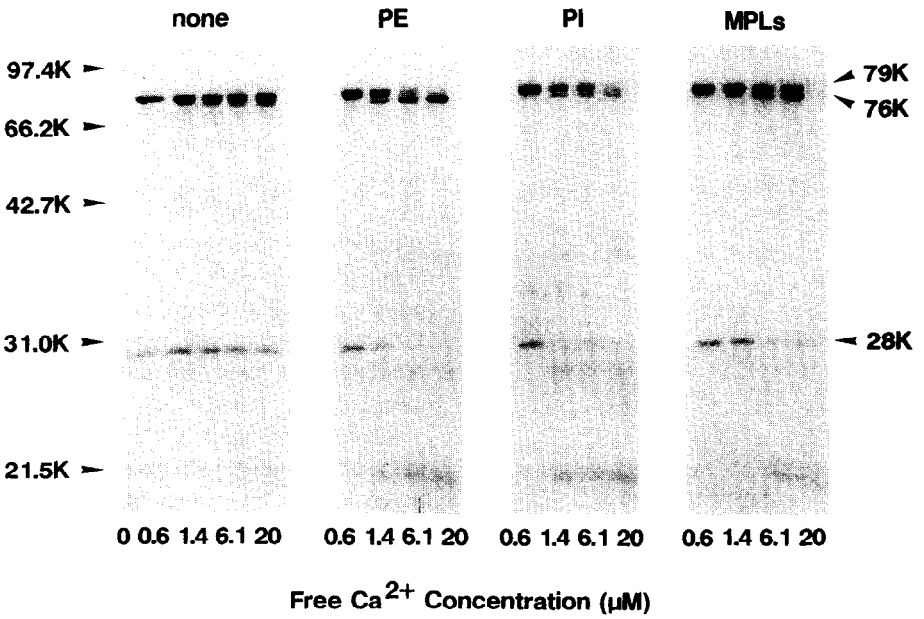

Fig. 8. Effect of phospholipids on the autoproteolysis of μ-calpain. 5 μg of native μ-calpain was incubated at 25° C for 2 min in 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol containing Ca^{2+}-EGTA buffer with various concentrations of free Ca^{2+} in the presence or absence of phospholipids. The reaction was stopped by the addition of the sample buffer for SDS-PAGE. The samples were electrophoresed on 12.5% SDS-polyacrylamide gels and the proteins were stained with Coomassie brilliant blue. none, with Ca^{2+} only; PE, with Ca^{2+} and phosphatidylethanolamine (100 μg/ml); PI, with Ca^{2+} and phosphatidylinositol (100 μg/ml); MPLs, with Ca^{2+} and a phospholipid mixture (100 μg/ml) from erythrocyte white ghosts.

tidylinositol even at low concentrations of calcium ions, and no significant accumulation of an active species was observed. The autoproteolytic behavior of μ-calpain in the presence of phosphatidylinositol is very similar to that observed with μ-calpain-modified membranes. The phospholipid mixture isolated from erythrocyte white ghosts showed the same effect as phosphatidylethanolamine rather than phosphatidylinositol. Although phosphatidylserine and phosphatidylcholine were also potent stimulators of the autoproteolytic activation of μ-calpain, it was much weaker than that of phosphatidylethanolamine (data not shown).

4. Discussion

It is desirable to use μ-calpain and membrane from the same source to analyze the mode of binding and subsequent event on the membranes. But membrane preparations, especially inside-out vesicles, from rabbit erythrocytes were not satisfactory in purity and could not be used in our experiments. Previously, we compared the action of rabbit μ-calpain on unsealed white ghosts from human and rabbit erythrocytes and obtained similar results for the effects of substrate proteins and calpastatin except for some differences in the amount of μ-calpain bound Ca^{2+}-dependently to the membranes [11]. Furthermore, we observed that same membrane proteins on both membranes such as spectrin, band 3 and band 4.1 are degraded by rabbit μ-calpain (data not shown). We therefore concluded that the results obtained in the present experiments using a heterologous combination of μ-calpain and membranes reflects those obtained using homologous systems.

Most animal cells and tissues contain two species of calpain and its specific inhibitor protein, calpastatin. There have also been a few reports that activator proteins for calpain can be found in several tissues and cells [23–26]. Thus, it is considered that calpain activity is regulated by calcium ions and those endogenous regulatory proteins. However, the precise regulatory mechanism of calpain activity in living cells is only poorly understood.

In this report, we describe an interesting phenomenon that seems to be related to a novel regulatory mechanism of calpain activity by cell membranes. Namely, we have verified that erythrocyte membranes obtain the ability to accelerate the degradation of μ-calpain by pretreatment with μ-calpain. Although, the degradation of μ-calpain in the presence of μ-calpain-modified membranes was not inhibited by various proteinase inhibitors, it was strongly inhibited by the addition of digestible substrates that possess the ability to prevent the binding of calpain to membranes (Figs. 4 and 5). When μ-calpain inactivated with *N*-ethylmaleimide was incubated with μ-calpain-modified membranes, no degradation was observed (Fig. 6). These results strongly suggest that the degradation of μ-calpain proceeds on the surface of the μ-calpain-modified membranes and it depends on the autoproteolytic activity of μ-calpain itself. We could not detect significant amounts of typical autoproteolytic fragments of μ-calpain during the incubation of μ-calpain with the μ-calpain-modified membranes. But it was verified that μ-calpain was degraded autoproteolytically without significant accumulation of the typical fragments in the presence of phosphatidylinositol (Fig. 8). It seems quite probable that this type of 'unusual' degradation of μ-calpain occurs as a result of extensive acceleration of its autoproteolytic activity by some component that becomes exposed on the surface of membranes by treatment with μ-calpain. On the basis of the existing data, we believe that the above phenomenon functions as a mechanism for the down-regulation for calpain, in which calpain molecules excessively bound and/or having already functioned on membranes are removed.

It is thought that calpain usually exists as an inactive proenzyme in the cytosolic part of living cells and binds to membranes in response to mobilized calcium ions. Then, it autoproteolyzes immediately to an active form on membranes, and hydrolyzes various substrate proteins such as cytoskeletal proteins and membrane proteins. Therefore, if the cell membranes were attacked continuously by calpain without restriction, it would cause great damage to cellular function. Accordingly, it is conceivable that cell membranes are provided with a function to protect themselves from excessive attack by calpain. In many cells, however, calpastatin is present in concentrations sufficient to completely block all calpain activity. Thus, further investigation is required to clarify whether this down-regulation system actually functions in living cells. It has also been reported that calpastatin can not interact with membrane-associated calpain [27].

The activity that acts to accelerate the autoproteolytic degradation of μ-calpain was mostly removed from μ-calpain-modified membranes by treatment with Triton X-100 (Fig. 7) together with most integral membrane proteins and glycerophospholipids. Thus, we first examined the effect of various glycerophospholipids on the autoproteolysis of μ-calpain under membrane-free conditions. It is generally accepted that phosphatidylinositol can reduce the Ca^{2+} concentration needed for autoproteolytic activation of calpain. Under our experimental conditions, however, the factor(s) that accelerates the autoproteolytic degradation of μ-calpain in μ-calpain-modified membranes was mimicked by phosphatidylinositol (Fig. 8). It was demonstrated by Cong et al. [28] that phosphatidylinositol markedly reduces the specific activity of autoproteolyzed μ-calpain (active form) without changing the Ca^{2+}-sensitivity. This phenomenon might be regarded as the down-regulation of an active form of μ-calpain by phosphatidylinositol. Phosphatidylinositol might act to accelerate the degradation velocity of the active form leading to inactivation. But, pretreatment of μ-calpain-modified membranes with phospholipase C also has no effect on the autoproteolytic degradation of μ-calpain (data not shown). Conse-

quently, it seems unlikely that phosphatidylinositol alone causes the autoproteolytic degradation of μ-calpain. Thus, it is interesting and of significance to examine the components that are extracted from membranes by treatment with Triton X-100. Such work is currently underway in our laboratory.

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