The Calpain Cascade

µ-CALPAIN ACTIVATES m-CALPAIN

(Received for publication, October 1, 1996)

Peter Tompa, Andrea Baki, Éva Schád, and Peter Friedrich

From the Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapest, Hungary

m-Calpain, which usually requires near-millimolar Ca\(^{2+}\) for activation, undergoes autolysis at 25 µM Ca\(^{2+}\) in the presence of µ-calpain. m-Calpain in itself exhibits no sign of autolysis around this Ca\(^{2+}\) concentration. Half-maximal rate of the reaction occurs at 30 µM Ca\(^{2+}\), showing that it is µ-calpain that catalyzes the limited proteolysis of m-calpain in an intermolecular reaction (*“heterolysis”*). This heterolytic step is accompanied by the activation of m-calpain: µ- and m-calpain preincubated together at 25 µM Ca\(^{2+}\) show significantly higher activity than the sum of activities of µ- and m-calpains preincubated separately. m-Calpain is sensitized to Ca\(^{2+}\) by µ-calpain-mediated activation: the half-maximal value of 160 µM for activation is lowered to 64 µM, which is similar to the shift found in m-calpain autoactivation. We suggest that these *in vitro* observations are relevant *in vivo* and the calpain cascade may play an important role in coordinating the functioning of calpains in living cells.

Calpain (Ca\(^{2+}\)-activated cysteine protease) is one of the mediators of intracellular Ca\(^{2+}\) signal in animal cells (1, 2). It has two well characterized ubiquitous isoforms, µ- and m-calpain. Both consist of a large (80-kDa) catalytic and a small (30-kDa) regulatory subunit but differ considerably in their Ca\(^{2+}\) sensitivities: µ-calpain is activated at micromolar, m-calpain at millimolar free Ca\(^{2+}\) concentration. At the resting cytoplasmic Ca\(^{2+}\) concentration, both forms are inactive. Mandatory for their activation is an elevation in free Ca\(^{2+}\) concentration, which results in an autolytic cleavage at the N terminus of the calpain molecule. In principle, this would allow for a similar reaction between µ- and m-calpains as they coexist in most tissues (14) and share significant structural homology (15). Our results demonstrate that this reaction indeed takes place *in vitro*. We discuss the possible implications of this novel finding and propose that such a “calpain cascade” may explain the activation of m-calpain at least in some tissues.

EXPERIMENTAL PROCEDURES

Calpain Preparation—Human erythrocyte µ-calpain was purified as described previously (16). Pig kidney m-calpain was isolated by essentially the same method, with slight modifications. Pig kidneys, kept on ice, were used as fresh as possible, usually within one-half hour after slaughter. They were minced and homogenized with 4 volumes of homogenization buffer using a commercial blender. The homogenate was centrifuged at 25,000 × *g* for 30 min, and the supernatant was applied to the DEAE-cellulose column. m-Calpain was eluted with a linear gradient of 50 to 500 mM NaCl; activity appeared at 215 mM NaCl. Phenyl-Sepharose chromatography was then carried out as described in Ref. 17. In the subsequent step, gradient elution was used again: m-calpain appeared at 350 mM NaCl from the Q-Sepharose column. The rest of the procedure was used without modifications. µ- and m-calpains were dialyzed into the same batch of buffer to ensure that the EGTA concentration of the reaction mixtures be independent of their original composition.

Calpain Assay—Calpain activity was determined under initial rate conditions essentially as described in Ref. 18 in the following reaction mixture: 10 mM HEPES, pH 7.5, 1 mM EDTA, 0.5 mM dithioerythritol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine (calpain buffer), various concentrations of Ca\(^{2+}\); and 66 µg/ml [3H]casein (19) in a total volume of 75 µl. The reaction was started by the addition of calpain and terminated after 3 min at 30 °C by adding 58 µl of 30% trichloroacetic acid and 118 µl of 3.7 mg/ml bovine serum albumin. Trichloroacetic acid-soluble peptides were separated from precipitated casein by centrifugation (15,000 × *g* × 5 min), and 200 µl of the supernatant was tested for radioactivity. Calpain activity was calculated from the difference in counts between the sample and a control prepared without Ca\(^{2+}\).

Preparation of E-64\(^{1}\)-inactivated Calpains—For control purposes, calpains were irreversibly inactivated by E-64 applied at a molar excess of 350 (µ-calpain) or 250 (m-calpain). µ-Calpain was incubated with the inhibitor for 4 h at 0 °C in the presence of 400 µM Ca\(^{2+}\) whereas m-calpain for 1 h at room temperature in the presence of 1 mM Ca\(^{2+}\). Unreacted E-64 was removed by overnight dialysis at 4 °C against calpain buffer. Residual activity of calpains following this treatment was less than 1%.

Determination of the Apparent First Order Rate Constant of Autolysis or Heterolysis—m-Calpain was incubated either in the absence or in the presence of µ-calpain at various Ca\(^{2+}\) concentrations at 30 °C. Aliquots were withdrawn at various times, boiled for 5 min with SDS sample buffer, and run on SDS-PAGE. Optical density of the 30-kDa band was determined by densitometry. Natural logarithm of the optical density determination of activation have been identified, including proteins (10), nucleic acids (11), and lipids (12). The prevailing activation model, partially drawing on such studies, is that activation involves translocation of calpain to the plasma membrane where interaction with lipid head groups and/or membrane proteins along with high local Ca\(^{2+}\) concentration at Ca\(^{2+}\) channels would suffice (2). Experimental evidence in support of this model, however, is not conclusive.

In this work we raise the possibility of an alternative means of m-calpain activation. It is known that calpain autolysis may proceed by an intermolecular reaction (13), *i.e.* via the autolytic cleavage of native calpain by another, already activated, calpain molecule. In principle, this would allow for a similar reaction between µ- and m-calpains as they coexist in most tissues (14) and share significant structural homology (15). Our results demonstrate that this reaction indeed takes place *in vitro*. We discuss the possible implications of this novel finding and propose that such a “calpain cascade” may explain the activation of m-calpain at least in some tissues.

1 The abbreviations used are: E-64, trans-epoxysuccinyl-l-leucyl-amido-(4-guanidino)butane; PAGE, polyacrylamide gel electrophoresis.
within 1 min at the same Ca\(^{2+}\) subunit of m-calpain is converted into smaller fragments. However, significantly facilitates this interconversion. The small m- and E-64-inactivated m-calpain was incubated at 25 \(\mu\)M Ca\(^{2+}\); lanes 11–14, m- and E-64-inactivated m-calpain at 400 \(\mu\)M Ca\(^{2+}\); lanes 15–17, controls without Ca\(^{2+}\). Aliquots of the reaction mixtures were withdrawn at the times indicated, boiled for 5 min with SDS sample buffer containing 20 mM EGTA, and run on 11% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue.

The curve converges to that measured with m-calpain alone because at high Ca\(^{2+}\) concentrations m-calpain activity dominates in the system. The Ca\(^{2+}\) requirement of m-calpain autolysis is significantly higher, with an estimated half-maximal value at or above 500 \(\mu\)M. Autolysis of m-calpain becomes immeasurably fast at high Ca\(^{2+}\) concentrations, which prevents an accurate determination of the maximal autolytic rate constant.

Autolysis of m-calpain has been claimed to have two functional consequences: activation of the enzyme and sensitization to Ca\(^{2+}\) (5, 21). Our results indicate that the reaction catalyzed by \(\mu\)-calpain has similar consequences. Activation of m-calpain is demonstrated in Fig. 3. \(\mu\)- and m-calpain at a molar ratio of 1:5 were preincubated for various times at 25 \(\mu\)M Ca\(^{2+}\), and their activity was measured at 100 \(\mu\)M Ca\(^{2+}\). The activity of \(\mu\)-calpain, measured separately in the presence of E-64-inactivated m-calpain, was subtracted from this joint activity to get the contribution of m-calpain. The m-calpain activity calculated in this way increases with time and becomes almost 3 times that of m-calpain preincubated in the absence of \(\mu\)-calpain. In control experiments, \(\mu\)-calpain was also preincubated with heat-inactivated m-calpain or casein to ensure that this increment in activity was not due to inhibition of \(\mu\)-calpain autodegradation by m-calpain. A similar time course of \(\mu\)-calpain activity was observed in both instances, indicating that the increase in m-calpain activity is genuine.

The \(\mu\)-calpain-catalyzed conversion of m-calpain also results in a significant sensitization to Ca\(^{2+}\) (Fig. 4). Half-maximal activity of m-calpain is seen at 160 \(\mu\)M Ca\(^{2+}\) while that of m-calpain activated with \(\mu\)-calpain at 64 \(\mu\)M Ca\(^{2+}\). This shift is of the same magnitude as that brought about by autolysis of m-calpain from the same source (22). These results show that autolysis and heterolysis are mechanically closely related.

### RESULTS

Autolysis of m-calpain requires very high Ca\(^{2+}\) concentrations. At 25 \(\mu\)M Ca\(^{2+}\), virtually no conversion of its 30-kDa autolytic removal of the N-terminal segment of the large subunit with \(\mu\)-calpain, and of the small subunit with m-calpain, is instrumental in the activation of these enzymes. We have shown in kinetic experiments previously for \(\mu\)-calpain that the two reactions run in close parallel (7), which renders autolysis an adequate marker of activation. In fact, autolysis has become virtually synonymous to activation over the years and has been used to demonstrate activation of calpain in vivo.
The Calpain Cascade

33163

Fig. 3. Activation of m-calpain by μ-calpain. 0.3 μM m-calpain was preincubated in the absence (□) or in the presence (×) of 0.06 μM μ-calpain at 25 μM Ca^{2+} for the times indicated at 30 °C. Calpain activity, given in radioactivity of supernatant, was measured as described under "Experimental Procedures." Activity of μ-calpain, preincubated separately with 0.3 μM E-64-inactivated m-calpain (△), was subtracted from the joint activity of the two calpains to obtain activity due to m-calpain (●). μ-Calpain preincubated in the presence of 0.3 μM heat-inactivated m-calpain or 66.7 μg/ml casein showed a similar decay of activity (data not shown).

Fig. 4. Sensitization of m-calpain to Ca^{2+} by μ-calpain. 0.06 μM μ-calpain and 0.3 μM m-calpain were preincubated at 25 μM Ca^{2+} for 3 min, and their activity was determined at various Ca^{2+} concentrations. μ-Calpain activity was also determined separately under identical conditions and was subtracted from this joint activity to obtain the contribution of activated m-calpain (●). Half-maximal activity occurs at 64 μM Ca^{2+}, m-Calpain in itself (□) requires significantly higher Ca^{2+} concentration for activity (EC_{50%} = 160 μM). Data points are averages of duplicate experiments.

(23, 24). Mechanistic studies of autolysis have revealed that it may proceed in both intra- and intermolecular reactions. We asked the question whether intermolecular autolysis (i.e. heterolysis) existed between μ- and m-calpains.

Our results show that this reaction does take place in vitro: μ-calpain catalyzes the limited proteolytic interconversion of m-calpain small subunit at a low Ca^{2+} concentration where m-calpain exhibits no autolytic activity in itself. Apparently, the reaction is not due to sensitization of m-calpain to Ca^{2+} by a direct interaction with μ-calpain as the latter is applied in substoichiometric amounts, and its inactivation completely abolishes the reaction. The presence of activating factors, possibly altering Ca^{2+} sensitivity of m-calpain (cf. Refs. 10–12), in the μ-calpain preparation can also be excluded on this ground. Furthermore, the Ca^{2+} sensitivity of this heterolytic reaction is characteristic of μ-calpain: half-maximal rate is at 30 μM (cf. Refs. 25 and 26). This value provides good evidence that μ-calpain is responsible for the reaction, because autolysis of m-calpain by itself requires much higher Ca^{2+} concentrations (EC_{50%} about 500 μM). The end products of autolysis and heterolysis are apparently identical as judged by SDS-PAGE: both result in a truncated, 18-kDa small subunit. The course of heterolysis, however, is somewhat different from that of autolysis: it proceeds via two relatively stable intermediates of about 28 and 24 kDa. The prevalence of these intermediates may permit the demonstration of the calpain cascade in vivo.

We have checked if activation accompanies the heterolysis of m-calpain. Indeed, m-calpain is activated by μ-calpain at a low Ca^{2+} concentration. μ- and m-calpain preincubated at 25 μM Ca^{2+} exhibit an activity significantly greater than the sum of activities of μ- and m-calpain measured separately. This activity increment could not be due to subtracting spuriously low μ-calpain activities from the (μ + m) curve, because control experiments showed that the decrease in μ-calpain activity during incubation was not influenced by inactivated m-calpain or casein. The loss of μ-calpain activity results from autodegradation of the enzyme which follows its autolytic activation (7). The fast initial rise in activity, however, is not seen here as it cannot be resolved in the present assay which includes a 3-min incubation of the enzyme with the substrate.

These observations constitute a compelling body of evidence for the in vitro operation of a "calpain cascade," i.e. the activation of m-calpain by μ-calpain. As for the physiological relevance of this cascade, its functioning in vivo is to be tested. While this needs further studies, there are several considerations that make this organization likely and attractive. In the first place, it may alleviate the extreme Ca^{2+} demand for m-calpain activity. The coexistence and similar subcellular localization of the two ubiquitous calpains in many tissues render their physical contact possible. The well-known extracellular proteolytic cascades such as those in fibrinolysis and complement activation have two important functional characteristics: (i) they accomplish significant amplification, which results in a large response to a small stimulus, and (ii) they provide multiple control to prevent unwanted and deleterious activation. One may speculate that the calpain cascade possesses similar functional attributes. The calpain cascade, along with other factors (10–12), may provide for controlled m-calpain activation at low Ca^{2+} concentrations. This control may be tissue-specific as distribution of μ- and m-calpains is not uniform (14). In tissues where μ-calpain is scarce, activation by the calpain cascade may be limited or even negligible. In cells where μ-calpain abounds, the calpain cascade may dominate in m-calpain function. It is also conceivable, that the calpain cascade not only activates but also down-regulates m-calpain due to degradation of the activated enzyme. Such a mechanism has been attributed to μ-calpain in vivo (27). The calpain cascade may enable a further, more subtle, way of m-calpain control. Calpain action is thought to be controlled by cellular localization, e.g. by translocation to the plasma membrane (2), to the nucleus (28), or to mitotic chromosomes (29). Localized activation of μ-calpain may bring about a spatially restricted activation of m-calpain, leaving other parts of the cell unaffected.

In conclusion, the calpain cascade may contribute in various and incisive ways to coordinating μ- and m-calpain action. Further work is needed to assess the physiological implications.

Acknowledgment—We thank Csilla Farkas for her assistance in preparing the figures.
REFERENCES

1. Sorimachi, H., Saido, T. C., and Suzuki, K. (1994) FEBS Lett. 343, 1–5
2. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) FEBS J. 343, 814–822
3. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Sorimachi, H., Murofushi, H., Tsuji, S., Ito, H., and Suzuki, K. (1992) J. Biochem. (Tokyo) 111, 81–86
4. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Yoshizawa, T., Sorimachi, H., Ito, H., Tsuji, M., Kawashima, S., and Suzuki, K. (1994) FEBS Lett. 346, 263–267
5. Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y., and Imahori, K. (1981) J. Biochem. (Tokyo) 90, 275–278
6. Inomata, M., Imahori, K., and Kawashima, S. (1986) Biochem. Biophys. Res. Commun. 138, 638–641
7. Baki, A., Tompa, P., Alexa, A., Molnár, O., and Friedrich, P. (1996) Biochem. J. 318, 897–901
8. Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., and Murachi, T. (1983) J. Biol. Chem. 258, 8883–8889
9. Coolican, S. A., Haiech, J., and Hathaway, D. R. (1986) J. Biol. Chem. 261, 4170–4176
10. Pontremoli, S. Melloni, E., Michetti, M., Salamino, F., Sparatore, B., and Horecker, B. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1740–1743
11. Mellgren, R. L., Song, K., and Mericle, M. T. (1993) J. Biol. Chem. 268, 653–657
12. Coolican, S. A., and Hathaway, D. R. (1984) J. Biol. Chem. 259, 11627–11630
13. Inomata, M., Kashi, Y., Nakamura, M., and Kawashima, S. (1988) J. Biol. Chem. 263, 19783–19787
14. Murachi, T., Hatanaka, M., Yasumoto, Y., Nakayama, N., and Tanaka, K. (1981) Biochem. Int. 2, 651–656
15. Suzuki, K. (1990) in Intracellular Ca²⁺-dependent Proteolysis (Mellgren, R. L., and Murachi, T., eds) pp. 25–35, CRC Press Inc., Boca Raton, FL
16. Tompa, P., Schaid, E., Baki, A., Alexa, A., Batke, J., and Friedrich, P. (1995) Anal. Biochem. 228, 287–293
17. Karlsson, J.-O., Gustavsson, H., Hall, C., and Nilsson, E. (1985) Biochem. J. 231, 201–204
18. Pfetter, M., and Friedrich, P. (1992) Biochemistry 31, 8201–8206
19. Rice, R. M., and Means, G. E. (1971) J. Biol. Chem. 246, 831–832
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. DeMartino, G. N., Huff, C. A., and Croall, D. E. (1986) J. Biol. Chem. 261, 12047–12052
22. Brown, N., and Crawford, C. (1993) FEBS Lett. 322, 65–68
23. Saido, T. C., Suzuki, H., Yamazaki, M., Tanoue, K., and Suzuki, K. (1993) J. Biol. Chem. 268, 7422–7426
24. Saito, K.-I., Elce, J. S., Hames, J. E., and Nixon, R. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2628–2632
25. Inomata, M., Hayashi, M., Nakamura, M., Imahori, K., and Kawashima, S. (1993) J. Biochem. (Tokyo) 93, 291–294
26. Cong, J., Goll, D. E., Peterson, A. M., and Kapprell, H.-P. (1989) J. Biol. Chem. 264, 10096–10103
27. Inomata, M., and Kawashima, S. (1995) Biochim. Biophys. Acta 1235, 107–114
28. Mellgren, R. L., and Lu, Q. (1994) Biochem. Biophys. Res. Commun. 204, 544–550
29. Schollmeyer, J. E. (1988) Science 240, 911–913