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Calpastatin Subdomains A and C Are Activators of Calpain*

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Peter Tompa‡§, Zoltán Mucsi¶, György Orosz¶, and Peter Friedrich‡

From the ‡Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapet, P. O. Box 7, and ¶Research Group of Peptide Chemistry at Eötvös Loránd University, Hungarian Academy of Sciences, Budapest, Hungary

The inhibitory domains of calpastatin contain three highly conserved regions, A, B, and C, of which A and C bind calpain in a strictly Ca^{2+}-dependent manner but have no inhibitory activity whereas region B inhibits calpain on its own. We synthesized the 19-mer oligopeptides corresponding to regions A and C of human calpastatin domain I and tested their effect on human erythrocyte μ-calpain and rat m-calpain. The two peptides significantly activate both calpains: the Ca^{2+} concentration required for half-maximal activity is lowered from 4.3 to 2.4 μM for μ-calpain and from 250 to 140 μM for m-calpain. The EC_{50} concentration of the peptides is 7.5 μM for μ-calpain and 25 μM for m-calpain. It is noteworthy that at low Ca^{2+} concentrations (1–2 μM for μ-calpain and 70–110 μM for m-calpain) both enzymes are activated about 10-fold by the peptides. Based on these findings, it is suggested that calpastatin fragments may have a role in calpain activation *in vivo*. Furthermore, these activators open new avenues to cell biological studies of calpain function and eventually may alleviate pathological states caused by calpain malfunction.

Calpains represent a superfamily of proteases related by their homology in a papain-like protease domain (1, 2). Several members of this family, such as the best studied typical forms, μ- and m-calpain, are thought to be activated by a Ca^{2+} signal; their activation then leads to the limited proteolytic modification of a variety of substrate proteins. Recent x-ray crystallographic studies of rat (3) and human (4) m-calpain have revealed the structural background of the Ca^{2+} dependence of calpain activity. Without Ca^{2+} the structure of the active site is distorted; large scale conformational changes have to occur for the assembly of a catalytically competent active site. Although mechanistically clear, key aspects of the activation process *in vivo* are still enigmatic as calpains require non-physiologically high Ca^{2+} concentrations *in vitro*. μ- and m-Calpains reach half-maximal activation in the high μM to mM free Ca^{2+} concentration range (*cf*. Ref. 5), hardly attainable under normal cellular conditions.

Several suggestions have been put forward on how to close this gap. First, calpain undergoes autolytic activation with a concomitant sensitization to Ca^{2+} (5, 6); this, of course, does not alleviate the initial high Ca^{2+} requirement. Second, phospholipids increase calcium sensitivity of calpain (7, 8), and activation probably involves the translocation of the enzyme to the plasma membrane (9, 10). Third, calpain might also be sensitized to Ca^{2+} by a specific activator protein that affects membrane localization and autolysis of the enzyme (11). Nuclear DNA, shown to promote calpain effect on transcription factors and other nuclear proteins, may be another positive effector (12). The above factors have been characterized to different extents, and there is no consensus with respect to their contribution.

In this paper we raise the possibility of an additional, rather unexpected mode of calpain sensitization to Ca^{2+} by fragments of its endogenous inhibitor, calpastatin. Typical calpastatin molecules have five structural domains; the function of the N-terminal domain is not clear, the other four homologous domains are all capable of inhibiting a calpain molecule (13, 14). The presence of Ca^{2+} is mandatory for calpain binding and inhibition by calpastatin (15–17), and calpastatin binding invariably occurs at a Ca^{2+} concentration significantly lower than that needed for enzyme activity (18). Each inhibitory domain contains three short conserved stretches of about 20 amino acids, termed subdomains A, B, and C (*cf*. Fig. 1). In functional studies it was shown that subdomain B is responsible for inhibition (19–21), whereas subdomains A and C potentiate this inhibitory effect by binding to the calmodulin-like domains of the large and small subunit, respectively, in a strictly Ca^{2+}-dependent manner (17, 20–22). Subdomains A and C have no inhibitory effect on calpain (20–23).

These observations allow for a very intriguing possibility, the *activation* of calpain by calpastatin fragments. Namely, as binding of calpastatin and its subdomains to calpain is facilitated by Ca^{2+}, thermodynamic necessity demands that the reverse be also true: calpastatin should facilitate Ca^{2+} binding to calpain. In other words, calpastatin or its subdomains must lower the Ca^{2+} demand of calpain. This is clearly seen with whole calpastatin, which binds at a Ca^{2+} concentration significantly lower than that needed for calpain activity (18). In essence, calpastatin shifts the conformational equilibrium of calpain toward the active form; activation with whole calpastatin, of course, is not seen because of blocking of the active site by subdomain B. Subdomains A and C, on the other hand, lack this inhibitory potential. In earlier experiments they did not inhibit calpain at high Ca^{2+} concentrations (20–23). Our results here show that they in fact potentiate Ca^{2+} binding and thus activate the enzyme at subsaturating Ca^{2+} levels.

EXPERIMENTAL PROCEDURES

Enzymes—Human erythrocyte μ-calpain was purchased from Calbiochem (catalog no. 208713). Rat m-calpain composed of an 80-kDa large subunit and a 21-kDa truncated small subunit was expressed in *Escherichia coli* and purified via the C-terminal His tag attached to its large subunit as given in Ref. 24. The enzymes were dialyzed against three changes of 10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 15 mM β-mercaptoethanol (calpain buffer) and stored at 4 °C until use. On the day of use a

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§ Supported by a Bolyai János Scholarship. To whom correspondence should be addressed. Tel.: 361-466-5633; Fax: 361-466-5465; E-mail: tompa@enzim.hu.

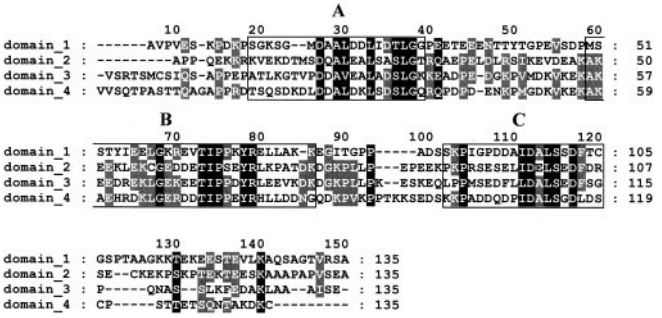

FIG. 1. **Alignment of human calpastatin inhibitory domains.** The four domains of human calpastatin inhibitory to calpain are shown aligned. Identity or strong similarity of residues extending to four (*black*) or three (*gray*) domains are marked by *shading*. Subdomains A, B, and C, as defined in Ref. 21, are *boxed*.

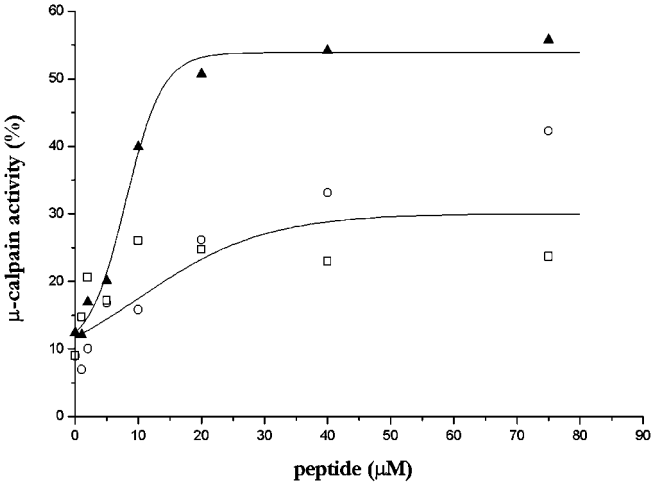

FIG. 2. **Activation of human μ-calpain by calpastatin subdomains A and C.** The activity of μ-calpain was measured by a fluorescent peptide substrate, LY-AMC, at 2.4 μM free Ca^{2+} concentration as given under "Experimental Procedures." To the assay, either peptide A (○) or C (□) or both A and C (▲) were added at the concentrations indicated. The initial rate of substrate consumption was determined and plotted in percentage of the activity measured at saturating (2 mM) Ca^{2+} concentration. Data points represent the mean of three separate experiments.

small aliquot was taken out and reactivated with 15 mM β-mercaptoethanol for 1 h prior to the experiments.

Peptides—The acetylated oligopeptide amides used in the present study correspond to subdomains A and C of human calpastatin inhibitory domain I (*cf.* Fig. 1 and Ref. 21) and have the sequence as follows. A, SGKSGMDAALDDLIDTLGG; C, SKPIGPDDAIDALSSDFTS. The peptides were synthesized on 4-methylbenzhydrylamine resin by solid phase method using the Boc[1] technique. The side protection groups were as follows: Boc-Asp(cHex), Boc-Lys(2-Cl-Z), Boc-Ser(Bzl), Boc-Thr(Bzl). All Boc derivatives were obtained from Reanal, Budapest, Hungary. The coupling was carried out by using 2 eq of protected amino acid derivative and 1.9 eq of *O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (dissolved in 1-methyl-2-pyrrolidone or in *N,N*-dimethylformamide), in the presence of *N,N*-diisopropylethylamine for 30 min. In case of positive ninhydrin assay the coupling was repeated until no free amino groups were detected. The Boc group was cleaved by 100% trifluoroacetic acid for 1 × 1 min. After the cleavage the resin was washed with 5% *N,N*-diisopropylethylamine in dichloromethane and with dichloromethane. The N-terminal aminoacetyl group was introduced by reacting the resin-bound peptide with 10 eq of 4-nitrophenyl acetate.

The peptides were cleaved from the resin by liquid HF (20 ml) at −5 °C, in the presence of 2 ml of anisole and 100 mg of dithiothreitol for 1.5 h. The peptides were purified by gel filtration in 30% acetic acid

[1] The abbreviations used are: Boc, *t*-butoxycarbonyl; LY-AMC, *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin.

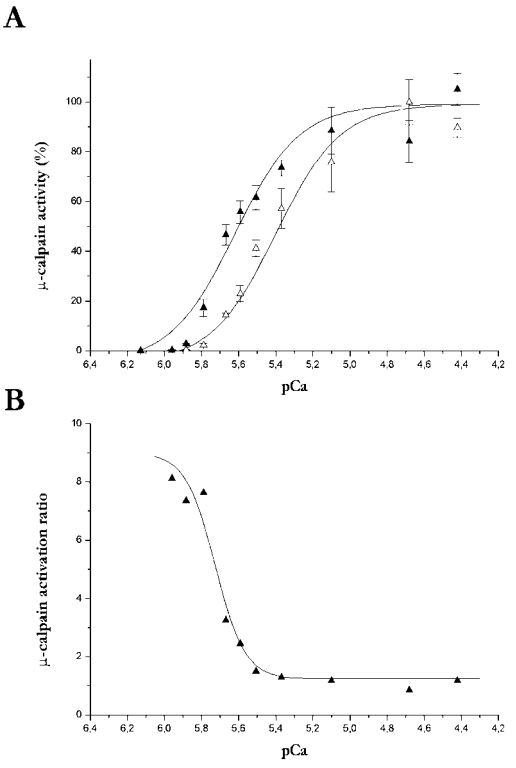

FIG. 3. **Ca^{2+} dependence of human μ-calpain activation by calpastatin subdomains A and C.** *A*, the activity of μ-calpain was measured by LY-AMC at the free Ca^{2+} concentrations indicated in the absence (△) or presence (▲) of 40 μM peptides A and C. The initial rate of substrate consumption was determined and plotted in percentage of the activity measured at saturating (2 mM) Ca^{2+} concentration. Data points represent the mean ± S.D. for three or four separate measurements. Free Ca^{2+} concentration needed for half-maximal activation is shifted from 4.3 to 2.4 μM by the peptides. *B*, the extent of activation exerted by the peptides at various Ca^{2+} concentrations is calculated by dividing enzyme activity measured in the presence and absence of the peptides, taken from A.

followed by reverse phase-high performance liquid chromatography on a Vydac 5-μm C18 column (250 × 4.6 mm) (Hesperia, CA) using 0.045% trifluoroacetic acid in water as eluent A, 0.036% trifluoroacetic acid in acetonitrile as eluent B, and linear gradient 30–55% B in 60 min and 20–52% B in 30 min for peptide A or 8.5–30% B in 100 min and 5–40% B in 30 min for peptide C. The homogeneity of peptide A was >98% and of peptide C was >92%.

The peptides were checked by amino acid analysis using a Beckman model 6300 amino acid analyzer, and mass spectra were recorded on a PerkinElmer Sciex API2000 triple quadrupole instrument (Toronto, Canada) equipped with TurboIonspray source. Both peptides were dissolved in calpain buffer at 5 mg/ml concentration and stored in aliquots at −20 °C until use.

Calpain Activity Measurements—Calpain activity was measured in a Jasco FP 777 spectrofluorometer at excitation and emission wavelengths of 380 and 460 nm in a 3 × 3 mm quartz cuvette. The reaction mixture in 50 μl of calpain buffer contained 1 mM LY-AMC as substrate at various peptide and Ca^{2+} concentrations as indicated. The actual free Ca^{2+} concentration was calculated from the total concentrations by using the stability constant log K_{app} = 7.815 for Ca^{2+}-EDTA. The reaction was started by the rapid mixing of the enzyme at a final concentration of 0.1 μM. The fluorescent trace recorded was transferred to a PC and evaluated by the MicroCal Origin data analysis software for determining the initial slope of fluorescence change; it was checked that this slope was proportional to enzyme amount/activity under the given conditions.

Materials—The fluorescent substrate LY-AMC (catalog no. S 1153) and all other chemicals were purchased from Sigma. The buffer was prepared with ion-exchanged distilled water.

RESULTS

To see if oligopeptides A and C have an effect on μ-calpain activity, an intermediate Ca^{2+} concentration was used at

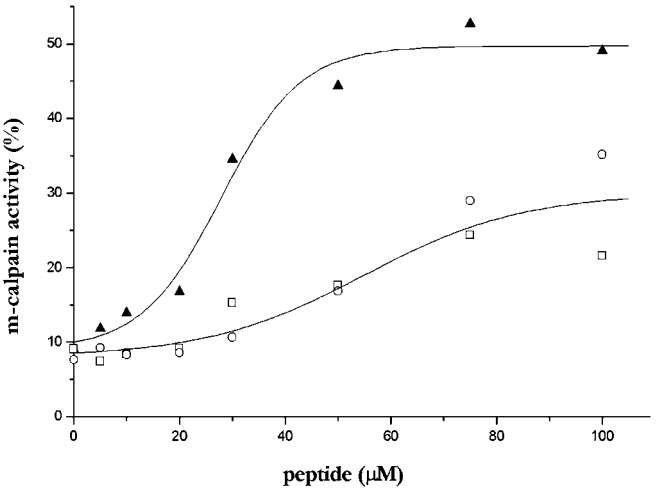

FIG. 4. **Activation of rat m-calpain by calpastatin subdomains A and C.** The activity of m-calpain was measured by a fluorescent peptide substrate, LY-AMC, at 140 μM free Ca^{2+} concentration as given under "Experimental Procedures." To the assay, peptide A (○) or C (□) or both A and C (▲) were added at the concentrations indicated. The initial rate of substrate consumption was determined and plotted in percentage of the activity measured at saturating (3 mM) Ca^{2+} concentration. Data points represent the mean for two to three separate experiments.

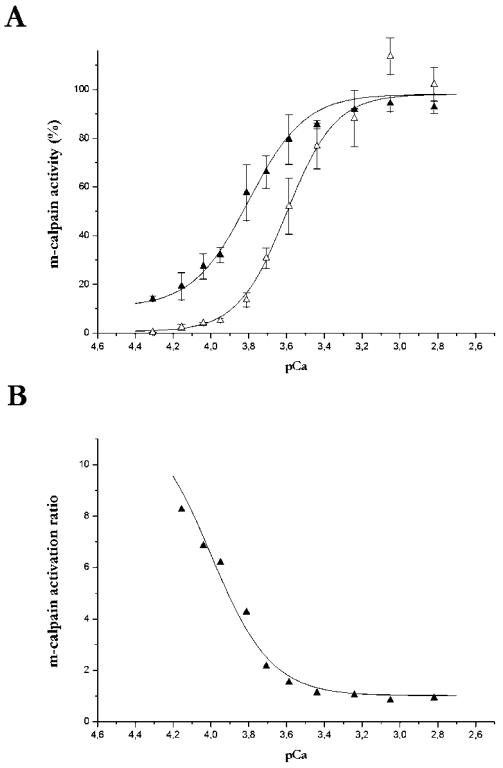

FIG. 5. **Ca^{2+} dependence of rat m-calpain activation by calpastatin subdomains A and C.** *A*, the activity of m-calpain was measured by LY-AMC at the free Ca^{2+} concentrations indicated in the absence (△) or presence (▲) of 75 μM peptides A and C. The initial rate of substrate consumption in the percentage of the activity measured at saturating (3 mM) Ca^{2+} concentration is given. Free Ca^{2+} concentration needed for half-maximal activation shifted from 250 to 140 μM by the peptides. *B*, the extent of activation exerted by the peptides at various Ca^{2+} concentrations is calculated as given in Fig. 3*B* for μ-calpain.

which the enzyme activity could be conveniently measured but was significantly lower than that at saturating Ca^{2+} concentrations (13% at 2.4 μM free Ca^{2+}). At this Ca^{2+} level the enzyme was titrated with increasing concentrations of a mixture of peptides A + C. Fig. 2 shows that the oligopeptides increase calpain activity about 5-fold with an EC_{50} concentration around 7.5 μM. The concentration dependence of activation has a slight sigmoidal shape, which hints at a cooperative effect between the two peptides. Subdomains A and C, when applied separately, also activate the enzyme (Fig. 2). Under the conditions used both increase the activity about 2–3-fold, with a half-maximal effect around 15 μM concentration.

Activation of calpain at subsaturating Ca^{2+} concentration might result from the expected sensitization to Ca^{2+}, from a net increase in maximal activity without affecting Ca^{2+} sensitivity, or both. To distinguish between these alternatives, the Ca^{2+} dependence of μ-calpain in the absence of peptides and in the presence of peptides at saturating concentration (40 μM each) was measured (Fig. 3*A*). Activation is clearly due to a significant increase in Ca^{2+} sensitivity of the enzyme, the concentration of half-maximal activity shifts from 4.3 to 2.4 μM upon the addition of the peptide mixture. The maximal activity at saturating Ca^{2+} concentrations, on the other hand, is unchanged within experimental error. With respect to the physiological activation of the enzyme, it is potentially very significant that an activation of about an order of magnitude is seen at low Ca^{2+} concentrations (Fig. 3*B*).

A similar set of experiments was carried out with m-calpain, *i.e.* with rat 80/21. Subdomains A and C exert a qualitatively similar activating effect: at 140 μM free Ca^{2+}, where enzyme activity is 10% of that at saturating Ca^{2+} concentrations, the peptides cause an about 5-fold activation of the enzyme (Fig. 4). Half-maximal peptide concentration is somewhat higher (25 μM), and the course of activation has a more distinct sigmoidal shape than with μ-calpain. The peptides themselves activate the enzyme about 2–3-fold with a half-effective concentration of 55 μM. The activation again can be accounted for by an increase in the Ca^{2+} sensitivity of the enzyme: the half-maximal Ca^{2+} concentration shifts from 250 to 140 μM at saturating (75 μM) concentrations of the peptides with no effect on maximal activity (Fig. 5*A*). At low Ca^{2+} concentrations the activation approaches one order of magnitude (Fig. 5*B*).

DISCUSSION

The results presented unequivocally demonstrate that peptides corresponding to subdomains A and C of human calpastatin domain I markedly sensitize both μ- and m-calpain to Ca^{2+}. At saturating peptide concentrations the peptides lower the Ca^{2+} concentration needed for half-maximal activity from 4.3 to 2.4 μM for μ-calpain and from 250 to 140 μM for m-calpain. Perhaps more significantly, at low Ca^{2+} concentrations approaching the physiological range both μ- and m-calpain are activated by an order of magnitude. The activation shows a sigmoidal concentration dependence, which indicates cooperativity between binding of the two peptides. In structural terms this implies that the conformational change of the two subunits is functionally linked and occurs in a concerted manner. The peptide concentration needed for half-maximal effect is somewhat lower for μ-calpain (7.5 μM) than m-calpain (25 μM), which might be due to the different sensitivity of isoforms, but may also reflect species difference as the peptides are derived from human calpastatin. Either way, this difference indicates that the development of isoform-specific activator peptides is conceivable. Overall, our observations have three important implications.

The first implication relates to the issue of the activation of calpains under physiological Ca^{2+} concentrations. As outlined in the introduction, several factors may be involved in lowering the Ca^{2+} demand of calpains. Although this issue is not settled yet, it seems that phospholipids (7, 8), nuclear DNA (12), a specific activator protein (11) and autolytic processing of cal-

pain itself (5, 6) may all contribute to shifting the Ca^{2+} need of calpain toward the physiological range. Our findings, perhaps paradoxically, bring calpastatin, the specific calpain inhibitor, into this picture. In principle, it is possible that calpastatin is fragmented in such a way *in vivo* that degrades its inhibitory subdomain (B) but leaves activator subdomains A and C more or less intact. Under such circumstances activation might override inhibition and calpastatin fragments stimulate calpain. Calpastatin is very sensitive to proteolysis *in vitro* (25) and is degraded by caspases (26) and calpain itself (27) *in vivo*. A significant difference in the sequence of calpastatin subdomains A, B, and C suggests that the action of a protease with a trypsin-like specificity may result in such a fragmentation: subdomain B has several Lys and Arg residues making this part of calpastatin sensitive whereas subdomains A and C have only a single N-terminal Lys residue offering limited access to proteolysis.

The other condition for this mechanism to operate *in vivo* is that the intracellular concentration of calpastatin be high enough for the peptides to reach a level sufficient for activation. Quantitative considerations argue that in some tissues this condition is met. The physiological concentration of calpastatin varies in different tissues from about 0.035 to 4.4 μM (28, 29); as calpastatin consists of four homologous inhibitory domains (*cf.* Fig. 1), each capable of inhibiting one calpain molecule in a Ca^{2+}-dependent manner (13, 14), the concentration of inhibitory domains, and the potential concentration of activator peptides, is four times higher, *i.e.* is in the range of 0.14 to 17.6 μM. Thus, in certain tissues specific fragmentation of calpastatin may produce the activator peptides in concentrations commensurable with the range used in our studies. Furthermore, two other considerations support this conclusion in a wider range. First, it seems that calpastatin concentrations at certain locations within the cell is markedly higher than the average values just mentioned. In an unstimulated cell calpastatin localizes in an aggregated state close to the cell nucleus (30), at local concentrations which significantly exceed the values calculated for homogeneous distribution. Second, macromolecular crowding caused by the extreme protein concentration in the cytoplasm favors interactions to such an extent that reaction rates and equilibrium constants of interactions may be orders of magnitude higher *in vivo* than under test tube conditions (31). Because of this mechanism, fragments corresponding to the same amount of calpastatin subdomains A and C may give a much higher effective concentration in the cell than in dilute solution in the test tube and activate calpain at concentrations significantly lower than the values reported in this work.

The second general implication of our work is related to previous efforts at characterizing the physiological function of calpains. It is known from knockout experiments that calpains play an essential role as deletion of the ubiquitous small subunit is lethal in mice (32). The role of various calpain forms has been extensively studied, almost invariably by the application of calpain inhibitors. From such studies we know that calpain(s) play a regulatory role in basic cellular processes such as the cell cycle (33), cell motility (34), cell spreading (35), apoptosis (36), and many more. The inhibitors, however, lack strict specificity. They may react with the proteosome, lysosomal proteinases, or even non-proteolytic enzymes (37, 38). These findings have prompted the development of strictly specific calpain inhibitors (39) or the use of a cell-permeable variety of calpastatin subdomain B for cell biological studies. In general, the results with calpain inhibitors cannot always be unequivocally associated with the action of calpain. Thus, it is desirable to develop activators for calpain based on our observations with subdomains A and C. As these peptides are highly charged, both subdomains have to be fused with "carrier" peptides that make other substances membrane-permeable. The development of such constructs, which will probably complement the use of calpain inhibitors in further analysis of calpain function, is under way.[2]

The third implication worth considering is related to the pathological role of calpain. Overactivation of calpain seems to be important in a range of disorders (37, 40); more recent findings, however, point to the link of diminished activity and the development of pathological states. Positional cloning in patients with limb girdle muscular dystrophy 2A linked the gene of calpain 3 (p94) with this disease (41). Later studies revealed that disease is associated with mutations in this gene, which invariably leads to a partial or complete loss of enzyme activity (42). In a similar approach, diabetes mellitus type 2, which accounts for about 90% of the incidence of diabetes, was linked to the gene of calpain 10 (43); a single-nucleotide polymorphism within one intron of the gene is associated with a significantly reduced mRNA level (44). Thus, the enzyme in this case is fully active, but it is expressed at a lower level. The two diseases, and possibly others, have in common a diminution in calpain activity; apparently, drugs that could boost activity might be beneficial.

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