Acyl-CoA-binding Protein Is a Potent m-Calpain Activator*

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Acyl-CoA-binding protein, a 20-kDa homodimer that exerts many physiological functions, promotes activation of the classic calpain forms, most markedly that of the m-isozyme. This protein factor was purified from rat skeletal muscle and was also expressed in Escherichia coli. Both native and recombinant acyl-CoA-binding proteins show the same molecular properties and an identical capacity to decrease the [Ca^{2+}] required for m-calpain activity. The binding of long-chain acyl-CoAs to acyl-CoA-binding protein does not modify the activating effect on calpains. Acyl-CoA-binding protein seems to be involved in the m-calpain regulation process, whereas the previously identified UK114 activator is a specific modulator of m-calpain. Acyl-CoA-binding protein is proposed as a new component of the Ca^{2+}-dependent proteolytic system. A comparative analysis among levels of classic calpains and their activator proteins is also reported.

How the calcium-dependent proteolytic system is activated is an intriguing question in terms of understanding its general properties, physiological functions, and involvement in the occurrence of tissue damage (1–4). Calpains, the enzymatic components of the system, are primarily regulated by calcium ions. The binding of Ca^{2+} to calmodulin-like domains of the proteinases induces a conformational change that constitutes the initial limiting step of the overall sequential activation process (5). In this new conformation calpains undergo limited auto-proteolysis that irreversibly removes the molecular constraints responsible for stabilizing the native inactive form (1–4). In their autoproteolyzed active forms, calpains acquire the highest affinity for calpastatin, which becomes the only negative modulator of calpain activity (6). In rat brain we have recently identified a protein factor, called UK114, which specifically interacts with m-calpains and accelerates their activation steps, increasing the affinity for calcium ions (7). The reduced time of calpain activation is consistent with the proposed involvement of the Ca^{2+}-dependent proteolytic system in specific signal transduction pathways, resulting, for instance, in granule secretion in various cell types (8) or in the conversion of protein kinase C into an autoproteolyzed form produced during the differentiation of murine erythroleukemia cells (9).

The rat brain m-calpain activator shows a strict specificity for all m-calpain isoforms, whereas it has no effect on the classic m-calpains (10), the activation of which requires a calcium concentration at least two orders of magnitude higher than that required by m-calpain. Despite reports indicating that free calcium ions can be accumulated in specific cell regions at high micromolar concentrations (11), the calcium requirement for m-calpain activation can apparently be satisfied only during tissue necrosis, a situation far removed from physiological conditions (12).

We now report that a highly conserved protein, called acyl-CoA-binding protein (ACBP) (13–15), shows a potent m-calpain-activating property. This 20-kDa protein, which is involved in various physiological processes including binding to the type-Α γ-aminobutyric acid receptor (16), acyl-CoA binding and transport (17), and steroidogenesis (18), reduces the optimal calcium requirement of m-calpain from 300 μM to approximately 10 μM. The two calpain activators, UK114 and ACBP, are diversely expressed by many rat tissues, and their levels are inversely correlated with those of the corresponding target calpains.

EXPERIMENTAL PROCEDURES

Materials

Trypsin, leupeptin, phenylmethylsulfonyl fluoride, isopropyl β-D-thiogalactopyranoside, glutathione, glutathione-Sepharose, and butyl agarose were obtained from Sigma. Moloney murine leukemia virus reverse transcriptase, BamHI, and EcoRI were obtained from Promega; AmpliTag DNA polymerase was from Perkin-Elmer;NuSieve agarose gel was from FMC Corp. Bio Products. pGEX-6P-1 vector, PreScission protease, Escherichia coli BL21 cells, Superdex 75 column, Source 15Q column, CNBr-Sepharose, and Sephadex G-100 were purchased from Amersham Pharmacia Biotech. A Sequenase® 2.0 kit was obtained from U. S. Biochemical Corp. C-18 reverse-phase LUNA column was purchased from Phenomenex. N23 murine erythroleukemia is a subclone of DS19 cells (19), a murine erythroleukemia cell line obtained as described previously (20).

Methods

Calpain Purification—Calpain from human erythrocytes was purified and assayed as described previously (21). Purification of μ-calpain and m-calpain from rat skeletal muscle was carried out as described (22). 1 unit of calpain activity is defined as the amount of enzyme producing 1 nmol of free NH₃ groups in standard conditions (23).

Assay of Calpain Activator Activity—Calpain activator activity was measured by adding the appropriate amounts of the activator sources to the routine calpain assay mixture, which contained 50 mM sodium borate buffer, pH 7.5, 5 μM CaCl₂, 2 mg/ml of denatured globin, and 0.01 nmol of erythrocyte calpain (19). 1 unit of calpain activator activity is defined as the amount that elicits 1 unit of calpain activity in the presence of 5 μM Ca^{2+} (7).

Purification of Calpain Activators from Rat Skeletal Muscle—Samples of rat skeletal muscle (100 g) were minced and homogenized in a Waring blender by means of 3 bursts (30 s each) in 10 volumes of ice-cold 0.25% sucrose containing 1 mM EDTA, 0.5 mM 2-mercaptoethanol.

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anol, 0.1 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonic oscillation for six 10-s bursts, with a 10-s pause between bursts. The disrupted cell suspension was centrifuged at 20,000 × g for 15 min, and the supernatant was collected. The purification of calpain activators was then carried out as described previously for calpain activator purification from rat brain (7). Briefly, the pH value of the supernatant was adjusted to 5.8, and the sample was heated to 90 °C for 3 min and centrifuged. The clear supernatant, adjusted to a pH value of 7.5, was subjected to chromatography on Source 15Q column (18 × 60 mm) equilibrated in 50 mM sodium borate buffer, pH 7.5, containing Buffer A (0.5 mM 2-mercaptoethanol, 0.1 mM EDTA). The calpain activator did not bind to the resin and emerged in the run-through and wash fractions. Fractions containing calpain-activating activity were pooled and applied to a butyl-agarose column (1 × 10 cm) equilibrated in Buffer A. The unbound material, containing calpain activator proteins, was collected, concentrated by (NH₄)₂SO₄ (80% final concentration) precipitation, dissolved in Buffer A, and applied to a Sephadex G-100 column (1.8 × 160 cm) equilibrated in the same buffer. Two peaks of calpain activator activity, named CA I and CA II, were recovered, separately collected, and further purified by affinity chromatography.

Affinity Chromatography of Calpain Activators—Erythrocyte calpain was purified and inactivated with iodoacetamide as described previously (6). A calpain-Sepharose column (5 × 15 mm) was prepared by coupling carboxymethylated calpain (0.4 mg) with CNBr-Sepharose following the manufacturer’s instructions and equilibrated in 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol, 20 μM CaCl₂. CA I and CA II were separately loaded on the column and eluted with 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM β-mercaptoethanol, 0.1 mM EDTA at a flow rate of 0.5 ml/min. Fractions of 0.3 ml were collected, and calpain activator activity was assayed on each fraction using 0.010 ml of sample.

Superdex 75 Chromatography of Calpain Activators—Purified calpain activators (0.2 ml), obtained by affinity chromatography on an immobilized-calpain column, were separately loaded on a Superdex 75 column (10 × 300 mm) equilibrated in Buffer A at a flow rate of 0.4 ml/min. Fractions of 0.4 ml were collected, and calpain activator activity was determined on each fraction.

Amino Acid Sequence Determination of Calpain Activators—Purified calpain activators (25 pmol) were concentrated at 0.2 ml in the presence of 0.05% SDS to avoid protein insolubilization, and the pH value of the solution was adjusted to 8.3. The samples were digested with trypsin (1:25 trypsin to activator, weight/weight ratio) for 18 h at 37 °C and then lyophilized, suspended in 0.1% trifluoroacetic acid, and applied to a C-18 reverse-phase column (1 × 100 mm) equilibrated with 0.1% trifluoroacetic acid. Absorbed peptides were eluted with a linear gradient of CH₃CN (from 0 to 70%), and fractions of 0.03 ml were collected. The tryptic peptides were directly applied to a Beckman LF30000 protein sequencer.

RNA Isolation and ACBP cDNA Synthesis—Total RNA was isolated from 10⁴ N23 murine erythroleukemia cells by extraction with guanidinium thiocyanate (23), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase as described (23). The primers for PCR were selected externally to the translated sequence of ACBP (accession number X61431). The sense primer was 5'-CTTGGATGCTCTGCTG-TG' (nucleotides 1–20); the antisense primer was 5'-TATGGTCCAAGGTCAGC-3' (complementary to nucleotides 353–373). PCR was carried out by using AmpliTaq DNA polymerase and by performing 30 cycles of amplification as described previously (24). The product of amplification was analyzed on a 4% NuSieve agarose gel and consisted of a single fragment of approximately 370 base pairs that was eluted from the gel. An aliquot (0.1 μg) of this PCR product was subsequently subjected to 25 cycles of amplification using a sense primer, 5'-GGGATCCGTGGCTCTAAGTTT-GACAAAG-3' (nucleotides 66–87) containing a BamHI restriction site at the 5'-end; the antisense primer was 5'-AGTAATTCTACAGGTCACAGTGCC-3' (complementary to nucleotides 345–366) containing an EcoRI restriction site at the 5'-end.

Expression of Recombinant ACBP—The product of amplification was cloned into pGEX-6P-1 vector, and the sequence of the insert was determined by the dideoxynucleotide chain termination method (25) from a SuperScript II cDNA Synthesis Kit (Gibco BRL, Life Technologies, Inc.), E. coli BL21 cells were transfected with the recombinant plasmid by electroporation (Bio-Rad gene pulser) and the expression of glutathione S-transferase-ACBP fusion protein was induced by addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. After 3 h at 20 °C, cells were lysed, and the fusion protein was bound to a glutathione-Sepharose column (5 × 10 mm) as specified by the manufacturer. Recombinant ACBP was eluted from the column following digestion with 2 μl of PreScission protease (Amersham Pharmacia Biotech) in 0.5 ml of 50 mM Tris·Cl, pH 7.5, containing 1 mM 1,4-dithiothreitol.

Effect of Acyl-CoAs on Calpain-activating Properties of ACBP—ACBP was preincubated at 37 °C for 10 min directly in the calpain assay mixture with different concentrations (from 0.5 to 100 μM), final concentrations of palmitoyl-CoA (C16:0), palmitoleoyl-CoA (C16:1), mireistoyl-CoA (C14:0), or stearyl-CoA (C18:0); rat muscle m-calpain was then added in a 1:1 molar ratio ACBP/ enzyme. The activator activity was assayed as previously reported (7) using 20 μM calcium concentration. The same experiments were also carried out using increasing calcium concentrations (from 1 to 100 μM).

RESULTS

Identification of Two Calpain Activator Forms in Rat Skeletal Muscle—To purify calpain activator from rat skeletal muscle, samples of tissue were treated as described under “Experimental Procedures,” and the extracted proteins were subjected to the same procedure as that described for the purification of calpain activator from rat brain (7); however, instead of the Superose 12 chromatography used for brain activator purification, gel chromatography was carried out on a Sephadex G-100 column. As shown in Fig. 1, this column separates the calpain activator activity into two peaks, both having activating properties toward human erythrocyte calpain. This isozyme was used as it shows the functional properties of both μ-calpains and m-calpains and is therefore more responsive to modulators. Gel filtration data indicate that the two activators are present in similar amounts.

Molecular Properties—The two protein factors were further purified by affinity chromatography on an immobilized-calpain column (data not shown) and subjected to comparative analysis of their molecular properties using non-denaturing Superose 75 chromatography and one-dimensional SDS-PAGE (Fig. 2). The first calpain activator (CA I) displays a native molecular mass of 30 kDa (Fig. 2A) and is composed of two 15-kDa polypeptide chains (Fig. 2B). The amino acid sequence of CA I is almost identical to that of UK114, the specific activator of μ-calpain (Ref. 7 and data not shown). The second activator (CA II) is a homodimeric protein consisting of two 10-kDa subunits (Fig. 2, C and D).

CA II was digested with trypsin, and the tryptic peptides were isolated. One of these, which contained 18 residues, proved to be identical to the 15–32-residue segment of ACBP (rat ACBP; Swiss Protein Database accession number P11030),
an 86-residue polypeptide with a 10-kDa molecular mass. Sequences of three other tryptic peptides corresponded to those of the ACBP fragments containing amino acid residues 1–6, 63–66, and 77–81 (Fig. 3).

To confirm that ACBP is really a calpain activator protein, ACBP-encoding cDNA was prepared as described under “Experimental Procedures,” and the corresponding protein was expressed in E. coli and purified. During SDS-PAGE analysis, the recombinant ACBP (Fig. 4, inset) displayed a single band corresponding to a 10-kDa molecular mass, whereas its elution volume from the Sephadex G-75 column (Fig. 4) was the same as that of CA II (see Fig. 2), corresponding to a 20-kDa molecular mass. On the basis of these data, the new calpain activator shows identical molecular properties to ACBP.

**Activating Properties of CA II and ACBP**—To provide final evidence that CA II and ACBP are the same protein and to define their calpain-activating properties, the effect of both CA II purified from rat muscle and recombinant ACBP on rat muscle calpains is shown (Table I). In the presence of CA II or recombinant ACBP, a 50-fold reduction is seen in the amount of Ca\(^{2+}\) required to activate m-calpain, which expresses \(\frac{1}{2}V_{\text{max}}\) at 6 \(\mu\)M Ca\(^{2+}\). Both CA II and ACBP are also active toward \(\mu\)-calpain, although to a lesser degree, with its Ca\(^{2+}\) requirement being lowered only 6-fold. Taken together, these data indicate that ACBP and CA II display identical effects in reducing the Ca\(^{2+}\) requirement of \(\mu\)-calpain and m-calpain. Pre-incubation of ACBP with long-chain acyl-CoAs (C14:0, C16:0, C16:1, C18:0) does not modify the calpain-activating properties of the protein.

We also analyzed the degree of efficiency of ACBP on both calpain isozymes. As shown in Fig. 5, the amount of protein required to induce the maximal effect on \(\mu\)-calpain is 3.5 times higher than that required by m-calpain (Fig. 5); the optimal molar ratio of ACBP/m-calpain is 1.1, whereas a 4-fold molar excess of ACBP is required to obtain the maximal effect on m-calpain.

### Table I

| Calpain isofrom | Control | CA II | ACBP | \(\frac{V_{\text{max}}}{V_{\text{max}}^\text{m-calpain}}\) ratio |
|-----------------|---------|-------|------|-------------------|
| \(\mu\)-Calpain | 10.0 ± 2.0 | 1.7 ± 0.5 | 1.8 ± 0.5 | 1.03 |
| m-Calpain       | 295 ± 30  | 7.0 ± 1.0 | 6.0 ± 2.0 | 1.01 |

\(a\) Ca\(^{2+}\) requirement to promote \(\frac{1}{2}V_{\text{max}}\) on calpain isozymes. 

\(b\) Expressed as the ratio between the \(V_{\text{max}}\) measured in the absence or in the presence of the optimal amount of activator (see Fig. 5).
In the bulk cytosol (11), the measured free \([\text{Ca}^{2+}\]) may be higher than previously estimated from measurements of actual concentration of ions of this metal in cells. Although it pain but to a lesser degree. Furthermore, the greatest decrease in its activating effect on calpains, suggesting that the conformational changes occurring in the acyl-CoA:ACBP complex (26) are not crucial for the interaction with these proteinases. However, as this interaction is calcium-dependent, we can postulate that recognition between the two proteins occurs when calpains expose hydrophobic regions following Ca\(^{2+}\) binding (27).

It has been shown that ACBP is implicated in many physiological processes including binding to the type-A receptor for \(\gamma\)-aminobutyric acid (16), binding and transport of acyl-CoA (17), steroidogenesis, and peptide hormone release (18). How this new function of ACBP is connected with the other important roles of the protein has not yet been determined, but recent reports (28) concerning the involvement of ACBP in Ca\(^{2+}\) release from muscle sarcoplasmic reticulum suggest that the activation of m-calpains might be complementary in promoting these cellular effects. As part of the calcium-dependent proteolytic system, calpain activators may adapt the rate and site of calpain activation to specific needs for cell responses.

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![Figure 5](https://example.com/figure5.png)

**FIG. 5.** ACBP/calpain ratio required for maximal activating effect. \(\mu\)-Calpain (○) and m-calpain (●), purified from rat skeletal muscle as described under "Experimental Procedures," were incubated with increasing amounts of ACBP to reach the indicated ACBP/calpain molar ratio. \(\mu\)-Calpain and m-calpain activities were assayed in the presence of 5 \(\mu\)M \(\text{Ca}^{2+}\) and 20 \(\mu\)M \(\text{Ca}^{2+}\), respectively. The values were expressed as the percentage of total proteolytic activity measured in the presence of 1 mM \(\text{Ca}^{2+}\). The values are arithmetical means of four separate experiments.
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