Adaptive Modifications in the Calpain/Calpastatin System in Brain Cells after Persistent Alteration in Ca\(^{2+}\) Homeostasis* 

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Persistent dysregulation in Ca\(^{2+}\) homeostasis is a pervasive pathogenic mechanism in most neurodegenerative diseases, and accordingly, calpain activation has been implicated in neuronal cells dysfunction and death. In this study we examined the intracellular functional state of the calpain-calpastatin system in −G93A(+) SOD1 transgenic mice to establish if and how uncontrolled activation of calpain can be prevented in vivo during the course of prolonged [Ca\(^{2+}\)], elevation. The presented data indicate that 1) calpain activation is more extensive in motor cortex, in lumbar, and sacral spinal cord segments compared with the lower or almost undetectable activation of the protease in other brain areas, 2) direct measurements of the variations of Ca\(^{2+}\) levels established that the degree of the protease activation is correlated to the extent of elevation of [Ca\(^{2+}\)], 3) intracellular activation of calpain is always associated with diffusion of calpastatin from perinuclear aggregated forms into the cytosol and the formation of a calpain-calpastatin complex, and 4) a conservative fragmentation of calpastatin is accompanied by its increased expression and inhibitory capacity in conditions of prolonged increase in [Ca\(^{2+}\)]. Thus, calpastatin diffusion and formation of the calpain-calpastatin complex together with an increased synthesis of the inhibitor protein represent a cellular defense response to conditions of prolonged dysregulation in intracellular Ca\(^{2+}\) homeostasis. Altogether these findings provide a new understanding of the in vivo molecular mechanisms governing calpain activation that can be extended to many neurodegenerative diseases, potentially useful for the development of new therapeutic approaches.

An increasing number of reports have indicated that calpain activity exerts a vital physiological role in several neuronal functions (1–5). This implies that activation of the protease must be under a tight control generally attributed to the ubiquitous presence of its natural inhibitor calpastatin (2, 3, 6–12). However, several questions must still be precisely defined particularly because it has also been established that calpain activity can undergo a transition from physiological to pathological function and that this condition occurs in most neurodegenerative diseases due to a dysregulation in Ca\(^{2+}\) homeostasis (1, 13–20). However, to reconcile regulation of calpain both in physiological and pathological conditions to the inhibitory capacity of calpastatin, it seemed necessary to explore additional pertinent topics. These include the observation that Ca\(^{2+}\)-mediated activation of calpain occurs also in cells in which calpastatin is in large excess over the protease (2, 3, 7, 21). Moreover, it has been established that calpastatin normally present in perinuclear aggregates diffuses into cytosol, becoming available for the association with calpain upon elevation of Ca\(^{2+}\) (9, 21, 22). However, this obligatory step in controlling calpain activation has never been demonstrated in in vivo conditions after Ca\(^{2+}\) overload. An additional point to be clarified is that calpastatin, being a substrate of calpain (7, 23–25), is progressively degraded in the course of calpain activation, and as a consequence, the cell inhibitory capacity should be progressively lost (7, 23, 24). In previous reports we have observed that in rat brain, in the course of a mild intracellular elevation of Ca\(^{2+}\), a resulting limited activation of calpain was efficiently compensated by calpastatin through an integrated mechanism that involved a conservative degradation and an increased expression of the inhibitor molecule (7, 23–25). In these experiments a persistent alteration in intracellular free [Ca\(^{2+}\)], was obtained by administration of a high sodium diet to normoten- sive and hypertensive rats (7).

In the present study we extended these observations using as an experimental model a transgenic mouse strain overexpressing the point mutated −G93A(+) human soluble copper/zinc superoxide dismutase 1 (SOD1)\(^2\) form, generally utilized to investigate the familial amyotrophic lateral sclerosis (26–32). To assure a long term dysregulation of Ca\(^{2+}\) homeostasis, animals were sacrificed after 4 months from birth (29, 30, 33–38). Furthermore, the experimental procedure involved the simultaneous analysis of both calpain activation and the intervening changes in the functional properties of the calpain-calpastatin system. Activation of the protease was assessed by determining its consumption and substrate degradation, whereas immunofluorescence analysis was carried out to identify both cellular calpastatin localization and formation of a binary enzyme-inhibitor complex. Our findings confirmed that prolonged dysregulation of Ca\(^{2+}\) homeostasis occurs in specific brain areas of transgenic mice with an intensity correlated to the extent of...
calpain activation. These findings were significantly evident in motor cortex, lumbar and sacral segments of spinal cord as well as in skeletal muscle. We also demonstrate that calpain activation was counteracted by a multistep sequential mechanism involving a diffusion of calpastatin in cytosol to generate a complex with calpain, a conservative degradation of the inhibitor producing still active fragments, and a large increase in the synthesis of calpastatin mRNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—4-(2-Aminoethyl)benzenesulfonyl fluoride was obtained from Calbiochem. The SuperSignal® West Pico detection system was purchased from Pierce. Leupeptin and aprotinin were obtained from Sigma. KILLIK frozen section medium was purchased from Bio Optica. Calcium Green-1/AM was obtained from Molecular Probes. Source 15Q resin was purchased from GE Healthcare. Human erythrocyte calpain was purified and assayed as reported previously (12). Rat brain recombinant RNCAST104 calpastatin form, containing the L- and four repetitive inhibitory domains, was expressed and purified according to a previously reported procedure (25).

**Animals**—B6SJL-TgN SOD1-G93A(+)/Gur mice expressing a high copy number of mutant human SOD1 with G93A substitution (SOD1-G93A(+)) (defined as “transgenic” throughout the text) and B6SJL-TgN (SOD1)2Gur mice expressing wild-type human SOD1 (SOD1(+)) (defined as “control” throughout the text) were obtained from The Jackson Laboratory and bred at the animal facility of the Pharmacology and Toxicology Section, Department of Experimental Medicine in Genoa. Selective breeding maintained each transgene in the hemizygous state on an F1 hybrid C57Bl6 × SJL genetic background (26). All transgenic mice were identified analyzing crude extracts obtained from tail tips. Tail tips were homogenized in phosphate-buffered saline (PBS) solution, lysed by 2 cycles of freezing and thawing, and centrifuged at 23,000 × g for 15 min at 4 °C. The SOD1 level was evaluated by staining for its enzymatic activity after 10% SDS-PAGE (39). Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular dark-light schedule (light on from November 24th, 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

**Antibodies**—Mouse monoclonal anti-calpastatin antibody (mAb 35.23) was produced as reported (22). Mouse monoclonal anti-µ-calpain antibody (mAb 56.3) was produced as reported (40). Rabbit polyclonal anti-µ-calpain antibody, mouse monoclonal anti-m-calpain antibody, and mouse monoclonal anti-β-actin antibody were purchased from Sigma. Mouse monoclonal anti-α-spectrin II antibody was obtained from Chemicon. Mouse monoclonal anti-nNOS antibody was purchased from BD Transduction Laboratories. Horseradish peroxidase (HRP)-linked anti-mouse and HRP-linked anti-rabbit secondary antibodies were purchased from Cell Signaling.

**Methods**

**Immunoblot Analysis**—Control and transgenic mice were sacrificed by decapitation, and the cerebellum, motor cortex, spinal cord, skeletal muscle, and the remaining brain part (deprived of cerebellum and motor cortex) were rapidly removed, quickly frozen in liquid nitrogen, and stored at −80 °C. Aliquots of the indicated tissues were resuspended in 5 volumes (w/v) of 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol (buffer A), 2 mM EDTA, 0.1 mg/ml leupeptin, 10 µg/ml aprotinin, and 2 mM 4-(2-amino-ethyl)benzenesulfonyl fluoride, minced, and homogenized in a Potter Elvejem homogenizer and lysed by sonication. Aliquots of crude extracts (41), obtained from the tissues considered above, were submitted to SDS-PAGE (39). Proteins were then transferred to a nitrocellulose membrane (Bio-Rad) by electroblothing (42). To detect μ- and μ-calpain isoforms as well as nNOS and α-spectrin II, the nitrocellulose membranes were probed with the specific antibodies. The immunoreactive material was developed with ECL® detection system (43), detected with a Bio-Rad Chemi Doc XRS apparatus, and quantified using the Quantity One 4.6.1 software (Bio-Rad).

**Calcium Imaging**—Control and transgenic mice were sacrificed by decapitation, and the cerebellum, motor cortex, spinal cord, skeletal muscle, and the remaining brain part (deprived of cerebellum and motor cortex) were rapidly removed. Intracellular calcium levels were identified after the procedure described in Averna et al. (7) with the following modifications. Tissues were cut into thin slices that were immediately collected, transferred into a 96-well plate, and incubated in darkness at 37 °C for 30 min in 10 mM HEPES containing 0.14 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl2, pH 7.4 (buffer B), in the presence of 15 µM Calcium Green-1/AM (44). The slices were then washed three times with buffer B to remove excess fluorophore, and the green fluorescence was detected with a Bio-Rad MRC1024 confocal microscope connected to a Nikon Diaphot 200 microscope equipped with a 60× Plan Apo objective with numerical aperture 1.4. The excitation/emission wavelengths were 488/522 nm, respectively. Calcium Green-mediated green fluorescence was quantified with Laser Pix Software (Bio-Rad) as reported (7, 45).

**Calpastatin Levels and Forms**—Aliquots (20 µg of protein) of crude extracts (41) obtained from different tissues of control and transgenic mice as previously described in this Section were heated for 5 min at 100 °C. The particulate material was discarded by centrifugation (60,000 × g for 10 min at 4 °C), and the clear supernatants were separately collected. Calpastatin forms present in the indicated tissues were identified after the procedure described (7, 23). Briefly, aliquots (1.5 mg of protein) of the clear supernatants (46) were distributed in 10 lanes and submitted to 12% SDS-PAGE (39). Calpastatin species were identified after protein extraction from the gel (7, 23). Calpastatin activity was measured in the presence of human erythrocyte calpain, and human-denatured globin as substrate as reported (7, 12, 23, 25, 45). Calpastatin levels were calculated from the area underlying the calpastatin activity peak. One unit of cal-
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Aliquots of crude extracts prepared from the tissues collected from control (black bars) and transgenic (gray bars) mice were submitted to 8% SDS-PAGE followed by immunoblotting. \( \mu \)-Calpain and m-calpain were detected using the specific antibody (Sigma). The immunoreactive material was developed with ECL\textsuperscript{®} detection system, detected with a Bio-Rad Chemi Doc XRS apparatus, and quantified using Quantity One 4.6.1 software (Bio-Rad). The calpain protein level detected in control mice was taken as 100%.

**Assay of Calpain Activity**— Aliquots of the frozen tissues obtained from control and transgenic mice as previously described in this section were resuspended in 5 volumes (w/v) of ice-cold buffer A containing 2 mM EDTA, minced, homogenized in a Potter Elvejem homogenizer, and lysed by sonication. The particulate material was discarded by centrifugation (60,000 \( \times \) g for 10 min at 4 °C) of the crude extract, and the clear supernatants were separately collected. Protein quantification was performed as reported (46). Equal amounts (35 mg of protein) of the clear supernatants were collected and separately submitted to ion-exchange chromatography onto a Source 15 Q column (1 \( \times \) 5 cm) previously equilibrated in buffer A containing 0.1 mM Tris-HCl, pH 6.8, containing 20% glycerol, 10 mM 2-mercaptoethanol, and 10 mM EDTA and loaded on a 10% polyacrylamide gel containing 1 mg/ml casein. The gel was not performed, this protein substrate was still added to the gel, in accordance with the original procedure (11).

The electrophoretic run was carried out in 25 mM Tris-HCl, pH 8.0, containing 125 mM glycine, 1 mM EDTA, and 10 mM 2-mercaptopoethanol for 2 h at 4 °C and 125 V.

**Identification of the Calpain-Calpastatin Complex Formation**—The following experimental strategy was used: (i) preparation of crude extracts from different brain regions and skeletal muscle in a Ca\textsuperscript{2+}-free buffer; (ii) electrophoresis in non-denaturing conditions of the extract samples (under these conditions, free calpain and calpastatin as well as the calpain-calpastatin complex showed different mobility); (iii) transfer of proteins to nitrocellulose membranes; (iv) identification of both calpain and calpastatin and their complex by specific antibodies. Briefly, aliquots of the fresh tissues (90–300 mg) collected from control and transgenic mice as described above were resuspended in 5 volumes (w/v) of ice-cold buffer A containing 2 mM EDTA, minced in tissue grinder, and lysed by sonication. Zymogram analysis was carried out as described (11, 47, 48) with the following modifications. Aliquots of the crude extracts (corresponding to 50 \( \mu \)g of protein) were diluted in 60 \( \mu \)l of 0.1 M Tris-HCl, pH 6.8, containing 20% glycerol, 10 mM 2-mercaptoethanol, and 10 mM EDTA and loaded on a 10% polyacrylamide gel containing 1 mg/ml casein. Although the step required to measure calpain activity after the digestion of casein was not performed, this protein substrate was still added to the gel, in accordance with the original procedure (11). Moreover, the presence of casein during electrophoresis did not modify the mobility of both calpain and calpastatin as well as their association capacity. The electrophoretic run was carried out in 25 mM Tris-HCl, pH 8.0, containing 125 mM glycine, 1 mM EDTA, and 10 mM 2-mercaptopoethanol for 2 h at 4 °C and 125 V. Proteins were then transferred to a nitrocellulose membrane (42). Calpain was identified with the polyclonal antibody anti-\( \mu \)-calpain antibody (Sigma). Calpastatin was identified by using mAb 35.23 (11). The immunoreactive material was identified by ECL\textsuperscript{®} detection system (43) and detected by the Bio-Rad Chemi Doc XRS apparatus.

**Confocal Microscopy**—Control and transgenic mice were sacrificed by decapitation, and the cerebellum, motor cortex, spinal cord, and skeletal muscle were rapidly removed, immediately frozen in liquid nitrogen, and stored at −80 °C. The frozen tissues were then included in KILLIK frozen section medium and cut into 10-\( \mu \)-m-thick slices by using a Reichert-Jung Frigocut 2800 cryostat (Cambridge Instruments GmbH).
**RESULTS**

Assessment of Calpain Activation in Brain Areas of Transgenic Mice—To establish the extent of calpain activation in brain of control and transgenic mice, we first relied on two well established methods such as the consumption of the calpain isoforms and the degradation of known calpain substrates (1, 3, 21, 45, 51–54). As shown in Fig. 1, in motor cortex (Fig. 1A) and

**FIGURE 2. Digestion of calpain protein substrates in brain regions of control and transgenic (Transg.) mice.** Aliquots (25 μg of protein) of crude extracts, prepared from the tissues collected from control and transgenic mice, were submitted to 6% SDS-PAGE followed by immunoblotting. α-spectrin II and nNOS were detected using the specific mAbs. The immunoreactive material was developed, detected, and quantified as in the legend to Fig. 1. In motor cortex (A), because no accumulation of the 130-kDa nNOS form was detected, the level of the 160-kDa nNOS form evaluated in control mice was taken as 100%. The pictures in the upper panels represent one of the experiments performed. The reported values are the means ± S.D. of three independent experiments carried out on three different control and transgenic animals. Protein quantification was normalized versus β-actin immunoreactive band intensity, used as loading control protein. A, motor cortex; B, spinal cord; C, cerebellum; D, remaining brain regions after the removal of motor cortex and cerebellum.

Slices of the indicated tissues (five slices for each experiment) were layered on glass microscope slides and washed three times with ice-cold PBS. Cells were fixed and permeabilized using the Triton/paraformaldehyde method (22) and treated with PBS containing 5% (v/v) fetal-calf serum (Euroclone). After 1 h at 4 °C, slices were washed 3 times with ice-cold PBS. Calpastatin and calpain were detected using the anti-calpastatin 35.23 mAb and the anti-α-calpain 56.3 mAb as primary antibodies, respectively. After incubation overnight at 4 °C, the slices were washed 3 times with PBS and incubated for 1 h at 4 °C in the presence of a fluorescein isothiocyanate-conjugated sheep antimouse IgG (GE Healthcare), used as secondary antibody. Chromatin was stained with propidium iodide (49). Slices were then washed 3 times with ice-cold PBS, and images were captured by a Bio-Rad MRC1024 confocal microscope connected to a Nikon Diaphot 200 microscope equipped with a 60 × Plan Apo objective with numerical aperture 1.4. The excitation/emission wavelengths were 488/522 nm for fluorescein-labeled antibodies and 488–568/605 nm for propidium iodide-stained chromatin. Calpastatin- and calpain-mediated green fluorescence was quantified with Laser Pix Software (Bio-Rad) as previously reported (7, 45).
in total spinal cord (Fig. 1B), \(\mu\)- and \(m\)-calpain levels were significantly reduced in transgenic mice, indicating a large extent of the proteases consumption, a marker of their activation after an increase in \([Ca^{2+}]_i\) (1, 45). This process was less pronounced in cerebellum (Fig. 1C) and almost undetectable in the remaining brain regions (Fig. 1D) deprived of cerebellum and of motor cortex. The decrease in total calpain protein, detected in transgenic mice, was accompanied by an almost equivalent loss of its catalytic activity evaluated in the same brain areas (data not shown). Activation of calpain was also assessed in control and transgenic mice by measuring \(\alpha\)-spectrin II and nNOS degradation, both sensitive substrates of the protease (1, 3, 51–53). In transgenic animals the proteolytic conversion of native \(\alpha\)-spectrin II to a 150-kDa species was much higher in motor cortex (Fig. 2A) and in total spinal cord (Fig. 2B) than in cerebellum (Fig. 2C), and it was almost undetectable in the remaining brain regions after the removal of cerebellum and of motor cortex (Fig. 2D). Similarly, nNOS, the neuronal isoform of nitric-oxide synthase, was also found to be degraded in transgenic mice as revealed by the decrease of the native form accompanied by the formation of the still active 130-kDa nNOS form (51, 54), largely accumulated in spinal cord and, to a lower extent, in cerebellum (Fig. 2, B and C). In the motor cortex of transgenic mice (Fig. 2A), the 50% decrease in the level of the native 160-kDa form was not accompanied by the accumulation of the digested nNOS species. This observation is consistent with the higher rate of calpain activation detected in this brain area likely to produce more extensive degradation of the synthase. In the remaining brain regions, nNOS was not affected (Fig. 2D). Altogether these findings indicate the occurrence of a highly pronounced activation of calpain in motor cortex and spinal cord, thereby suggesting that dysregulation of \(Ca^{2+}\) homeostasis selectively occurs at a higher extent in these brain compartments of transgenic animals. To verify this hypothesis we have measured the variations in \([Ca^{2+}]_i\), levels by loading with Calcium Green tissue slices taken from brain, spinal cord, and skeletal muscle areas of control and transgenic mice.

As shown in Fig. 3, A–D, the level of the green fluorescence was much higher in motor cortex (A) and lower spinal cord segment (B) samples collected from transgenic mice, areas in which calpain activation was also identified. In the cerebellum (C) the extent of \(Ca^{2+}\) elevation was significantly lower.
Whereas, in the remaining brain regions as well as in the upper segments of spinal cord of transgenic mice, the alteration in Ca$^{2+}$/H$^{11001}$ homeostasis was almost undetectable. Although the actual concentration of Ca$^{2+}$/H$^{11001}$ cannot be quantitatively determined from these fluorescence data, it can be calculated that in those brain regions of transgenic mice affected by the pathology, a 3–4-fold increase in [Ca$^{2+}$/H$^{11001}$]$_i$ occurred. These changes are consistent with the large extent of calpain activation also detected in the same brain compartment of these animals.

**Intracellular Functional State of the Calpain-Calpastatin System in Brain Areas of Transgenic Mice**—To explore the functional state of the calpain-calpastatin system, we have used a previously well established methodology based on the observation that, upon elevation of [Ca$^{2+}$]$_i$, calpastatin diffuses into the cytosol from the perinuclear aggregated forms, becoming colocalized with calpain and, thus, available for association to the protease (9, 10, 21, 22, 45, 55). Accordingly, perturbation in [Ca$^{2+}$]$_i$ may be identified by changes in the intracellular distribution of calpastatin. Once associated to the inhibitor protein, calpain, as a result of a conformational transition, acquires a much higher affinity for mAb 56.3, detectable through a fluorescent secondary antibody (8, 45). Thus, the increase in intracellular calpain-mediated fluorescence is not due to an increase in calpain level but is indicative of the formation of the calpain-calpastatin complex after elevation in [Ca$^{2+}$]$_i$. The redistribution of calpastatin in the cell-soluble fraction and the formation of the enzyme-inhibitor complex is an essential step for the control of calpain translocation at its sites of activation, but at the same time it exposes calpastatin to degradation by calpain being the inhibitor protein also a substrate of the protease (7, 8, 11, 23, 24). Calpastatin diffusion and formation of the protease-

**FIGURE 4.** Changes in the calpain-calpastatin system in motor cortex of control and transgenic mice. Frozen motor cortices obtained from control (black bars) and transgenic (gray bars) mice were cut into thin slices that were immediately collected, layered on glass microscope slides, and treated as described under “Methods.” Calpastatin and m-calpain were detected using mAb 35.23 and 56.3, respectively. Calpain and calpastatin green fluorescence and propidium iodide-stained chromatin were detected and quantified as described under “Methods.” The images in the upper panel represent one of the experiments performed. The reported values are the means ± S.D. of five independent experiments carried out on three different control and transgenic animals.

**FIGURE 5.** Calpastatin intracellular localization in spinal cord of control and transgenic mice. A, spinal cord was collected from control and transgenic mice and dissected in four segments. Each section was labeled from the cervical region (Segment 1) to the sacral region (Segment 4) and treated as described under “Methods.” Calpastatin was detected using mAb 35.23. Calpastatin-mediated green fluorescence and propidium iodide-stained chromatin were detected and quantified as described under “Methods.” The images represent one of the experiments performed. B, calpastatin-mediated green fluorescence was detected in control (black bars) and in transgenic (gray bars) mice and quantified as reported under “Methods” and in the legend to Fig. 3. The reported values are the means ± S.D. of five independent experiments performed on three different control and transgenic animals.
inhibitor complex can, thus, be reasonably considered as an indication of a dysregulation of intracellular Ca\(^{2+}\) homeostasis.

In the present study confocal microscope inspection of neurons was performed on slices of brain areas obtained from control and transgenic mice. In motor cortex neurons of transgenic mice (Fig. 4) from a perinuclear aggregated form present in controls, calpastatin appeared to be largely detectable in a diffused cytosolic localization. Confocal imaging revealed that calpastatin-mediated fluorescence was 8–10-fold increased in motor neurons of transgenic mice, indicating a corresponding increase in the availability of the inhibitor. Concomitantly calpain-mediated immuno-fluorescence analysis revealed that the protease was preferentially localized in cytosol, that its mediated green fluorescence was up to 6–8-fold increased, and that it was colocalized with calpastatin. The large extent of binding of mAb 56.3, detected in transgenic mice neurons by this increase in fluorescence intensity, confirmed the formation of the protease-inhibitor complex and the acquirement by the protease of a preactive state (8, 11).

Because spinal cord motor neurons, depending on their localization, innervate different muscles in the mouse, this brain area was dissected into four segments starting from cervical to sacral regions (56), and each one has been separately analyzed. We further confirmed that in all control spinal cord segments, calpastatin was detected as perinuclear aggregates (Fig. 5A). In transgenic mice spinal cord segments 3 and 4, the inhibitor protein was largely present in cytosol (Fig. 5A) and, correspondingly, the cytosolic calpain fluorescence (Fig. 6A) was largely increased, indicating that colocalization of the protease and its inhibitor induced the formation of the complex. No significant differences between control and transgenic mice

FIGURE 6. Calpain activation in spinal cord of control and transgenic mice. A, spinal cord was collected from control and transgenic mice and treated as described under “Methods.” μ-calpain was identified using mAb 56.3. Calpain-mediated green fluorescence and propidium iodide-stained chromatin were detected as described under “Methods.” The images are representative of one of the experiments performed. The insets are 3-fold enlarged images collected from the same sections. B, calpain-mediated green fluorescence was detected in control (black bars) and transgenic (gray bars) and quantified as reported under “Methods” and in the legend to Fig. 3. The reported values are the means ± S.D. of five independent experiments performed on three different control and transgenic animals.

FIGURE 7. Changes in the calpain-calpastatin system in cerebellum of control and transgenic mice. Frozen cerebellum obtained from control and transgenic mice was treated as described under “Methods.” Calpastatin and μ-calpain were identified using mAb 35.23 and mAb 56.3, respectively. Calpastatin- and calpain-mediated green fluorescence and propidium iodide-stained chromatin were detected in control (black bars) and transgenic (gray bars), and quantified as described under “Methods,” and in legend to Fig. 3. The reported values are the means ± S.D. of five independent experiments performed on three different control and transgenic animals. The images in the upper panel represent one of the experiments carried out.
were detected in calpain and calpastatin fluorescence in spinal cord segments 1 and 2 (Figs. 5A and 6A).

In the cerebellum of transgenic mice (Fig. 7), the intracellular diffusion of calpastatin and the formation of the calpain-calpastatin complex were much less pronounced with respect to motor cortex and spinal cord, in agreement with the data showing a lower degree of calpain activation and consumption (see Figs. 1 and 2). Thus, in the motor cortex, lumbar, and sacral spinal cord segments of transgenic mice, all containing motor neurons, the calpain-calpastatin system resulted in being profoundly altered, in agreement with the increased \([\text{Ca}^{2+}]\)
, responsible for the activation of the protease and for the consequent cell damages.

The assumption that the large increase in calpain fluorescence accompanied by cytosolic relocation of calpastatin, both observed in brain areas of transgenic mice, has been interpreted as an indication of the formation of binary complexes between calpastatin and calpain. The occurrence of such complexes is supported by previous observations and by the colocalization of the protease and its inhibitor in the cytosol (9–11, 22). To provide additional experimental evidence in support of this event, we have measured the calpain-calpastatin interaction capacity in crude extracts prepared from different brain areas of transgenic mice. To this purpose the presence of complexes between these two proteins was evaluated by means of an electrophoretic run carried out in the absence of \([\text{Ca}^{2+}]\).

Under these conditions, we have previously demonstrated that free \(\mu\)-calpain and calpastatin show an electrophoretic mobility different from that of the enzyme-inhibitor complex (11). As shown in Fig. 8, we have here established that almost all this isoform co-migrated with calpastatin in a discrete band corresponding to the enzyme-inhibitor complex. Calpastatin was found to also be present in a second band corresponding to the free inhibitor form. These data establish that \(\mu\)-calpain can associate with calpastatin in the various brain areas to form binary complexes, thus confirming that the increased fluorescence observed in the confocal images of the brain regions from transgenic mice is due to the presence of the protease-inhibitor complex (see Figs. 4–7). Although this technique is poorly quantitative, it is interesting to note that the excess of free calpastatin appears to be more represented in those brain areas in which a lower degree of calpain activation occurred.

**Inhibitory Capacity and Molecular Properties of Calpastatin in Brain Areas of Transgenic Mice**—We have previously demonstrated that the cytosolic diffusion of calpastatin followed by the formation of the calpain-calpastatin complex is always accompanied by degradation of the inhibitor protein, leading to the appearance of still active low \(M_c\) (15 kDa) fragments not constitutively expressed in brain (2, 3, 7, 23, 24, 25, 57).
Although these low $M_c$ calpastatin species are produced after calpain activation, they assure the persistence of a still efficient cellular inhibitory capacity. Thereby, total calpastatin inhibitory efficiency and fragmentation were explored in selective brain areas of control and transgenic mice. Unfortunately, this analysis could not be carried out on motor cortex samples due to limitations in the amount of tissue. Calpastatin molecular species, present in brain areas, were separated by polyacrylamide gel electrophoresis and identified and quantified by measuring their inhibitory activity after extraction from gel slices (7, 23, 24). As shown in Table 1, total calpastatin inhibitory activity in brain areas of transgenic mice, characterized by calpain activation and calpastatin cytosolic diffusion, was slightly increased from ~1.25- to 1.5-fold. In the remaining brain regions of transgenic mice, there was no significant increase. In the remaining brain regions no changes in calpastatin mRNA expression were observed.

Thus, cytosolic redistribution of calpastatin and its fragmentation into free inhibitory domains, associated with an increased synthesis of the inhibitor, appear to be part of a complex defense mechanism that can prevent and control the extent of calpain activity. It still remains to be elucidated why in brain areas in which calpain activation and cell damages are more pronounced, this defense mechanism seems somehow defective.

**Functional State of the Calpain-Calpastatin System in Muscle of Transgenic Mice**—All the experimental evidence presented so far has indicated that the alteration in Ca$^{2+}$ homeostasis and the dysregulation of the intracellular Ca$^{2+}$-dependent proteolytic system occur to the highest extent in motor cortex, lumbar, and sacral regions of the spinal cord of transgenic animals. To explore if the loss of efficiency of these motor neurons affected the function of the related muscles, we have
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A

|            | \(\mu\)-calpain | m-calpain |
|------------|------------------|-----------|
| Control    | ![Control](image) | ![Control](image) |
| Transgenic | ![Transgenic](image) | ![Transgenic](image) |

Protein level (% of control)

- Control: Black
- Transgenic: Gray

B

|            | \(\alpha\)-Spectrin II | nNOS |
|------------|------------------------|------|
| Control    | ![Control](image)      | ![Control](image) |
| Transgenic | ![Transgenic](image)   | ![Transgenic](image) |

Protein level (% of total)

- Native: Bar graph
- Digested: Bar graph

C

|            | Control | Transgenic |
|------------|---------|------------|
| Longitudinal section | ![Control](image) | ![Transgenic](image) |
| Transversal section   | ![Control](image) | ![Transgenic](image) |

D

|            | \(\mu\)-calpain |
|------------|-----------------|
| Control    | ![Control](image) |
| Transgenic | ![Transgenic](image) |

E

|            | Control | Transgenic |
|------------|---------|------------|
| Transversal section | ![Control](image) | ![Transgenic](image) |

F

|            | C | T |
|------------|---|---|
| Free calpastatin | ![Free calpastatin](image) | ![Free calpastatin](image) |
| Complex | ![Complex](image) | ![Complex](image) |
| Free \(\mu\)-calpain | ![Free \(\mu\)-calpain](image) | ![Free \(\mu\)-calpain](image) |

\(\mu\)Cp. Cst \(\mu\)Cp. Cst
analyzed the functional state of the calpain-calpastatin system in skeletal muscle samples collected from the posterior legs of transgenic mice. As shown in Fig. 10, A and B, μ- and m-calpain consumption, α-spectrin II, and nNOS degradation occurred in these cells at an extent very similar to that observed in motor cortex and in lower spinal cord segments of transgenic animals. Confocal microscope images (Fig. 10, C and D) revealed that in both longitudinal and transversal skeletal muscle sections of transgenic mice, calpain-mediated fluorescence intensity was highly increased together with a large coincident diffusion of calpastatin into the cell cytosol. Calpain activation and intracellular redistribution of calpastatin was accompanied by a large perturbation of Ca^{2+} homeostasis (Fig. 10E). This conclusion is supported by the observation showing that, as in the brain areas (see Fig. 3), calpastatin is mostly associated with calpain and that free calpastatin was present in excess in transgenic mice (Fig. 10F). The level of calpastatin, its conservative proteolytic fragmentation, and the level of its mRNA expression were then determined. As shown in Fig. 11A, in posterior leg muscle of transgenic mice, total calpastatin inhibitory activity was 2.2-fold increased, and calpastatin mRNA was approximately 4 times higher than in controls (Fig. 11B), indicating that an identical defense mechanism against total activation of calpain was found to persistently operate also in the skeletal muscle of transgenic mice.

Altogether these findings indicate that under conditions of prolonged alteration of Ca^{2+} homeostasis, a still efficient control of calpain activation can be achieved through cytosolic diffusion of calpastatin in colocalization with calpain, the liberation of still active low Mr 15-kDa inhibitory domains, not normally expressed in muscle (2, 3, 58), and an increase in the inhibitor biosynthesis.

**DISCUSSION**

The present study was aimed to explore *in vivo*, using a transgenic mouse strain overexpressing the mutated -G93A(+) SOD1, the effects of long term and persistent elevation of [Ca^{2+}]_i (29, 30, 33–38) and the functional properties of the calpain-calpastatin system by evaluating calpain activation (3, 45, 51–53) as well as distribution, levels, and molecular species.

![FIGURE 10. Changes in the properties of the calpain-calpastatin system in skeletal muscle of control and transgenic (Transg.) mice.](image)

A, aliquots (100 μg of protein) of crude extracts prepared from skeletal muscle of control (black bars) and transgenic (gray bars) mice as described under “Methods” were submitted to electrophoresis on a 10% polyacrylamide gel containing 1 mg/ml casein as described in the legend to Table 1. The reported values are the means ± S.D. of three independent experiments performed on three different control and transgenic animals. B, CDNA was prepared from skeletal muscle total RNA (3 μg) isolated as described under “Methods” from control (black bars) and transgenic (gray bars) mice. Aliquots (0.8 μl) of CDNA were amplified using primers specific for GAPDH and calpastatin as described under “Methods.” To detect calpastatin and GAPDH, 37 and 24 cycles were, respectively, performed. Calpastatin PCR products amounts were detected, measured, and normalized with respect to GAPDH as described in the legend to Fig. 7. The amount of normalized calpastatin PCR product detected in control mice was taken as 100%. To minimize tube-to-tube variation, each experiment was carried out in triplicate. The inverted images in the upper panel represent one of the experiments. The reported values are the means ± S.D. of three independent experiments performed on three different control and transgenic animals.

![FIGURE 11. Calpastatin levels, molecular species, and mRNA expression in skeletal muscle of control and transgenic (Transg.) mice.](image)

A, aliquots (150 μg of protein) of the clear supernatants, prepared from skeletal muscle of control and transgenic mice as described under “Methods,” were submitted to electrophoresis on a 10% polyacrylamide gel containing 1 mg/ml casein as described in the legend to Table 1. The reported values are the means ± S.D. of three independent experiments carried out on three different control and transgenic animals. B, CDNA was prepared from skeletal muscle total RNA (3 μg) isolated as described under “Methods” from control (black bars) and transgenic (gray bars) mice. Aliquots (0.8 μl) of CDNA were amplified using primers specific for GAPDH and calpastatin as described under “Methods.” To detect calpastatin and GAPDH, 37 and 24 cycles were, respectively, performed. Calpastatin PCR products amounts were detected, measured, and normalized with respect to GAPDH as described in the legend to Fig. 7. The amount of normalized calpastatin PCR product detected in control mice was taken as 100%. To minimize tube-to-tube variation, each experiment was carried out in triplicate. The inverted images in the upper panel represent one of the experiments. The reported values are the means ± S.D. of three independent experiments performed on three different control and transgenic animals.
of calpastatin. Beside confirming the occurrence of a high degree of calpain activation, we also established that in transgenic mice this event was detectable mostly in motor cortex, lumbar, and sacral segments of the spinal cord and was of limited intensity in cerebellum and almost undetectable in the upper segments of the spinal cord and in the remaining brain. Of relevant interest, the confocal microscope images revealed that only in motor neurons in which activation of calpain occurred, calpastatin was diffused into the cytosol, becoming colocalized and associated with calpain. These findings provide first evidence that even under conditions of a persistent high elevation of \([\text{Ca}^{2+}]_c\), a massive and uncontrolled activation of calpain is prevented by its association to the inhibitor protein in a binary complex form. Furthermore, by detecting the products of calpastatin degradation and by measuring the level of calpastatin mRNA expression, we demonstrate that, despite its continuous proteolytic degradation, the overall calpastatin inhibitory capacity on calpain was sustained by two independent but integrated mechanisms. The first one involved in a conservative degradation of calpastatin into still active low molecular weight (15 kDa) fragments, whereas the second one consisted of an increased expression of the inhibitor. To confirm that this putative protective mechanism could be considered of general significance, all analyses were extended to skeletal muscle of transgenic mice.

Also in these cells extensive calpain activation was found to be associated with intracellular calpastatin diffusion and the formation of the calpain-calpastatin complex. Moreover, large accumulation of the active low \(M_r\), 15-kDa fragments and an ~4-fold increase in the level of calpastatin mRNA provided the basic conditions for the persistence of a efficient inhibitory capacity even in the presence of a continuous proteolytic degradation of the inhibitor protein. Altogether these findings provide the first evidence that in conditions of an in vivo long term and persistent elevation of \([\text{Ca}^{2+}]_c\), in motor neurons and in muscle cells, calpain activation remains under continuous control to avoid a potentially highly destructive proteolysis. The fact that these findings have been obtained in an experimental transgenic mice model, generally used for studies of familial amyotrophic lateral sclerosis (26–32), seems to us of particular relevance for the understanding of the pathological events that occur in this and, more generally, in other neurodegenerative diseases vulnerable to alteration in \(\text{Ca}^{2+}\) homeostasis (1, 3, 13, 59–61). These results further suggest that the calpain-calpastatin system may be a suitable target for new therapeutic approaches to neurodegenerative diseases.

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