

Regulation of Calpain Activity in Rat Brain with Altered Ca^{2+} Homeostasis*

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Activation of calpain occurs as an early event in correlation with an increase in $[\text{Ca}^{2+}]_i$ induced in rat brain upon treatment with a high salt diet for a prolonged period of time. The resulting sequential events have been monitored in the brain of normal and hypertensive rats of the Milan strain, diverging for a constitutive alteration in the level of $[\text{Ca}^{2+}]_i$ found to be present in nerve cells of hypertensive animals. After 2 weeks of treatment, the levels of the plasma membrane Ca^{2+} -ATPase and of native calpastatin are profoundly decreased. These degradative processes, more pronounced in the brain of hypertensive rats, are progressively and efficiently compensated in the brain of both rat strains by different incoming mechanisms. Along with calpastatin degradation, 15-kDa still-active inhibitory fragments are accumulated, capable of efficiently replacing the loss of native inhibitor molecules. A partial return to a more efficient control of Ca^{2+} homeostasis occurs in parallel, assured by an early increase in the expression of Ca^{2+} -ATPase and of calpastatin, both producing, after 12 weeks of a high salt (sodium) diet, the restoration of almost original levels of the Ca^{2+} pump and of significant amounts of native inhibitor molecules. Thus, conservative calpastatin fragmentation, associated with an increased expression of Ca^{2+} -ATPase and of the calpain natural inhibitor, has been demonstrated to occur *in vivo* in rat brain. This represents a sequential adaptive response capable of overcoming the effects of calpain activation induced by a moderate long term elevation of $[\text{Ca}^{2+}]_i$.

The regulatory mechanism of calpain activity is primarily based on the physiological control of intracellular Ca^{2+} homeostasis, predominantly exerted by the plasma membrane Ca^{2+} -ATPase and, more specifically, by the expression in all mammalian cells of a natural protein inhibitor named calpastatin (1–7).

Calpastatin is a protein endowed with peculiar molecular properties characterized by the presence of four identical inhibitory domains, each one possessing, in its free form, an inhibitory capacity almost equivalent to that expressed by the native

calpastatin molecule (2–4, 8–11). Moreover, calpastatin also behaves as a substrate of calpain, which is degraded to single inhibitory domains as well as to inactive products as a result of more extensive digestion (8, 12–16). Conservative fragmentation has been attributed to μ -calpain activity, and degradation to inactive peptides attributed to digestion by m-calpain (8, 16).

Despite this information and the numerous reports on the structural properties of calpain (17–22), including the presence of different isoforms in various tissues (23–27), the biological function of this protease and the efficiency of its “*in vivo*” intracellular regulation remain uncertain.

It is often suggested (28–34) that in brain, calpain is directly implicated in neurodegeneration and neuronal cell death as a consequence of a massive Ca^{2+} influx occurring under pathological conditions, such as hypoxia and ischemia. This statement has been largely based on the protective effects exerted by synthetic protease inhibitors and by overexpression of calpastatin (35–40). However, in these reports, the fact that a large excess of calpastatin is normally present in brain is not considered, and no explanation has been given on the possible ineffectiveness of the inhibitor under the experimental conditions used (31). It must also be considered that the severe and irreversible cell damage observed under these pathological conditions occurs in the presence of a massive Ca^{2+} influx, associated with the collapse of the membrane potential. On the basis of these considerations, it seems therefore reasonable to assume that in brain cells, as well as in other cell types, a moderate alteration in Ca^{2+} homeostasis, even prolonged for a long period of time, could evoke adaptive compensatory defense mechanisms capable of preserving cell integrity.

To explore *in vivo* the existence in the brain of such mechanisms and to characterize their molecular aspects, we have treated normal normotensive Milan strain rats (NMS)² with a high salt (sodium) diet (HSD) known to promote elevation in blood pressure in response to a mild increase in intracellular free $[\text{Ca}^{2+}]$ in vascular smooth muscle, via an alteration in the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (41–43). A role of the exchanger in the alteration of calcium homeostasis in brain has been previously suggested (44, 45). To amplify the effects of this treatment we have exposed hypertensive rats (HMS) of the same Milan strain (46) to the same diet, because these animals are characterized

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²The abbreviations used are: NMS, normotensive Milan strain rats; HMS, hypertensive Milan strain rats; Cl-1, calpain inhibitor 1; PM, plasma membrane; HSD, high salt (sodium) diet; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

by an unbalanced calpain-calpastatin system in heart, kidney, and erythrocytes (13), probably due to a genetically determined deregulation of intracellular Ca^{2+} homeostasis.

In the present study, we report that after 2 weeks of HSD treatment, as expected, $[Ca^{2+}]_i$ increases in the aorta and also in brain cells of NMS rats. These modifications induced upon HSD treatment are more pronounced in the brain of HMS rats, probably because of a constitutively slightly elevated basal level of $[Ca^{2+}]_i$.

Thus, on the basis of these differences, both animals represent appropriate models to study *in vivo* the effects of the increase in $[Ca^{2+}]_i$ on the calpain-calpastatin system. This relationship has been explored on the basis of: (i) changes in the level of calpain activity during HSD treatment; (ii) appearance of the activated calpain form; (iii) degradation of calpain protein substrate such as plasma membrane Ca^{2+} -ATPase and fodrin; and (iv) levels and molecular properties of calpastatin. The results obtained provide a new understanding of how the activity of the calpain-calpastatin system is regulated *in vivo* to prevent irreversible cell damage.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin, aprotinin, calpain inhibitor 1 (CI-1), monoclonal anti-fodrin antibody, and monoclonal anti-m-calpain (Domain III/IV) were purchased from Sigma. Calpain inhibitor PD151746 and 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) were obtained from Calbiochem. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Amersham Biosciences.

Human erythrocyte calpain was purified and assayed as reported previously (47). One unit of calpain activity is defined as the amount causing the production of 1 nmol of acid-soluble NH_2 groups per hour under the conditions reported previously (48). Calcium Green-1/AM was purchased from Molecular Probes.

Animals—To induce experimental hypertension, adult male normotensive (NMS) and hypertensive (HMS) Milan strain rats (46) of about 60 days old were housed under controlled conditions ($22\text{ }^\circ\text{C} \pm 1$; humidity $50\% \pm 5\%$; lighting 8–20 h), were fed on a standard rat chow, and drank *ad libitum* NaCl dissolved in tap water at a concentration of 10 g/liter for 15, 30, or 90 days. Each rat received ~ 0.7 g of NaCl every day. Alternatively, $25\ \mu\text{M}$ CI-1 was dissolved in tap water in the presence or in the absence of 10 g/liter NaCl and administered to NMS and HMS rats at the beginning of the experiment for 2 or 4 weeks. Each rat received ~ 0.7 mg of CI-1 every day. Experiments were conducted in accordance with the institutional ethical guidelines. During the course of the experiments, no appreciable changes were observed either in food consumption or body weight.

Systolic blood pressure was measured by tail-cuff plethysmography (W + W Electronic, BP recorder 8005) on preheated rats ($37\text{ }^\circ\text{C}$) following the procedure originally described by Byrom and Wilson (49). Experiments were performed using five animals for each treatment.

Ca^{2+} Imaging—NMS and HMS rats untreated and treated with HSD (high salt diet) for 2 weeks, were sacrificed by decapitation. Brains were immediately collected, and the tissue was dispersed by gently squeezing in 10 mM oxygenated HEPES containing 0.14 M NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl_2 , 1 mM CaCl_2 and 1% bovine serum albumin, pH 7.4 (buffer A). The suspensions were then filtered through a gauze, and the

tissue fragments were incubated at $37\text{ }^\circ\text{C}$ for 30 min with $15\ \mu\text{M}$ calcium green 1-AM (50). Cells were then washed with buffer, A to remove the fluorophore excess, and fluorescence was detected with a Bio-Rad MRC 1024 confocal microscope, connected to a Nikon Diaphot 200 microscope equipped with an N.A. 0.75 objective ($\times 40$).

Thoracic aorta were rapidly excised from the same animals, placed in oxygenated buffer A and stripped of connective tissue. Arterial segments (0.2×0.5 cm) were incubated in buffer A with $15\ \mu\text{M}$ calcium green 1-AM. After 30 min at $37\text{ }^\circ\text{C}$, the dye excess was removed by several washes in Buffer A and dye-loaded arteries, prepared as described in Refs. 43 and 51, were imaged by means of a Bio-Rad MRC 1024 confocal microscope connected to a Nikon Diaphot 200 microscope equipped with N.A. 0.75 objective ($\times 40$).

The fluorescence intensity in each collected image was quantified using LaserPix software (Bio-Rad). Each image was divided in ten sections, and the fluorescence value of each part was determined and the arithmetical mean was evaluated. The mean intensity value for each animal tissue utilized for these were calculated from the analysis of ten different images. Variation of the values was taken as an indication of corresponding changes in $[Ca^{2+}]_i$.

Determination of Ca^{2+} -ATPase Activity in Rat Brain—NMS and HMS rats were sacrificed by decapitation, the brains were rapidly removed, quickly frozen in liquid nitrogen, and stored at $-80\text{ }^\circ\text{C}$. To detect both plasma membrane and endoplasmic reticulum Ca^{2+} -ATPase activities, aliquots (0.3 g) were minced and re-suspended in 10 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4 containing 0.5 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 2 mM AEBSF. The samples were homogenized in a Potter-Elvehjem homogenizer and lysed by sonication, and the particulate material was collected by centrifugation ($100,000 \times g$ for 10 min). Membranes were washed, and Ca^{2+} -ATPase activity was measured as described (52).

Separation of Calpastatin Forms by Ion-exchange Chromatography—Freshly collected brains (2 g) from NMS and HMS rats were minced, homogenized in a Potter-Elvehjem homogenizer, and lysed by sonication in 3 volumes of chilled 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 2 mM AEBSF (buffer B). The particulate material was discarded by centrifugation ($100,000 \times g$ for 10 min), the clear supernatant (crude extract) was heated at $100\text{ }^\circ\text{C}$ for 3 min, and centrifuged at $100,000 \times g$ for 10 min. The soluble material (heated extract) was collected and loaded onto a Source 15 Q (Amersham Biosciences) column (1.5×3 cm) equilibrated in 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol. The adsorbed proteins were eluted with a linear gradient (40 ml) 0–0.35 M NaCl and collected in 1-ml fractions. Calpastatin activity was measured on aliquots of each fraction, as previously described (48). One unit of calpastatin activity is defined as the amount of inhibitor required to inhibit one unit of human erythrocyte calpain.

Separation of Calpain Isoforms by Ion-exchange Chromatography—Freshly collected brains (2 g) from NMS and HMS rats were minced, homogenized in a Potter-Elvehjem homogenizer and lysed by sonication in 3 volumes of chilled

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buffer B without protease inhibitors. Crude extracts prepared as described above were collected and loaded onto a Source 15 Q under the same conditions described for calpastatin ion-exchange chromatography. Calpain activity was assayed as reported (47).

Identification of Rat Brain Calpain and Calpastatin Species by Immunoblot Analysis—Crude extracts from brains (50 μ g) of NMS and HMS rats prepared as described above, underwent SDS-polyacrylamide gel electrophoresis (12%). Proteins were then transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. Membranes were probed with anti- μ -calpain monoclonal antibody 56.3 (53) or anti-m-calpain (Domain III/IV) monoclonal antibody (Sigma) or with anti-calpastatin monoclonal antibody 35.23 (54), followed by a peroxidase-conjugated secondary antibody as described (55) and then developed with an ECL detection system (Amersham Biosciences).

Separation and Quantification of Calpastatin Species in Rat Brain, following SDS-Polyacrylamide Gel Electrophoresis—Aliquots (1.5 mg of protein) of brain extract obtained from NMS and HMS were submitted to SDS-polyacrylamide gel electrophoresis (12%) divided in ten lanes. Calpastatin species were identified following protein extraction from the gel, as previously described (13). Calpastatin activity was measured as described in Ref. 48.

Determination of Total Calpain Activity in Brain Crude Extracts from NMS and HMS Rats—Crude extracts from brains of NMS and HMS rats (25 mg) untreated and treated with HSD for 4 weeks were prepared, as described above, without protease inhibitors. Calpain activity was measured directly on samples of each crude extract in the usual assay mixture containing 1 mM $CaCl_2$ (56).

Alternatively, to determine total calpain activity, 25 mg of the same samples were immediately loaded onto a Phenyl-Sepharose column (Amersham Biosciences). This chromatographic step separates calpastatin from calpain (57).

Levels of Calpastatin mRNA in the Brain of NMS and HMS Rats—Total RNA was isolated from brain of both NMS and HMS rats by extraction with guanidium thiocyanate (58), quantified, and immediately reverse-transcribed. First strand cDNA synthesis was performed by means of the Superscript RNase H⁻ Reverse Transcriptase kit (Invitrogen) using random examer primers.

Equal amounts (1 μ l) of each sample were co-amplified in the presence of primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calpastatin. The primers and the amplification conditions were the same as reported in Refs. 9 and 13, and mRNA levels were quantitatively determined with the method described by Ref. 59, following the modifications reported in Ref. 13. To minimize tube-to-tube variation, each experiment was carried out in triplicate.

Levels of Plasma Membrane Ca^{2+} -ATPase mRNA in the Brain of NMS and HMS Rats—Equal amounts (1 μ l) of the same cDNA samples prepared for the quantification of calpastatin mRNA levels were co-amplified in the presence of primers specific for GAPDH and plasma membrane Ca^{2+} -ATPase. Ca^{2+} -ATPase primers were chosen to detect all the forms of plasma membrane calcium pumps present in the brain tissue (60). The oligonucleotide used were: sense primer

5'-AAGAAAATGATGAAGGACAACAAC-3' and antisense primer 5'-CCTTCCAGGCACAGGAA-3'. The PCR conditions were: denaturation (94 °C for 40 s), annealing (52 °C for 30 s) and extension (70 °C for 60 s). Amplification was carried out as described in Ref. 13 and Ca^{2+} -ATPase mRNA levels were quantitatively determined with the method described by Ref. 59, following the modifications reported in Ref. 13. To minimize tube-to-tube variation, each experiment was carried out in triplicate.

RESULTS

Effect of HSD Treatment on Ca^{2+} Homeostasis in Aorta and in the Brain of NMS and HMS Rats—The effect of an increased $[Ca^{2+}]_i$ in normal rat brain (normotensive Milan strain, NMS), was studied using animals treated for 2 weeks with a high salt (NaCl) diet (HSD), known to promote aorta contraction accompanied by an elevation of its $[Ca^{2+}]_i$ (41–43). In parallel we have submitted the same treatment to genetically determined hypertensive rats of the same strain (HMS).

According to previous reports (43), we have observed that in NMS rats, HSD treatment produces elevation in blood pressure (Fig. 1A) from 105 to 145–150 mm Hg, accompanied by a 1.4–1.5 increase in fluorescence recorded following exposure of aorta slices to calcium green fluorescence (Fig. 1B). These data indicate that HSD treatment promotes a contraction of aorta smooth muscle mediated by an alteration in Ca^{2+} homeostasis. In HMS rats the elevated blood pressure (Fig. 1A) is associated to a constitutively increased $[Ca^{2+}]_i$ ranging from 1.8–1.9-fold (Fig. 1B). This calcium concentration is further increased (2.3-fold) by 2-week treatment with HSD (Fig. 1B).

Because the calcium green loading of aorta slices proved to be efficient in detecting changes in $[Ca^{2+}]_i$, this procedure was also utilized for the same purpose on brain tissues to avoid longer methodologies that might by themselves affect the level of $[Ca^{2+}]_i$.

Of particular interest was the finding that also in the brain of HMS rats $[Ca^{2+}]_i$ was constitutively increased, as 1.4–1.5-fold higher than that in normal rats. These levels were further increased 1.9-fold following HSD.

Altogether these findings are consistent with the following conclusions: 1) HSD is a suitable condition to induce a moderate and prolonged increase in $[Ca^{2+}]_i$ in the brain of NMS rats, thus providing an appropriate model to study the correlation between changes in Ca^{2+} homeostasis and calpain activation. 2) In the brain of HMS rats this alteration is constitutively present and is further enhanced by HSD treatment; thus, representing an appropriate and additional model to evaluate possible cell damage.

Evaluation of Calpain Activation in the Brain of NMS and HMS Rats during HSD Treatment—On the basis of the results described above, we then explored if alteration in Ca^{2+} homeostasis induced calpain activation in rat brain. For this purpose, the following parameters were selected: (a) digestion of fodrin, a calpain target protein in nerve cells (61); (b) consumption of calpain activity; (c) appearance of the autoproteolyzed low M_r calpain form.

It was found that, following HSD treatment, the accumulation of the digested form of fodrin occurred at very low

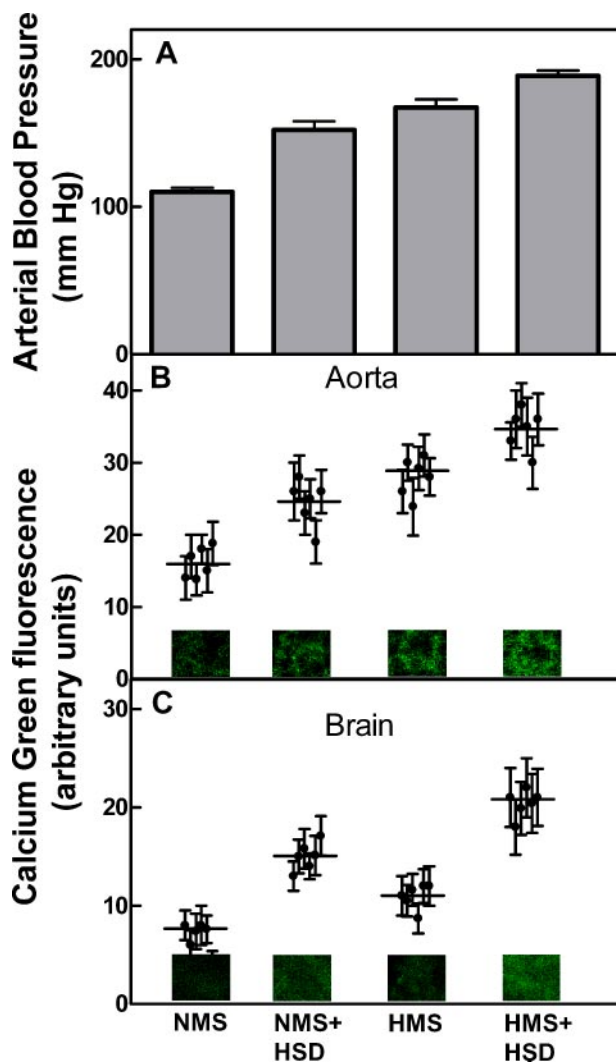


FIGURE 1. Effect of HSD treatment on $[Ca^{2+}]_i$ in aortic smooth muscle and brain cells of NMS and HMS rats. *A*, rats (group of six animals) were treated with high sodium diet as described under "Experimental Procedures." Arterial blood pressure of NMS and HMS rats, untreated or treated with HSD for 2 weeks, was measured as reported in Ref. 49. The values are reported as the arithmetical mean \pm S.D. *B*, aortic segments prepared as described in Refs. 43 and 51 or (*C*) brain tissue aggregates prepared as described under "Experimental Procedures" from NMS and HMS rats untreated or treated with HSD for 2 weeks were incubated with calcium green 1-AM for 30 min at 37 °C. The fluorescence was analyzed as reported under "Experimental Procedures." The images are representative of six different experiments analyzed by means of a Bio-Rad confocal microscope (see "Experimental Procedures"). Quantification of fluorescence, carried out as described under "Experimental Procedures," is reported in each panel as the arithmetical mean \pm S.D.

levels (Fig. 2), as indicated by a slight increase in the ratio between the digested and the native fodrin forms (from 0.17–0.22 to 0.28–0.33). Furthermore, as shown in Table 1, the levels of μ - and m-calpains, as well as their catalytic activity, did not reveal appreciable modification in the brain of NMS and HMS rats, following HSD treatment. Thus, it appeared that the alteration in $[Ca^{2+}]_i$ did not affect the consumption or the expression of both μ - and m-calpain isoforms. Furthermore, the formation of autolyzed or low $[Ca^{2+}]_i$ -requiring forms could not be detected in brain following HSD. Thus, under these experimental conditions the low extent of fodrin digestion indicated that activation of

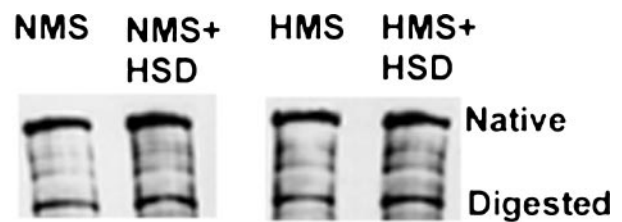


FIGURE 2. Digestion of fodrin in the brain of NMS and HMS rats following HSD treatment. Aliquots (50 μ g) of brain crude extract of untreated or 2-week-treated NMS and HMS rats were submitted to 12% SDS-PAGE; proteins were then transferred to a nitrocellulose membrane and stained with an anti-fodrin mAb (61). The immunoreactive material was detected as reported under "Experimental Procedures." The autoradiography was scanned in a densitometer, and the ratio between the digested and the native fodrin forms were calculated from the areas of the peaks.

calpain occurred at a slow rate involving a very limited amount of the protease.

Activation of Calpain and Degradation of Plasma Membrane Ca^{2+} -ATPase in the Brain of NMS and HMS Rats during HSD Treatment—To extend the search for the activation of calpain, this was evaluated at the level of the plasma membrane, by establishing the occurrence of a proteolytic degradation of a highly sensitive calpain substrate, such as Ca^{2+} -ATPase. This high susceptibility to degradation may be ascribed to the presence of a calmodulin-like domain in calpain and of a calmodulin binding site in Ca^{2+} -ATPase (62, 63).

As shown in Fig. 3A, HSD treatment promoted in the brain of NMS rats a 25–30% decrease in PM- Ca^{2+} -ATPase, suggesting that a slow and modest activation of calpain occurred under these conditions. Also, in the brain of HMS rats, the levels of the PM- Ca^{2+} -ATPase were significantly reduced, even though a 30–35% decrease in the activity of the Ca^{2+} pump is constitutively present in untreated rats. Thus, an elevation of $[Ca^{2+}]_i$ seems to be always associated with the degradation of PM- Ca^{2+} -ATPase. This digestion process appears to be highly specific since SERCA is not affected by HSD treatment in the brain of both NMS and HMS rats.

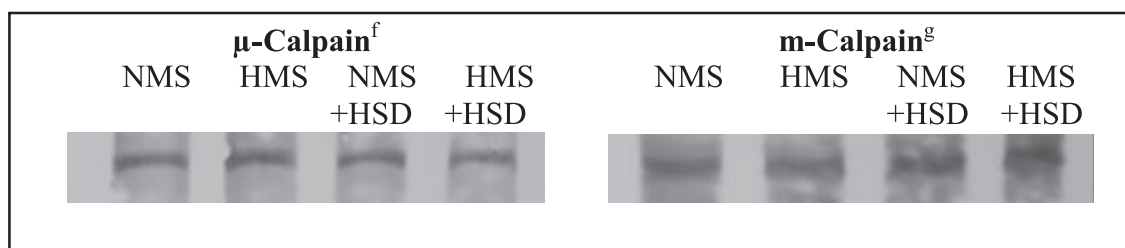
To further confirm this assessment, the levels of Ca^{2+} -ATPase were evaluated at different times in isolated brain cells from both NMS and HMS rats enriched in their $[Ca^{2+}]_i$ by exposure to the ionophore A23187. In both animal strains, Ca^{2+} -ATPase progressively decreased, whereas when cells were preincubated with the synthetic calpain inhibitor CI-1 (64) or with PD151746, a more selective calpain inhibitor (38, 39), no decrease in the Ca^{2+} pump activity was observed during the entire period of the experiments (Fig. 3B).

A final demonstration of the involvement of calpain activity in the disappearance of Ca^{2+} -ATPase in NMS rat brain following HSD treatment was obtained by the concomitant administration for 2 or 4 weeks of the calpain inhibitor CI-1. Under these conditions, no increase in blood pressure was observed (Fig. 3C), and no decrease in the level of PM- Ca^{2+} -ATPase occurred (Fig. 3D) in the brain of NMS rats. In the brain of HMS rats, the administration of CI-1 induced after 2 weeks a partial recovery of PM- Ca^{2+} -ATPase activity (Fig. 3D); however after 4 weeks, the activity of PM- Ca^{2+} -ATPase was returned to \sim 80% of control values. A longer treatment with the synthetic calpain inhibitor caused a recovery of PM- Ca^{2+} -ATPase also in the brain of HMS rats treated with

TABLE 1

Identification of calpain activation in brain of NMS and HMS rats during HSD treatment

Calpain isoform ^a	Animal ^b	Treatment ^c	Specific Activity in the presence of		Ratio B/A (x 100)
			mM Ca^{2+} (A) ^d (units/mg)	μ M Ca^{2+} (B) ^e (units/mg)	
μ	normotensive	none	1050 \pm 45	12.0 \pm 0.2	1.14 \pm 0.25
μ	hypertensive	none	985 \pm 40	10.4 \pm 0.3	1.05 \pm 0.2
μ	hypertensive	HSD	1090 \pm 30	9.5 \pm 0.3	0.87 \pm 0.25
m	normotensive	none	650 \pm 20	5.5 \pm 0.05	0.80 \pm 0.1
m	normotensive	HSD	625 \pm 20	6.0 \pm 0.4	0.96 \pm 0.2
m	hypertensive	none	680 \pm 15	4.8 \pm 0.06	0.70 \pm 0.1
m	hypertensive	HSD	668 \pm 22	5.0 \pm 0.3	0.73 \pm 0.1



^a The calpain isoforms were separated by ion-exchange chromatography as described under "Experimental Procedures."

^b Normotensive and hypertensive rats of the Milan strain were used.

^c The animals were fed *ad libitum* with water containing 10 g/liter sodium chloride.

^d Assay of total calpain activity was carried out as described under "Experimental Procedures" in the presence of 1 mM Ca^{2+} .

^e The Ca^{2+} concentration used in the assay of μ -calpain activity was 2 μ M, a saturating concentration for the autolyzed μ -calpain form (47). For the assay of m-calpain activity the concentration of Ca^{2+} was 20 μ M, a saturating concentration for the autolyzed m-calpain form (2).

^f Equal amounts (50 μ g) of brain crude extract of NMS and HMS rats, untreated and treated for 2 weeks with HSD were submitted to 12% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and stained by anti- μ -calpain monoclonal antibody 56.3. The immunoreactive material was detected as reported under "Experimental Procedures."

^g Equal amounts (50 μ g) of brain crude extract of NMS and HMS rats, untreated and treated for 2 weeks with HSD were submitted to 12% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and stained by anti-m-calpain mAb. The immunoreactive material was detected as reported under "Experimental Procedures."

HSD (Fig. 3D), accompanied by a limited decrease in the blood pressure. These findings suggest that hypertension genetically determined or produced by high sodium intake is evoked by different mechanisms, having as a common characteristic the alteration of Ca^{2+} homeostasis. It may be interesting to underline that these findings provide direct demonstration that *in vivo* intracellular activation of calpain can be efficiently monitored by following the inactivation of Ca^{2+} -ATPase.

Degradation of Calpastatin in the Brain of NMS and HMS Rats during HSD Treatment—Although the data reported in Fig. 3 indicate activation of calpain at the plasma membrane, no apparent activation of the protease seems to occur at the cytosolic level (see Table 1). This probably was due to the formation of an inactive calpain-calpastatin complex. In this associated form and in the presence of adequate $[Ca^{2+}]_i$, calpastatin underwent a slow degradation, almost completely prevented by the presence of synthetic calpain inhibitors (8, 16). The occurrence and the characteristics of such a degradation process were explored in our experimental model. In the brain of untreated NMS and HMS rats, the total levels of calpastatin activity were almost identical. Following 2 weeks of HSD treatment, the level of calpastatin was reduced

20–25 and 50% in the brain of NMS and HMS rats, respectively (Fig. 4A); this being consistent with a calpain-mediated calpastatin degradation. Inactivation was accompanied by a decrease in the amount of the native (high M_r) calpastatin and by the accumulation of fragments with a mass of ~15 kDa (Fig. 4B). To establish the inhibitory efficiency of the newly generated calpastatin forms, proteins were separated on SDS-PAGE, eluted from the gel and then assayed for their inhibitory activity (Fig. 4C). This procedure allowed us to quantify more precisely the low M_r species, because not all these fragments could be recognized by the mAb used for the immunostaining. Approximately 50% of calpastatin in the brain of HSD-treated normal rats was found to be present in the low 15-kDa form (Fig. 4C).

In the brain of HMS rats, a similar pattern of calpastatin digestion occurred but, because of the higher level of $[Ca^{2+}]_i$, the calpastatin fragments are virtually the only inhibitory species (Fig. 4D). As indicated in Fig. 4D, in the brain of untreated HMS rats, about 5% of total inhibitory activity was represented by these 15-kDa fragments, almost completely absent in normal rats. The presence of these new calpastatin forms did not derive from the expression of mRNAs coding for the single calpastatin inhibitory domain, as seems to occur in human testis (65). This

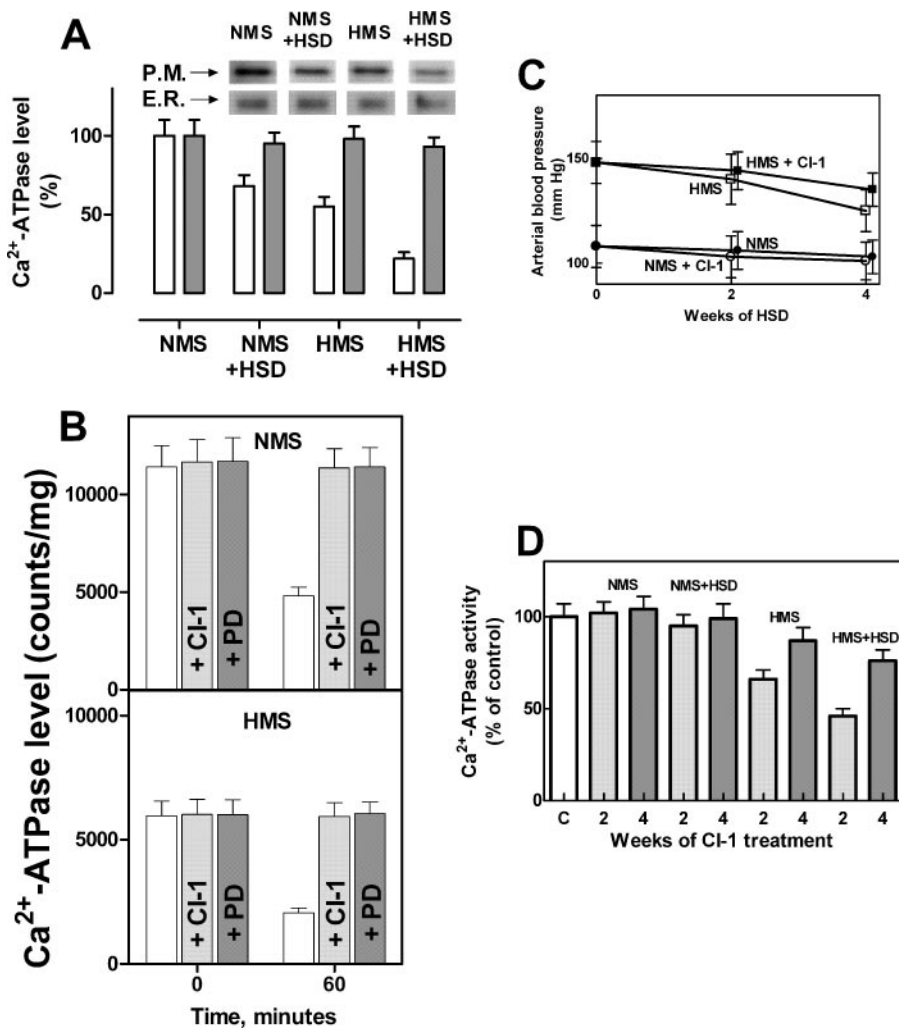


FIGURE 3. Calpain activation revealed by digestion of plasma membrane Ca^{2+} -ATPase pump. *A*, plasma membrane (P.M.) Ca^{2+} -ATPase activity (open bars) and endoplasmic reticulum (E.R.) Ca^{2+} -ATPase activity (gray bars), were detected in the brain of NMS and HMS rats before and after 2 weeks of treatment with HSD. The calcium pumps were identified by the formation of the ^{32}P -labeled acyl-phosphate intermediate in the presence of Ca^{2+} . In the absence of the metal ion, no incorporation of ^{32}P -labeled acyl-phosphate was observed (not shown). The two Ca^{2+} pumps were distinguished on the basis of their different molecular size (molecular mass of the Ca^{2+} -ATPase was 138 kDa and 90 kDa for the P.M. and E.R. forms, respectively.) The bands revealed in the autoradiography (top panel) have been quantified following densitometric analysis with the Shimadzu densitometer. The level of Ca^{2+} -ATPase activity in untreated NMS rats was taken as 100%. The data reported are the arithmetical mean \pm S.D. of five different experiments. *B*, brain tissue fragments, isolated as described under "Experimental Procedures" from NMS (upper panel) and HMS (lower panel) rats were prepared as reported under "Experimental Procedures" and incubated for the indicated times in buffer A containing 1 mM Ca^{2+} and 0.1 μ M calcium ionophore A23187, in the absence (open bars) or in the presence of CI-1 and PD151746 (PD). At the end of each experiment, cells were lysed by sonication, and the membrane fractions collected by centrifugation (52). Ca^{2+} -ATPase bands were identified as described in *A*. The labeled bands were cut from the gel and counted in a Multi-Purpose Scintillator (Beckman Coulter β -counter). The values reported are the arithmetical mean \pm S.D. of five different experiments. *C*, NMS and HMS rats were fed with HSD for the indicated times with (unfilled circles and squares) or without (filled circles and squares) CI-1. Arterial blood pressure was measured as reported in Ref. 49. The values reported are the arithmetical mean \pm S.D. of six different experiments. *D*, plasma membrane Ca^{2+} -ATPase levels were quantified as reported in *B*. 100% was taken as the level evaluated in the brain of normal rats. The indicated values are the arithmetical mean \pm S.D. of six different experiments carried out on six different animals of each strain.

explanation can be ruled out under our conditions, because all attempts to isolate such messengers were unsuccessful (data not shown).

Effect of Prolonged HSD Treatment on the Level of Calpastatin and of Plasma Membrane Ca^{2+} -ATPase in the Brain of NMS and HMS Rats—To establish if longer times of high salt intake could further modify the functional aspects of the regulation of the calpain-calpastatin system, the levels of calpastatin

and of Ca^{2+} -ATPase were evaluated after 12 weeks of HSD. In both animal strains (Fig. 5, *A* and *B*), the level of the calpastatin inhibitory activity were increased, reaching values close to the normal ones. In the brain of normal rats, the level of native (high M_r) calpastatin increased 1.6-fold, compared with the level detected after 2 weeks of HSD, and thus explained the normalization of the inhibitory activity. In the brain of HMS rats, the level of the native (high M_r) calpastatin was more than 25-fold higher than that of 2-week HSD-treated rats. The persistence in these conditions of the 15-kDa calpastatin fragments in association with appreciable amounts of native calpastatin could be an indication of a still active proteolysis, although at much lower rate, and of an enhanced calpastatin expression. This hypothesis was confirmed by the results shown in Fig. 5*C*, indicating that the level of calpastatin mRNA was increased, reaching after 12-week values 1.2–1.4-fold higher in the brain from normal rats. In the brain of HMS rats (Fig. 5*D*), calpastatin expression was enhanced to a much higher extent (2.5-fold).

The postulated decrease in calpain-mediated proteolysis should require the onset of a more efficient control of Ca^{2+} homeostasis. This occurrence was demonstrated by the reappearance of Ca^{2+} -ATPase activity (Fig. 6*A*) largely attributable to an increase in the level of Ca^{2+} -ATPase mRNA (Fig. 6*B*), which temporally preceded the increase in calpastatin mRNA and hence the accumulation of native calpastatin molecules. The kinetics of all these processes suggest that the increased expression of Ca^{2+} -ATPase, which reached its maximum after 4 weeks

of HSD treatment (Fig. 7*A*), is a crucial component of the overall cell defense process, because it also favors the accumulation of new calpastatin molecules whose synthesis was triggered only later (Fig. 7*B*).

DISCUSSION

The assumption that an enhanced calpain-mediated proteolytic activity was involved in neurodegeneration and neuronal

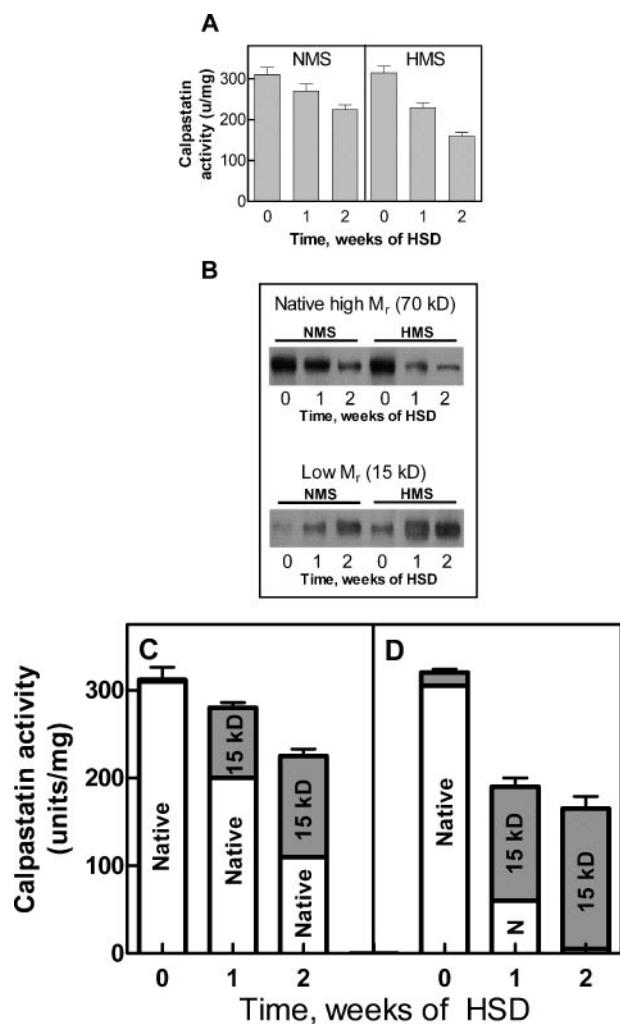


FIGURE 4. Calpastatin activity and its molecular species present in brain of NMS and HMS rats following HSD treatment. *A*, NMS and HMS rats were fed with HSD for the indicated times. Brain crude extracts (40 mg), prepared as described under "Experimental Procedures," were submitted to ion-exchange chromatography. Calpastatin activity was assayed as described in Ref. 48, in the presence of purified human erythrocyte calpain. Calpastatin activity is expressed as units/mg of brain crude extract. The values reported are the arithmetical mean \pm S.D. of five different experiments carried out on five different animals of each strain. *B*, aliquots (50 μ g) of brain crude extract obtained from untreated or HSD-treated NMS and HMS rats for the indicated times, were submitted to 12% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane (see "Experimental Procedures") and probed using the specific anti-calpastatin mAb 35.23 as described in Ref. 53 and under "Experimental Procedures." The immunoreactive material was detected as reported under "Experimental Procedures." Similar results were obtained from five different animals. *C*, aliquots (150 μ g) of brain crude extract of untreated or HSD-treated NMS rats for the indicated times were submitted to 12% SDS-PAGE. The gel was cut in 2-mm slices, and protein was eluted as described under "Experimental Procedures." Calpastatin molecular species were identified assaying the inhibitory activity reported in Ref. 48. The *open bars* refer to the native calpastatin molecular species, whereas the *gray bars* refer to the newly generated 15-kDa inhibitory species. Calpastatin activity is expressed as units/mg of brain crude extract. The values reported are the arithmetical mean \pm S.D. of five different experiments carried out on five different animals of each strain. *D*, aliquots (150 μ g) of brain extracts from untreated or HSD-treated HMS rats were analyzed as in *C* for brain of NMS rats. The values reported are the arithmetical mean \pm S.D. of five different experiments carried out on five different animals of each strain.

cell death following traumatic brain injury (66–69) is essentially based on the proteolytic effects displayed by synthetic inhibitors or by overexpression of calpastatin (the natural calpain inhibitor) (8, 16, 40, 70, 71). Accordingly, it seemed rea-

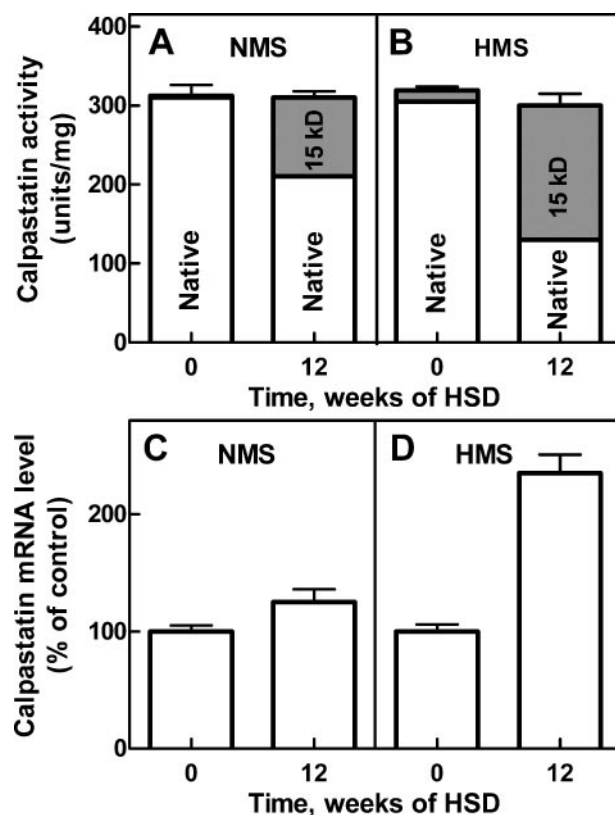


FIGURE 5. Level of calpastatin activity and of mRNA in the brain of untreated or 12-week HSD-treated NMS and HMS rats. *A* and *B*, aliquots (150 μ g) of brain crude extracts from untreated or 12-week HSD-treated NMS and HMS rats were submitted to SDS-PAGE as described under "Experimental Procedures" and in the legend to Fig. 4. The gel was cut in 2-mm slices, and protein was eluted as previously described. Calpastatin activity was then assayed on an aliquot of each fraction. The values are the arithmetical mean \pm S.D. of five different experiments carried out on five different animals. *C* and *D*, cDNA were prepared from the brain of untreated and treated rats of both strains described under "Experimental Procedures." Aliquots (1 μ l) of cDNA were co-amplified using primers specific for GAPDH and calpastatin (9, 13). PCR was performed by adding the sense primers 5' 32 P-labeled, and calpastatin mRNA amounts were determined as described in Refs. 13 and 59. mRNA levels are expressed as a percentage of the amount measured in untreated animals. All the values reported are the arithmetical mean \pm S.D. of five different experiments performed in triplicate.

sonable to assume that under these drastic experimental conditions, the regulation of Ca^{2+} homeostasis as well as the level of endogenous calpastatin had been severely affected, resulting in the loss of an endogenous control mechanism.

In the present study, this hypothesis has been explored by the use of an experimental model involving a moderate elevation in brain cells of $[Ca^{2+}]_i$ induced by HSD treatment shown to promote an increase in Ca^{2+} influx via the Na^+/Ca^{2+} exchanger (41–43). At different periods of time, animals were sacrificed, and the distinct contribution of calpastatin and of Ca^{2+} -ATPase in the regulation *in vivo* of calpain activation in brain tissue was evaluated. The results obtained have indicated that in nerve cells after a 2-week treatment, an increase in free $[Ca^{2+}]_i$ occurred accompanied by activation of calpain, as revealed by a decrease in the level of Ca^{2+} -ATPase and of the endogenous calpastatin. These observations are consistent with the existence of a correlation *in vivo* between the level of calpain activation and activity with the occurrence of a

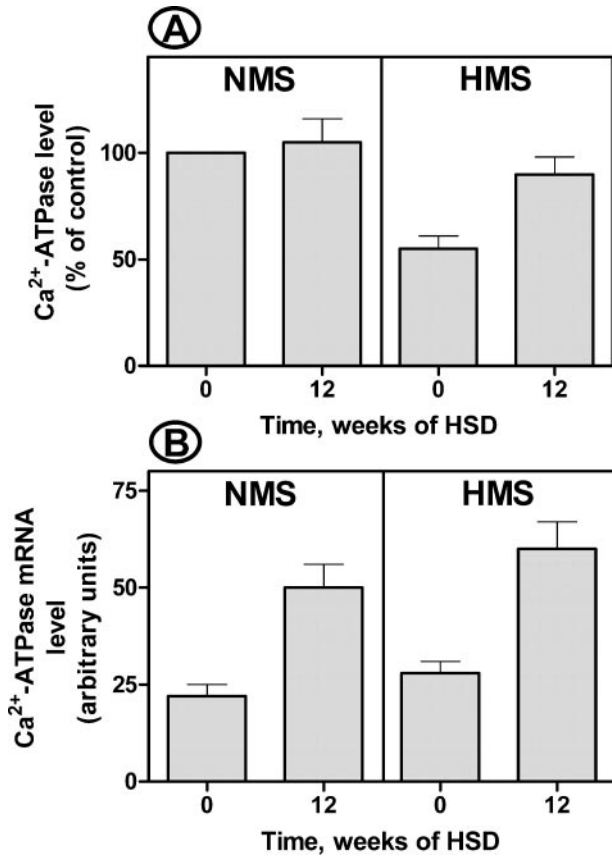


FIGURE 6. Plasma membrane Ca^{2+} -ATPase activity and mRNA levels in brain of untreated or 12-week HSD-treated NMS and HMS rats. *A*, Ca^{2+} -ATPase activity was detected in brain plasma membranes of NMS and HMS rats untreated and treated with HSD for 12 weeks, as previously described in the legend to Fig. 3 and under "Experimental Procedures." Ca^{2+} -ATPase quantification was carried out as described in the legend to Fig. 3. The data reported are the arithmetical mean \pm S.D. of different assays carried out on five animals of each strain. *B*, to measure Ca^{2+} -ATPase mRNA levels, equal amounts of brain cDNA (1 μ l) from NMS and HMS rats were co-amplified in the presence of primers specific for GAPDH and plasma membrane Ca^{2+} -ATPase (see "Experimental Procedures"). PCR was performed by adding the sense primers 5' ³²P-labeled, and Ca^{2+} -ATPase mRNA amounts were determined as described in Refs. 13 and 59. All the values reported are the arithmetical mean \pm S.D. of five different experiments carried out on five different animals of each strain.

decreased efficiency in the control of the intracellular Ca^{2+} level.

Furthermore, the demonstration of a direct involvement of calpain activity was provided by the protection against these degradative processes induced by the concomitant administration of the synthetic calpain inhibitor CI-1 to animals treated with HSD. Further support of this conclusion was obtained in reconstituted systems in which the plasma membrane Ca^{2+} pump has been shown to be almost completely protected from digestion following the addition of PD151746, a selective calpain inhibitor (38). The findings that under these conditions ER- Ca^{2+} pump (SERCA) remained unaffected give further evidence of the crucial role of calpain-mediated proteolysis, following alteration in Ca^{2+} homeostasis.

A very significant observation in our studies concerns the concomitant formation and accumulation of free 15-kDa inhibitory units found to occur during calpastatin degradation. This limited proteolysis becomes a compensatory mechanism

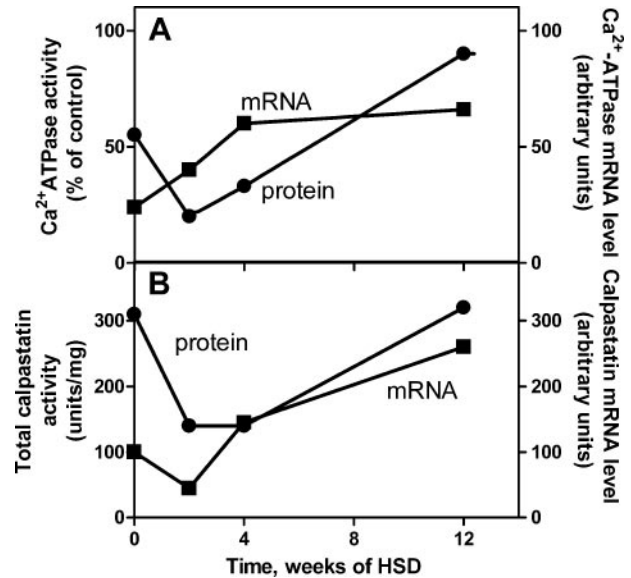
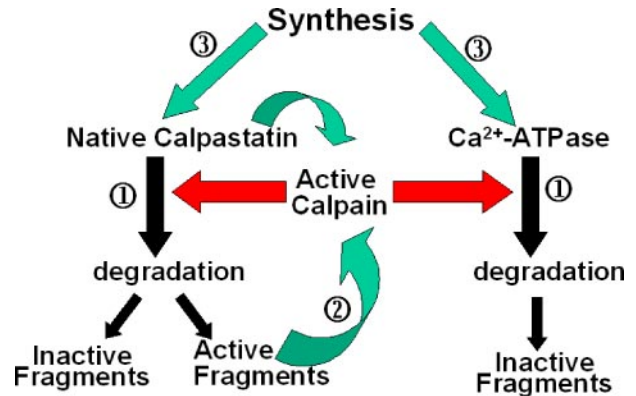


FIGURE 7. Comparative changes in activity and mRNA levels of plasma membrane Ca^{2+} -ATPase and calpastatin in the brain of HMS rats during HSD treatment. *A*, Ca^{2+} -ATPase activity (filled circles) and mRNA (filled squares) levels were quantified as described in the legend to Fig. 3, Fig. 6, and under "Experimental Procedures." All the values reported are the arithmetical mean \pm S.D. of four different experiments. *B*, calpastatin activity (filled circles) and mRNA (filled squares) levels were quantified as described under "Experimental Procedures" and in the legend to Fig. 4, Fig. 5, and under "Experimental Procedures." All the values reported are the arithmetical mean \pm S.D. of four different experiments.



SCHEME 1. Step 1, following an increase in $[Ca^{2+}]_i$, calpain becomes active, slowly degrading both calpastatin and Ca^{2+} -ATPase. Step 2, formation of active calpastatin fragments prevents the complete loss of regulation, reducing the extent of protease activation as well as its catalytic activity. Step 3, at longer times, the increase in synthesis of Ca^{2+} -ATPase and of calpastatin favors the recovery of a more controlled condition. Thus, extensive brain damage could be produced when the rate of Step 1 largely exceeds that of Step 3.

because, after 2 weeks of HSD treatment, the accumulation of these active inhibitor fragments represents an efficient device to overcome the degradation of native calpastatin and thus to assure a still functional mechanism for the control of calpain activity.

After 12 weeks of HSD treatment, brain cells appear to have developed an appropriate and efficient mechanism to compensate the initial imbalance in the calpain-calpastatin system based on an almost complete recovery of the level of Ca^{2+} -ATPase and of native calpastatin in the brain of NMS rats and with lower but still adequate efficiency in the brain of HMS rats.

This is accompanied by an early increased expression of Ca^{2+} -ATPase followed by an up-regulation of the level of native protein inhibitor synthesis. Taken together, these adaptive defense mechanisms seem to include three functionally correlated processes: (i) restoration of an efficient control of intracellular Ca^{2+} homeostasis, (ii) conservative fragmentation of calpastatin into single 15-kDa active inhibitory units, and (iii) increased expression of both Ca^{2+} -ATPase and calpastatin. The combined action of these biochemical processes restores lower intracellular Ca^{2+} levels, reduces the activation and activity of calpain, and thus allows brain cells to return progressively close to normal conditions. These processes are summarized in Scheme 1. According to this model, it can be suggested that in neurodegenerative pathologies, the rate of degradation of Ca^{2+} -ATPase and of calpastatin could largely exceed that of their synthesis, thereby unbalancing the brain defense process.

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