In this study, the effects of oxidation on calpain I autolysis and calpain-mediated proteolysis were examined. Calpain I was incubated with increasing concentrations of free calcium in the presence or absence of oxidant, and autolytic conversion of both the 80- and 30-kDa subunits was measured by immunoblotting utilizing monoclonal antibodies which recognize both autolyzed and non-autolyzed forms of each subunit, respectively. Autolytic conversion of the 80-kDa subunit of calpain I was not detected until free calcium concentration was greater than 40 μM, whereas autolysis of the 30-kDa subunit did not occur until the free calcium concentration was greater than 100 μM. In addition, autolytic conversion of either the 80- or 30-kDa subunit was not inhibited by the presence of oxidant. Calpain I activity was measured using the fluorescent peptide N-succinyl-[Leu](1)-[Leu](2)-[Leu](3)-[Val](4)-[Tyr](5)-7-amido-4-methylcoumarin or the microtubule-associated protein tau as substrate. Calpain I was found to have proteolytic activity at free calcium concentrations below that required for autolysis. Calpain I activity was strongly inhibited by oxidant at all calcium concentrations studied, suggesting that proteolytic activity of both the non-autolyzed 80-kDa and autolyzed 76-kDa forms was susceptible to oxidation. Interestingly, whereas oxidation did not inhibit autolytic conversion, the presence of high substrate concentrations did result in a significant reduction of autolysis without altering calpain proteolytic activity. Calpain I activity that had been inhibited by the presence of oxidant was recovered immediately by addition of the reducing agent dithiothreitol.

Calpains are a family of calcium-dependent thiol proteases that require both calcium and a reduced environment for activity. Calpains are present in virtually all vertebrate cells and have been postulated to play a role in many physiological processes (1–4). Calpains I and II are ubiquitously expressed, whereas the remaining isoforms are tissue-specific and are found predominantly in muscle (5, 6). Although homologous, calpains I and II require different concentrations of calcium for activity in vitro. Calpain II requires 200–1000 μM calcium (7), and calpain I requires 3–50 μM calcium (7) for half-maximal activity, a level which has been shown to be reached in the presynaptic terminals of neurons (8) and under pathological conditions (9). The focus of this study was calpain I because it is present in neurons and has been postulated to play a role in neuronal death associated with ischemia (10, 11) and certain neurodegenerative disorders (12–15).

Calpain I is a heterodimer composed of a unique 80-kDa catalytic subunit and a 30-kDa regulatory subunit which is identical to the 30-kDa subunit of calpain II (16). How these subunits interact and what regulatory mechanisms are involved in the process of calpain activation remain unresolved. Initially it was proposed that the 80-kDa form of calpain I was an inactive pro-enzyme that must undergo calcium-dependent conformational changes exposing the catalytic, thiol-protease domain, resulting in autolytic conversion to an intermediate 78-kDa form, then to a 76-kDa form in order to be an active protease (17). However, there is now substantial evidence to indicate that calpain I is active in its non-autolyzed 80-kDa form (7, 18, 19). The disparate findings concerning the proteolytic activity of the native 80-kDa form of calpain I may be due to the process of autolysis being modulated by more than calcium concentration alone (e.g. the presence of phospholipids, calpastatin, or other proteins) (20). It is also unclear as to the specific role of the 30-kDa noncatalytic subunit in modulating calpain autolysis and proteolytic activity. Several studies have shown that the calcium-dependent conversion of the 30-kDa to the 18-kDa form occurs subsequent to both autolysis of the 80-kDa subunit and proteolytic activity, suggesting conversion of the small subunit is unlikely to be required for calpain activation (21, 22). However, it has also been demonstrated that the presence of the 30-kDa subunit is required to reconstitute a proteolytically active, bacterially expressed, 80-kDa calpain II subunit (23). Although this study suggests that the catalytic subunit is essential to “enhance” the activity of the catalytic subunit, other studies indicate that the role of the smaller subunit may be as a chaperone and aid in folding of the 80-kDa inactive conformation (19). It has also been hypothesized that the 30-kDa is an inhibitor of the 80-kDa subunit and that dissociation of the two subunits is the crucial activation event (19, 24).

An interesting feature of calpain I-mediated proteolysis is that processing of most substrates is limited, resulting in the production of a few large polypeptide fragments. Generally, cleavage of enzyme substrates by calpain I results in a modification of activity rather than inactivation or complete digestion. For example, protein kinase C remains fully active after calpain cleavage but no longer requires calcium and phospholipid for activity (25). Likewise, calpain cleavage of calcineurin results in a phosphatase that is active but no longer requires calcium and calmodulin (26). Several structural proteins have also been identified as calpain substrates, including neurofilaments (27), spectrin (28, 29), and microtubule-associated pro-
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Proteins and Chemicals—cDNA clones encoding the longest isoform of human brain tau (T4L) were kindly provided by Dr. M. Goedert. T4L was expressed and purified as described previously (39). Porcine calpain I was expressed and purified as described previously (39). Fura-2 was from Molecular Probes, and the fluorescent peptide N-succinyl-I-leucyl-I-leucyl-I-valyl-L-tyrosine-7-amido-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC) was from Bachem. Trypsin, calcium chloride, DTT, nonstabilized hydrogen peroxide, and sodium hypochlorite were from Sigma.

Free Calcium Determination—Free calcium concentrations greater than 100 μM were determined using a calcium-sensitive ion selective probe (Orion Research model EA940) after calibration per manufacturer's instructions. Free calcium concentrations below 100 μM were determined in all reaction mixtures using the calcium indicator dye, fura-2. Fura-2 was excited at alternating wavelengths of 340- and 380-nm using a 75-watt xenon light source, monochromators, and a charge-coupled device-based PTI Deltasonic System. Emitted wavelengths passed through a monochromator set at 510 nm before detection by a photometer. Data were stored and processed using PTI software. Calcium determinations were made using the calcium indicator dye, fura-2, 50 mM Hepes, pH 7.5, and substrate concentrations of 0.1 mg/ml (2 μM) for T4L and 0.117 mg/ml (170 μM) for Suc-Leu-Leu-Val-Tyr-AMC. Ratios were initiated by the addition of calcium at the concentrations indicated. Oxidation studies included the addition of 100 μM hydrogen peroxide or 100 μM sodium hypochlorite immediately prior, unless otherwise stated, to addition of calcium. Measurement of calpain activity for the fluorescent peptide was performed as described previously (41) using the PTI Deltasonic System at an excitation wavelength of 340 nm and emission wavelength of 460 nm. For tau proteolysis and calpain autoylation, aliquots were removed at the designated times, added to a sodium dodecyl sulfate (SDS) Laemmli stop solution (42) composed of 500 mM Tris, pH 6.8, 10 mM EDTA, 10 mM EGTA, 4% SDS, 20% DTT, and 20% glycerol, incubated for 5 min in a boiling water bath, and stored at −20 °C until use. Aliquots were separated on either 7.5% (tau and 80-kDa calpain I subunit) or 12.5% (30-kDa calpain I subunit) SDS-polyacrylamide gels, transferred to nitrocellulose (43), and immunoblotted with a monoclonal antibody to tau, Tau1 (44), or a monoclonal antibody to calpain I’s 80-kDa (45) or 30-kDa (Chemicon) subunit. A bacterially expressed protein (21-kDa) corresponding to the natural autolytic product of the 30-kDa subunit (23) was utilized as a positive control for 30-kDa immunoreactivity. After incubation with the primary antibody, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and developed in 3,3'-diaminobenzidine in the presence of hydrogen peroxide or with enhanced chemiluminescence (Amersham Corp.) per manufacturer's instructions. The resulting immunoblots were quantitated using a Bio-Rad imaging densitometer (model GS-670). Data were evaluated using analysis of variance, and values were considered significantly different when p < 0.05.

RESULTS

Calpain I Proteolysis at Various Calcium Concentrations—Two previously identified calpain I substrates, the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC (41) and the microtubule-associated protein tau (46), were utilized to determine calpain I activity at various calcium concentrations from 0 to 2 mM. In the case of the fluorescent peptide the Km was determined to be 110 μM. A concentration slightly greater than the Km value was chosen (170 μM) to determine the effects of calcium concentration on proteolysis. Fig. 1A shows the change in the initial rate of calpain I proteolysis of the fluorescent peptide with increasing calcium concentration. No proteolytic activity was observed in the absence of calcium. Calpain I hydrolysis of the fluorescent peptide was observed at a concentration of calcium as low as 5 μM, although the rate was very low. The initial rate of calpain I activity dramatically increased between 5 and 100 μM calcium and approached maximal activity between 100 and 500 μM calcium. Half-maximal activity of calpain I was calculated by Hanes-Woolf analysis to occur at 86 μM calcium.

To determine the effects of calcium concentration on calpain I proteolysis of a potential physiological substrate, calpain I activity was also measured using tau as a substrate. In these studies recombinant tau representing the longest human isoform (T4L) was used. Fig. 1B shows calpain I-mediated proteolysis of tau at various calcium concentrations for 30 s of incubation. Calpain I-induced degradation of tau was not detected at 4 °C until the calcium concentration was greater than 20 μM. However, a profile of hydrolytic activity similar to that obtained with the fluorescent peptide was observed at calcium concentrations greater than 40 μM.

Calpain I Autoylation at Various Calcium Concentrations—Because autoylation of calpain I has been correlated with activation, samples were analyzed to determine the autolytic state of calpain I at various calcium concentrations. Fig. 2 shows representative immunoblots of calpain I autoylation during calpain I-mediated proteolysis of tau at the calcium concentrations indicated. No autoylation of calpain I was detected until the

1 The abbreviations used are: DTT, dithiothreitol; PHP, paired helical filament; Suc-Leu-Leu-Val-Tyr-AMC, N-succinyl-I-leucyl-I-leucyl-I-valyl-L-tyrosine-7-amido-4-methylcoumarin.
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76-kDa calpain I present at zero time was determined by comparing the immunoreactivity of the 76-kDa to the 80-kDa form using imaging densitometry (approximately 12% of total calpain I at zero time was 76 kDa). The calculated amount was then added as intact 80 kDa to a mixture containing the fluorescent compound, Suc-Leu-Leu-Val-Tyr-AMC, and activity was stimulated as described (see “Experimental Procedures”) at 2 mM free calcium to facilitate rapid autolytic conversion to the 76-kDa form and initiate maximal proteolytic activity. However, no measurable proteolytic activity was observed at this concentration of 76-kDa calpain I. The rate of autolytic conversion of the 30-kDa subunit of calpain I was also examined at various calcium concentrations. In good agreement with previous studies (21, 22), conversion of the 30-kDa subunit to 76 kDa was observed at a slower rate and at a higher minimal calcium concentration than observed for the 80-kDa subunit (data not shown).

Calpain I Autolysis at Various Substrate Concentrations—To determine whether calpain I autolysis was affected by substrate concentration, calpain I was incubated in the presence of various concentrations of the fluorescent peptide for up to 5 min at 2 mM calcium. At high substrate concentrations an inhibition of calpain I autolysis was observed (Fig. 3A). This was especially apparent at 150 μM Suc-Leu-Leu-Val-Tyr-AMC, where very little calpain I autolysis occurred compared with the autolysis that occurred in the absence of substrate. In contrast, there was no apparent substrate inhibition on calpain activity.

calcium concentration was greater than 40 μM, as determined by quantitation of the change in the ratio of the 78- and 76-kDa autolyzed forms to the intact 80-kDa form. However, proteolysis of tau, as well as the fluorescent peptide, was observed at 40 μM calcium (see Fig. 1, A and B, 40 μM calcium). Calpain I activity was also indicated by the degradation of calpain I fragments that are present at zero time (see Fig. 6A, 40 μM. Compare at arrowhead between − and + H2O2 at 5 min). Rapid autolysis was observed at calcium concentrations greater than 80 μM. Due to the presence of an immunoreactive band migrating at 76 kDa, which can be observed at zero time, separate experiments were carried out to determine whether this amount of 76-kDa calpain I could be responsible for proteolytic activity observed at low calcium concentrations. The amount of

FIG. 1. The effects of calcium concentration on calpain I-mediated proteolysis of the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC (A) and the human tau isoform T4L (B). A, quantitative analysis of the change in initial rate of the calpain mediated proteolysis of the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC with increasing calcium concentration. Initial rates were taken as the change in fluorescence over the first 30 s of incubation. Initial rates rapidly increase up to 100 μM with ½ maximal activity achieved at 86 μM calcium. Peak activity occurred between 500 μM and 2 mM calcium. Mean ± S.E., n = 3–6 separate experiments. B, immunoblots, representative of the results obtained in three separate experiments, demonstrating the change in the calpain-mediated proteolysis of human tau isoform T4L with increasing calcium concentration after 30 s of incubation. Rapid tau proteolysis was observed at calcium concentrations greater than 40 μM, although some proteolysis of tau was measurable at 40 μM calcium. 0.01 μg of T4L was run on each lane, and the blots were probed with Tau1 (1:2000).

FIG. 2. Immunoblots of the change in calpain I autolysis, in the presence of tau as substrate, with increasing calcium concentration, representative of the results obtained in three separate experiments. No autolysis was observed until the calcium concentration was greater than 40 μM, whereas rapid autolysis was observed at calcium concentrations greater than 80 μM. 0.1 μg of calpain I was run on each lane, and the blots were probed with the calpain I monoclonal antibody (1:2000).

FIG. 3. Immunoblots of the change in calpain I autolysis in the presence of increasing Suc-Leu-Leu-Val-Tyr-AMC [Substrate] concentrations at 2 mM calcium, representative of three separate experiments (A), and quantitative analysis of the change in initial rate of calpain I proteolysis of Suc-Leu-Leu-Val-Tyr-AMC (B) at 2 mM calcium. A decrease in calpain I autolysis was observed with increasing Suc-Leu-Leu-Val-Tyr-AMC concentration with substantial autolytic inhibition at 150 μM substrate (A). The initial rate of calpain I-mediated proteolysis of Suc-Leu-Leu-Val-Tyr-AMC rapidly increases over the substrate concentrations studied as shown in B. Initial rates were taken as the change in fluorescence over the first 30 s of incubation. Inset in (B) shows the Hanes-Woolf analysis plot with a correlation coefficient of 0.985. The Kₐ for the fluorescent peptide was calculated by Hanes-Woolf analysis to be 110 μM. Mean ± S.E., n = 5 separate experiments.
activity. Proteolytic activity increased up to 420 μM fluores-
cent peptide, and activity data exhibited classical Michaelis-
Menten kinetics (see Fig. 3B).

Effectsof Oxidation on Calpain I Activity—To determine
the effects of oxidation on calpain I-mediated proteolysis, both
the fluorescent peptide and tau were proteolyzed by calpain I in
the presence of 100 μM hydrogen peroxide. Calpain I proteolysis
of the fluorescent peptide was significantly decreased in the pres-
ence of 100 μM peroxide (Fig. 4A). Although the initial rates of
proteolysis of the substrate by peroxide-treated and control
were not significantly different, the extent of proteolysis was
significantly decreased by the presence of oxidant. Under con-
trol conditions, calpain I continued to cleave the substrate over
the time course studied, whereas in the presence of peroxide
the rate of substrate proteolysis by calpain I rapidly decreased
and was completely inhibited after 3 min. The results were
similar for all calcium concentrations between 40 μM and 2 mM.
In addition, the oxidant-induced inhibition of calpain I activity
was immediately recoverable by addition of the reducing agent
DTT (Fig. 4B). Similar experiments were carried out with tau
as the substrate. Fig. 5 shows the effects of oxidation on calpain
I-mediated proteolysis of tau at 40 μM calcium. The presence of
peroxide significantly inhibited calpain I-mediated proteolysis
of tau. Results with another oxidant, 100 μM sodium hypochlo-
rite, were similar to those observed with peroxide (data not
shown).

Because calpain I contains several cysteine residues
throughout its structure (47), experiments were carried out in
an attempt to elucidate whether the observed oxidative inhibi-
tion of calpain I activity was due to oxidation of the cysteine
group of the active site or simply a generalized oxidative event.
First, calpain I was preincubated with 40 or 100 μM calcium,
which should result in conformational changes that expos
the catalytic, thiol-protease domain to the environment, in the
presence or absence of 100 μM peroxide for 10 min, and then
added to a reaction mixture containing 0.1 mg/ml tau, resulting
in a final concentration of 10 μM peroxide. The reaction was
stopped at time points 30 and 50 min by addition of SDS stop,
immunoblotted for tau, and the amount of tau proteolysis
quantitated. Pretreatment with oxidant significantly reduced
the rate of calpain I-mediated proteolysis at both calcium con-
centrations compared with controls (data not shown). Second,
the same experiment was done except calpain I was preincu-
bated with no calcium in the presence or absence of 100 μM
peroxide for 10 min. When calpain I was added to the reaction
mixture containing tau as above, and then activated by calcium
addition, no difference in the rate of calpain I-mediated pro-
etolysis was observed between peroxide-treated and control sam-
pies (data not shown).

Effects of Oxidation on Calpain I Autolysis—To determine
whether the reduction in activity of calpain I by oxidation was
due to inhibition of autolysis of the 80-kDa to the 76-kDa form,
samples from fluorescent peptide and tau proteolytic experiments were analyzed for the state of calpain I autolysis. The presence of oxidant had no effect on the rate or extent of calpain I autolysis of either the 80- or 30-kDa subunits regardless of the calcium concentration (Fig. 6A). However, in the presence of oxidant, the proteolytic degradation of the 76-kDa form of calpain I was apparently inhibited (Fig. 6B).

**Effects of Oxidation on Other Proteases**—To determine whether the effects of oxidation on calpain I-mediated proteolysis were specific for thiol proteases, another thiol protease, papain, and a serine protease, trypsin, were examined using the fluorescent peptide as substrate. In the case of papain, the effects of peroxide were similar to those observed for calpain I, with substantial inhibition of papain activity after 2–3 min (Fig. 7A). Trypsin proteolysis, however, was unaffected by the presence of oxidant (Fig. 7B), and proteolysis of substrate continued throughout the time course studied.

**DISCUSSION**

Calpain I has been suggested to play a role in several pathogenic conditions including Alzheimer’s disease (12–15) and stroke (10, 11). In addition, oxidative stress has been postulated to be a significant factor in these same disease states (35, 38). It is important, therefore, to understand the mechanism(s) involved in calpain I activation as well as factors that modulate activity. In this study, several features of calpain I activation/activity were defined, *in vitro*, including the novel finding that oxidation of calpain I results in inhibition of activity without altering autolytic conversion of either the 80- or 30-kDa subunits.

Over the past several years it has become increasingly apparent that proteolytic processing of various substrates by calpain I most likely plays a crucial role in both physiological (4) and pathological conditions (12, 15, 48). Because of this, it is important to understand the mechanisms of autolysis and self-proteolysis and how exogenous regulators, e.g. calcium and phospholipids, modulate calpain I activity. It was originally proposed that calpain I activity is regulated through calcium-mediated autolysis, a proteolytic event that cleaves the N terminus of the 80-kDa protein resulting in either the 78- or 76-kDa form (49). Studies have indicated that autolysis of calpain I to the 76-kDa form apparently lowers the calcium concentration required for activity as well as increasing specific activity (50). However, interpretation of these data has recently been questioned (19, 24) as it has been suggested that it is dissociation of the heterodimer that is responsible for activation of the calpains. Regardless of these findings, it is clear that autolysis to the 76-kDa form is not a prerequisite for activity because in this study and others (7, 18) calcium-dependent proteolytic activity was observed in the absence of autolysis. Conflicting results regarding the necessity of calpain I autolysis for proteolytic processing are most likely due to different assay conditions. For example, in this study calpain autolysis...
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Figure 7. Quantitative analysis of papain (A) and trypsin (B) proteolysis of the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC in the absence (control) or presence (H₂O₂) of 100 μM hydrogen peroxide. The results are representative of three separate experiments. In the absence of peroxide papain continued to proteolyze the fluorescent peptide over the time course studied. In the presence of peroxide activity rapidly diminished and was completely inhibited after 2 min. Trypsin continued to proteolyze the fluorescent peptide with no diminution of proteolysis in both the absence and presence of peroxide over the time course studied.

was significantly inhibited by high concentrations of fluorescent substrate, although proteolytic activity was unaltered. It is likely that the presence of numerous factors such as calcium, calpastatin, phospholipids, and substrates modulate both autolytic and proteolytic activity of calpain I (20). Additionally, calpain I regulation occurs through inactivation by “self-proteolysis” into inactive polypeptides. Therefore, as previously demonstrated (50, 51), an initial increase in calcium concentration leads to autolysis of calpain I and the formation of an active protease, which is followed by enzyme inactivation through “self-proteolysis” which further complicates the issue.

Another important factor in calpain activation is the role of the noncatalytic 30-kDa subunit. It has been postulated that auto-conversion of the 30-kDa subunit to an 18-kDa polypeptide is involved in the activation of calpain I by lowering the calcium requirement for activity (52, 53). However, more studies are required since this study and others (7, 18) have shown that degradation of the 30-kDa subunit occurs subsequent to autolysis of the 80-kDa subunit, and/or at higher calcium concentrations. Recently, it has been suggested that the 30-kDa subunit acts as an inhibitor of the 80-kDa subunit and that dissociation of the heterodimer is required for calpain activation (19, 24). Furthermore, it has been speculated that although autolysis is not required for activity, autolysis of the calpain subunits accelerates dissociation and through this process facilitates activation (24). Although this is an intriguing hypothesis it requires further examination, and the current study does not address this issue.

There have been numerous studies examining the autolytic and activity state of calpain I in a variety of conditions (10, 12, 54, 55). The mechanism(s) involved in calpain activation and activity are complex and definitive interpretations of these findings are sometimes difficult. For example, to evaluate the role of calpain I in Alzheimer’s disease, calpain I was assayed by homogenization of tissue followed by incubation with substrate such as casein (14). In this case, no significant difference in the activity of calpain I was found between the pathological tissue compared with control. However, because these assays were done under reduced conditions the true oxidative state of the tissue was negated, which is relevant since oxidative stress has been demonstrated in Alzheimer’s disease (35). Based on the present study, oxidative state may be very important since calpain I activity was shown to be substantially decreased under oxidizing conditions. Furthermore, the present study also demonstrated that calpain I activity was recovered with addition of the reducing agent DTT, suggesting that assay of calpain I activity in the presence of a reducing agent may lead to an overestimation of in vivo calpain I activity.

Another method used to determine calpain I activity in pathological tissues is the ratio of the 76-kDa/78-kDa and 80-kDa forms of calpain I using immunoblot analysis (10, 12). This type of measurement is based on the hypothesis that the 80-kDa is “pro-calpain” while the 76-kDa form is the activated enzyme. Therefore, conversion of the 80-kDa to the 76-kDa changes their ratio and is an indication of activity. Because an increase in the ratio of the 76- to 80-kDa form was found in certain pathological states compared with controls, it was hypothesized that calpain I was overactivated in the diseased tissue (12). However, based on this study, these results may need to be re-evaluated since increased levels of the 76-kDa form may not necessarily translate into increased calpain I activity under conditions of oxidative stress. Indeed, since the 76-kDa calpain I form is susceptible to “self-proteolysis” and autolysis is not inhibited by an oxidizing environment but proteolytic activity is markedly reduced, the presence of increased levels of the 76-kDa form could indicate a condition of heightened oxidative stress.

The role of calpain I during oxidative stress is important to understand for several reasons. From the present study, it is apparent that oxidative stress could result in a net decrease in calpain-mediated proteolysis, resulting in the accumulation of the autolysed form of calpain I as well as calpain I substrates. Because calpain I has been proposed to be involved in many physiological functions (1–4), accumulation of autolysed calpain I may be of concern in pathological states which result in transient changes in intracellular calcium and/or redox potential such as ischemia where overactivation may lead to cell injury or cell death. The accumulation of substrate proteins may play a role in several disease states, such as Alzheimer’s disease, in which the pathological condition is defined in part by the formation of paired helical filaments which are aggregates of the tau protein (57, 58). The results of the present study suggest decreased activity of calpain I due to an oxidizing environment could contribute to the abnormal accumulation of tau in Alzheimer’s disease. In addition, oxidizing conditions have been shown to potentiate tau self-association (59) and the
formation of paired helical filament-like structures from tau constructs of just the microtubule-binding domains (60). Other substrates that could be affected by an oxidation-induced decreased calpain I activity include protein kinase C (17), calcineurin (26), and glutathione peroxidase (61), all of which can dramatically alter the ability of the cell to function properly.

Fig. 8 is a diagrammatic representation summarizing the potential pathways involved in the activation and activity of calpain I as suggested by the present study. Calpain I is active only in the presence of calcium as neither proteolytic nor autolytic activity has been observed in a calcium-free environment. Increasing calcium to levels below that required for autolysis activates the 80-kDa form resulting in proteolytic activity. Activity of the 80-kDa form was also suggested by Molinari et al. (18), who demonstrated that unautolyzed calpain I in erythrocytes localized to the plasma membrane was capable of proteolyzing calcium-ATPase. If calcium concentrations rise above the level required for autolysis, which may depend on the presence and type of substrate as well as other factors (20), calpain I would convert to a 78-kDa and then to the 76-kDa form. This conversion may be inhibited by the presence of high substrate concentrations. Because proteolytic activity was unaffected by increasing substrate concentrations, a variable that inhibited calpain I autolysis, it can be suggested that, in this case, it is the extent of calcium binding which controls the activation level of calpain I, not calpain I autolysis. However, it is clear that calpain I autolysis is rapid when substrate concentration is not excessive. In cases where calpain I is activated and capable of proteolytic activity, whether autolyzed or not, the presence of an oxidant, such as hydrogen peroxide, blocks the hydrolysis of substrate. The presence of oxidant had no effect on the rate or extent of calpain I autolysis of either the 80- or 30-kDa subunits. These data indicate that it is the cysteine residue within the active site of calpain I active site that is susceptible to oxidation, since inhibition only occurred when calpain was exposed to an oxidant in the presence of calcium.

Although these data clearly demonstrate that autolysis of calpain I is not a prerequisite for proteolytic activity, the role of autolysis in calpain function remains unknown. In addition, it is also clear that oxidation can reversibly inhibit calpain proteolytic activity with no effect on autolytic conversion. Clearly, much remains to be learned in order to fully understand the mechanism(s) involved in the process of calpain I-mediated proteolysis and its role in physiological and pathological conditions.

Note Added in Proof—Recently Baki et al. (Baki, A., Tompa, P., Alexa, A., Molnar, O., and Friedrich, P. (1996) Biochem. J. 318, 897–901) also examined the relationship between autolysis and calpain I activity.

REFERENCES

1. Lynch, G., and Baudry, M. (1987) Brain Res. Bul. 18, 809–815
2. Pontremoli, S., and Melloni, E. (1986) Annu. Rev. Biochem. 55, 445–481
3. Schollmeyer, J. E. (1988) Science 240, 911–913
4. Melloni, E., and Pontremoli, S. (1989) Trends Neurosci. 12, 438–444
5. Sorimachi, H., Ishiura, S., and Suzuki, K. (1993) J. Biol. Chem. 268, 19476–19482
6. Sorimachi, H., Saio, T. C., and Suzuki, K. (1994) FEBS Lett. 343, 1–5
7. Cong, J., Goll, D. E., Peterson, A. M., and Kapprell, H.-P. (1989) J. Biol. Chem. 264, 10986–10103
8. Llinas, R., Sugimori, M., and Silver, R. B. (1992) Science 256, 677–679
9. Kudo, Y., and Ogru, A. (1986) Br. J. Pharmacol. 89, 191–198
10. Neumar, R. W., Hagle, S. M., DeGracia, D. J., Krause, G. S., and White, B. C. (1996) J. Neurochem. 66, 421–424
11. Blomgren, K., Kawashima, S., Saio, T. C., Karlsson, J. O., Elmered, A., and Hagberg, H. (1995) Brain Res. 684, 143–149
12. Saito, K. I., Elce, J. S., Hames, J. E., and Nixon, R. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2628–2632
13. Bartus, R. T., Elliott, P. J., Hayward, N. J., Dean, R. L., Harbeson, S., Straub, J. A., Li, Z., and Powers, J. C. (1995) Neuror. Res. 17, 249–258
14. Nilsson, E., Alafuzoff, I., Blennow, K., Blomgren, K., Hall, C. M., Janssen, L., Karlsson, I., Wallin, A., Gottfries, C. G., and Karlsson, J. O. (1990) Neurobiol. Aging 11, 425–431
15. Nixon, R. A., Saito, K. I., Grynszpan, F., Griffin, W. R., Katayama, S., Honda, T., Mohan, P. S., Shea, T. B., and Beermann, M. (1994) Annu. N. Y. Acad. Sci. 747, 77–91
16. Ohno, S., Minoshima, S., Kudoh, J., Fukuyama, R., Shimizu, Y., Ohmi-Inajoh, S., Shimizu, N., and Suzuki, K. (1990) Cytogenet. Cell Genet. 53, 225–229
17. Melling, R. L. (1987) FASEB J. 1, 110–115
18. Molinari, M., Anaghi, J., and Carafoli, E. (1994) J. Biol. Chem. 269, 37992–37995
19. Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinhara, K., and Ishiura, S. (1995) Biol. Chem. Hoppe-Seyler 376, 523–529
20. Goll, D. E., Thompson, V. P., Taylor, R. G., and Zalewkska, T. (1992) BioEssays 14, 549–556
21. Cottin, P., Poussard, S., Desmaezes, J. P., Georgescaud, D., and Ducastaing, A. (1991) Biochem. Biophys. Res. Commun. 178, 254–259
22. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Yoshizawa, T., Kato, M., Mohan, P. S., and Beermann, M. (1994) FEBS Lett. 346, 263–267
23. Graham-Siegenthaler, K., Gauthier, S., Davies, P. L., and Elce, J. S. (1994) J. Biol. Chem. 269, 30457–30460
24. Yoshizawa, T., Sorimachi, H., Tomiska, S., Ishiura, S., and Suzuki, K. (1995) Biochem. Biophys. Res. Commun. 208, 376–383
25. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Spataro, B., Salamone, F., and Horecker, B. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6435–6439
26. Tallant, E. A., Brumley, L. M., and Wallace, R. W. (1988) Biochemistry 27, 2005–2011
27. Zimmerman, U.-J. P., and Schlaepfer, W. W. (1982) Biochemistry 21, 3977–3983
28. Siman, R., Baudry, M., and Lynch, G. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 901
3572–3576
29. Peterson, C., Vanderklish, P., Seubert, P., Cotman, C., and Lynch, G. (1991) *Neurosci. Lett.* 121, 239–243
30. Billger, M., Wallin, M., and Karlsson, J. O. (1988) *Cell Calcium* 9, 33–44
31. Nixon, R. A. (1989) *Ann. N. Y. Acad. Sci.* 568, 198–208
32. Johnson, G. V. W., Jope, R. S., and Binder, L. I. (1989) *Biochem. Biophys. Res. Commun.* 163, 1505–1511
33. Mehdi, S. (1991) *Trends Biochem. Sci.* 16, 150–153
34. Neumann, N. P. (1972) *Methods Enzymol.* 25, 393–400
35. Choi, B. H. (1995) *Neurobiol. Aging* 16, 675–678
36. Benzi, G., and Moretti, A. (1995) *Neurobiol. Aging* 16, 661–674
37. Bartus, R. T., Baker, K. L., Heiser, A. D., Sawyer, S. D., Dean, R. L., Elliott, P. J., and Straub, J. A. (1994) *J. Cereb. Blood Flow Metab.* 14, 537–544
38. Bronk, S. F., and Gores, G. J. (1993) *Am. J. Physiol.* 264, G744–G751
39. Laemmli, U. K., and Favre, M. (1973) *J. Mol. Biol.* 80, 575–599
40. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350–4354
41. Grynkievicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* 260, 3440–3450
42. Samis, J. A., Zboril, G., and Elce, J. S. (1987) *Biochem. J.* 246, 481–488
43. Johnson, G. V. W., Litersky, J. M., and Jope, R. S. (1991) *J. Neurochem.* 56, 1630–1638
44. Aoki, K., Imajoh, S., Ohno, S., Eneri, Y., Koike, M., Kosugi, K., and Suzuki, K. (1991) *FEBS Lett.* 250, 131–137
45. Perlmutter, L. S., Gull, C., Baudry, M., and Lynch, G. (1990) *J. Comp. Neurol.* 296, 269–276
46. Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y., and Imahori, K. (1981) *J. Biochem. (Tokyo)* 90, 275–278
47. Franzini, R. L., Repetti, A., Muck, T. C., and Easly, J. (1992) *J. Biol. Chem.* 257, 7293–7299
48. Johnson, G. V. W., Jope, R. S., and Binder, L. I. (1989) *Biochem. Biophys. Res. Commun.* 163, 1505–1511
49. Binder, L. I., and Rebhun, L. I. (1985) *J. Cell Biol.* 101, 1371–1378
50. Samis, J. A., Zboril, G., and Elce, J. S. (1987) *Biochem. J.* 246, 481–488