Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: A potential molecular basis for neuronal degeneration

(phosphorylation/ proteolysis/ membrane/ β -amyloid precursor protein/ cytoskeleton)

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Communicated by Richard L. Sidman, December 21, 1992 (received for review August 24, 1992)

ABSTRACT Calcium-activated neutral proteinases (CANPs or calpains) are believed to be key enzymes in intracellular signaling cascades and potential mediators of calcium-induced neuronal degeneration. To investigate their involvement in Alzheimer disease, we identified three isoforms of μ CANP (calpain I) in human postmortem brain corresponding to an 80-kDa precursor and two autolytically activated isoforms (78 and 76 kDa). As an index of changes in the *in vivo* activity of μ CANP in Alzheimer disease, the ratio of the 76-kDa activated isoform of μ CANP to its 80-kDa precursor was measured by immunoassay in selected brain regions from 22 individuals with Alzheimer disease and 18 normal controls. This μ CANP activation ratio was elevated 3-fold in the prefrontal cortex from patients with Alzheimer disease but not from patients with Huntington disease. The activation ratio was also significantly elevated, but to a lesser degree, in brain regions where Alzheimer pathology is milder and has not led to overt neuronal degeneration. These findings indicate that μ CANP activation is not simply a consequence of cellular degeneration but may be associated with dysfunction in many neurons before gross structural changes occur. The known influences of CANPs on cytoskeleton and membrane dynamics imply that persistent CANP activation may contribute to neurofibrillary pathology and abnormal amyloid precursor protein processing prior to causing synapse loss or cell death in the most vulnerable neuronal populations. Pharmacological modulation of the CANP system may merit consideration as a potential therapeutic strategy in Alzheimer disease.

Synaptic loss and neuronal cell death correlate strongly with the degree of cognitive impairment in Alzheimer disease (1–3). The molecular events underlying these losses have not yet been defined. Interest in the role of calcium has been rapidly growing because many putative etiologic factors in Alzheimer disease, including excitotoxicity, β -amyloid neurotoxicity, and free-radical injury, have in common the potential for disrupting cellular calcium homeostasis (4–6). Some studies suggest that such disruption may exist in fibroblasts from Alzheimer patients (7–11); however, evidence for altered calcium homeostasis in human brain is difficult to obtain due to postmortem influences on calcium levels.

One expected effect of altered calcium homeostasis is activation of calcium-activated neutral proteinases (CANPs), a family of proteases implicated in regulating aspects of signal transduction (12–14). Limited proteolysis by CANP is believed to regulate important enzymes, including neurotransmitter enzymes, calcium-dependent protein kinases, and phosphatases, and to modify the function of structural pro-

teins of the membrane and membrane skeleton (15). Effects of inappropriate CANP activation are, therefore, likely to be amplified through various metabolic cascades. While massive activation of CANP, as occurs in ischemia, induces rapid irreversible neuronal injury (16–18), persistent low-level activation by these or other mechanisms might be expected to have slowly progressive effects on phosphorylation and protein turnover, including some that are relevant to amyloid precursor protein (APP) processing (19) and cytoskeletal pathology (15). Since the activation of CANPs represents both a reflection of cellular calcium homeostasis and a direct pathway through which increased intracellular calcium may lead to neuronal dysfunction and degeneration, evidence for altered CANP activity has implications for the pathogenesis and treatment of Alzheimer disease.

CANPs exist as the isozymes μ CANP and mCANP, which require, respectively, micromolar or millimolar calcium for activation in their purified state. Both forms are present in cells principally as inactive precursor isoforms (20), which are activated by autoproteolytic cleavage of an N-terminal sequence in the presence of calcium (21–23). The action of CANP *in vivo* has been difficult to monitor by *in vitro* enzyme assay because the precursor forms are also activated by the assay procedure, and the observed enzyme activities are, in turn, influenced by instability of the enzyme as well as by various cytosolic inhibitory and activating factors (24). To circumvent some of these problems, we have measured distinct isoforms of μ CANP in postmortem human brain that correspond to the autolytically activated isoforms and uncleaved precursor previously demonstrated in platelets and erythrocytes (25, 26). Because the hydrolysis of protein substrates *in vivo* at physiological calcium levels requires μ CANP autolysis (27), the ratio of precursor to activated isoforms of the enzyme in tissues provides an index of μ CANP function *in vivo*.

MATERIALS AND METHODS

Tissues. Postmortem brain from 22 individuals with a premortem clinical diagnosis of probable Alzheimer disease (28), 16 individuals with Huntington disease, and 18 age-matched (65–85 yr old) neurologically normal individuals were used. Brain tissue was provided by E. D. Bird (McLean Hospital Brain Tissue Resource Center) and J. E. Hamos (University of Massachusetts Medical Center). Control brains, which were from patients with no history of neuropsychiatric disease, exhibited negligible microscopic neuropathology (0–2 senile plaques or 0–6 neurofibrillary tangles

Abbreviations: CANP, calcium-activated neutral proteinase; APP, amyloid precursor protein.

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per low-power field) in representative brain areas analyzed by the Nissl and Bielschowsky stains. All Alzheimer brains met the neuropathologic criteria developed by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (29). Huntington disease severity was assigned 1 of 5 grades from 0 to 4 by the criteria of Vonsattel *et al.* (30). Grade 3 cases (moderate-severe atrophy, neuron loss, astrocytosis) were chosen for study. The postmortem intervals for brain samples in the various groups were matched (see *Results* and figure legends). Agonal states of Alzheimer and Huntington patients were similar and overlapped in nature and severity with the range of agonal states experienced by the control group.

Protein Purification and Enzyme Assay. Human erythrocyte μ CANP was purified from blood of normal adults by anion-exchange and phenyl-Sepharose chromatographies (31, 32). Enzyme activity was determined by using [14 C]azocasein as substrate at a final free calcium concentration of 125 μ M (33). Human spectrin was isolated from fresh human erythrocyte ghost membranes by the low ionic strength buffer extraction method (34) and purified by chromatography on Sepharose 4B (35).

Immunoassay and Immunoblot Analyses. A 0.5-g quantity of tissue was homogenized with 10 vol of 20 mM Tris-HCl (pH 7.4) containing 2 mM EGTA, 1 mM EDTA, 1 mM benzamide, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.32 M sucrose (buffer A). The $15,000 \times g$ supernatant was applied to a column (1.0×15 cm) of DEAE-cellulose (DE-52; Whatman) that was developed with stepwise increments of KCl from 0 to 3 mM. μ CANP, eluted at 0.15 M KCl, was subjected to SDS/PAGE (7.5% acrylamide) and immunoblot analysis using a mouse monoclonal antibody to human μ CANP (25, 26) as described (33). In some experiments, homogenates of prefrontal cortex were centrifuged at $15,000 \times g$ for 30 min at 4°C to obtain cytosolic (supernatant) fractions and membrane (pellet) fractions before immunoblot analysis.

Total μ CANP content was quantified on immunoblots using a model 620 video densitometer (Bio-Rad). The weight of μ CANP on the immunoblot was calculated from standard

curves prepared by measuring the immunostaining intensity of various known amounts of human erythrocyte μ CANP on immunoblots (33). μ CANP standards were analyzed on every immunoblot that contained brain samples to control for possible minor immunostaining variations.

Protein Sequence Analysis. The 76-kDa isoform of μ CANP, generated by autolysis from 80-kDa μ CANP, was separated by SDS/PAGE and transferred to poly(vinylidene difluoride) Immobilon membranes (Millipore). N-terminal analysis by the Edman degradation method was performed by J. Kondo (Mitsubishi Kasei, Yokohama, Japan).

RESULTS

In human brain extracts, three isoforms of the catalytic subunit of μ CANP were detected by Western blot analysis with the μ CANP monoclonal antibody (25, 26). These forms exhibited molecular masses on SDS/polyacrylamide gels of 80, 78, and 76 kDa, identical to those in human erythrocytes (25, 26) (Fig. 1A). When purified μ CANP from human erythrocytes was activated *in vitro* by adding calcium, hydrolysis of spectrin was accompanied by N-terminal cleavage of the 80-kDa isoform at a bond not yet characterized to form the 78-kDa isoform and at Gly²⁶-Leu²⁷ to form the 76-kDa isoform (Fig. 1B). Conversion of the 80-kDa μ CANP isoform to 78- and 76-kDa isoforms was nearly complete within 30 sec. Enzyme activity was maintained in the absence of 80-kDa μ CANP and remained proportional to the amount of 76-kDa isoform (Fig. 1C), confirming that the 76-kDa isoform is enzymatically active (20, 37). Human brain μ CANP was unstable in cell-free extracts; however, conversion of the 80-kDa isoform of μ CANP to the smaller isoforms could be demonstrated in 1-mm slices of postmortem human neocortex incubated with calcium (Fig. 1D).

To evaluate the relative degree of μ CANP activation in the brain in Alzheimer disease, we quantitated the three μ CANP isoforms by immunoblot assay in brain regions from 22 individuals with Alzheimer disease and from 17 individuals free of clinical neurologic disease. The Alzheimer and control groups were matched with respect to age of the individual

FIG. 1. (A) Immunoblot analysis of μ CANP in prefrontal cortex from control (CONT) (lanes 1 and 2) and Alzheimer (AD) (lanes 3 and 4) patients. (B) Autolysis of μ CANP from human erythrocytes *in vitro*. μ CANP (lane 1) was incubated with 5.3 mM CaCl_2 (final free calcium, 50 μ M) and 2.2 μ g of human erythrocyte spectrin (36) for 30 sec at 30°C in the presence of 1 μ M leupeptin (lane 2), 10 μ M leupeptin (lane 3), or 100 μ M leupeptin (lane 4), or without leupeptin (lane 5). (C) Relationship between protease activity and 76-kDa μ CANP content. Human erythrocyte μ CANP was preincubated with 50 μ M calcium and spectrin for the indicated interval at 30°C , and reactions were terminated with 1 mM EGTA. The content of each μ CANP isoform was measured by immunoassay, and total μ CANP activity was measured by radiometric assay. The 80- and 78-kDa isoforms (not shown) completely disappeared after 1 min of incubation. ●, Protease activity; ○, 76-kDa μ CANP content. (D) μ CANP autolysis in human brain slices. Sections of postmortem prefrontal cortex were incubated at 32°C for 60 min in buffer A with or without 5 mM CaCl_2 . After homogenization, μ CANP isoforms were identified in a $15,000 \times g$ supernatant by immunoblot analysis. Lanes: 1, unincubated human erythrocyte (rbc) μ CANP; 2, unincubated brain slices; 3, incubation—no added calcium; 4, incubation with 5 mM CaCl_2 ; 5, incubation with 5 mM CaCl_2 plus 10 μ M leupeptin.

FIG. 2. Ratio of 76- to 80-kDa isoforms of μ CANP in prefrontal cortex from control individuals ($\circ$) and AD patients ($\bullet$) plotted against postmortem interval (PMI). Immunoassay of individual isoforms was performed as described.

(75.8 $\pm$ 2.0 yr vs. 68.9 $\pm$ 2.6 yr, respectively) and postmortem interval for brain samples (12.2 $\pm$ 1.3 hr vs. 12.0 $\pm$ 1.8 hr, respectively).

The ratio of 76- to 80-kDa μ CANP for individual brain samples was nearly 3-fold higher in the Alzheimer group than in the controls (2.20 $\pm$ 0.39 vs. 0.81 $\pm$ 0.10; P < 0.001; unpaired t test) (Figs. 2 and 3). This difference reflected a nearly 2-fold increase in the autolytically activated (76 kDa) μ CANP isoform in Alzheimer brain (41.2% $\pm$ 1.6%) with respect to controls (26.6% $\pm$ 2.2%) (P < 0.001) and a comparable decrease in the content of cytosolic 80-kDa μ CANP (22.7% $\pm$ 1.5% vs. 37.2% $\pm$ 2.2%; P < 0.001). The 78-kDa μ CANP isoform, which has an unclear relationship to the activation state of μ CANP in our studies, was unchanged. The sum of the three μ CANP isoforms was also not changed (Table 1).

The abnormal ratio of μ CANP isoforms in the cytosol was not due to a shift in their intracellular distribution. From 27% to 30% of the total μ CANP was associated with the particulate fraction in both control and Alzheimer groups, and the proportions of isoforms were also similar in each group. The high proportion of 76-kDa μ CANP (40.4 $\pm$ 1.8, control; 43.1 $\pm$ 1.7, Alzheimer disease; n = 9) and the low proportion of 80-kDa μ CANP (18.2 $\pm$ 1.3, control; 17.5 $\pm$ 1.3, Alzheimer disease; n = 9) in the particulate fraction are consistent with the hypothesis that μ CANP is activated mainly on the membrane (38).

The degree of abnormal μ CANP activation was comparable in Alzheimer brains at all postmortem intervals. The ratio of 76- to 80-kDa isoforms regressed against postmortem interval yielded nonsignificant correlations in control (r =

Table 1. Total μ CANP content (sum of three isoforms) in brain regions from normal control individuals and patients with Alzheimer disease or Huntington disease, measured by immunoassay (33)

	μ CANP content, ng per mg of protein		
	Prefrontal cortex	Putamen	Cerebellum
Control	567 $\pm$ 41 (18)	661 $\pm$ 53 (8)	589 $\pm$ 43 (14)
Alzheimer	536 $\pm$ 29 (22)	598 $\pm$ 33 (22)	574 $\pm$ 49 (21)
Huntington	597 $\pm$ 46 (12)	595 $\pm$ 47 (14)	590 $\pm$ 43 (16)

Results are means $\pm$ SEM for the number of cases in parentheses.

0.355), Alzheimer (r = 0.155), or combined (r = 0.148) groups (Fig. 2B). The possibility cannot be excluded, however, that isoform ratios change within the first few hours after death. The degree of μ CANP activation also did not significantly correlate with age within the age range studied.

Because some neurons are degenerating in Alzheimer neocortex, we also examined the degree of μ CANP activation in two brain areas where the neuronal dysfunction (37) has not advanced to evident neuronal degeneration (39). The ratio of 76- to 80-kDa μ CANP isoforms was elevated in both putamen (46%; P < 0.005) and cerebellum (58%; P < 0.005) in the Alzheimer cases (Fig. 3B and C), although less so than that observed in the neocortex. By contrast, the proportions of μ CANP isoforms were normal in the prefrontal cortex and cerebellum from patients with grade 3 Huntington disease (30) (Fig. 3A and C). Activation of μ CANP was increased 50% (P < 0.05) in samples of putamen from Huntington patients, where neuronal cell loss is considerable (30) (Fig. 3B).

DISCUSSION

These results, coupled with existing information on CANP, indicate that abnormally increased activity of μ CANP could be the molecular basis for the extensive synaptic degeneration and loss of neurons in Alzheimer disease. Activation of CANPs has been suggested to be a critical event underlying calcium-dependent degeneration in certain states, including the loss of axons in Wallerian degeneration (40), the resorption of synaptic terminals in neuromuscular junction development (41), and the death of hippocampal neurons by excitatory amino acids (16, 17). The relatively high vulnerability of neurons to μ CANP-mediated degeneration may be due partly to the relative enrichment of μ CANP in neurons (42, 43), including synaptic endings (42). Neurons at greatest risk for degeneration in Alzheimer disease are among those normally containing high levels of μ CANP (43). Alterations

FIG. 3. μ CANP isoform ratios (76/80 kDa) in brain regions from control (Cont), Alzheimer (AD), and Huntington (HD) patients measured by immunoassay (33). The number of cases is given in Table 1. (A) Prefrontal cortex: means $\pm$ SEM for postmortem interval (PMI) and age from control, AD, and HD cases were 12 $\pm$ 1.8 hr and 68.9 $\pm$ 2.6 yr, 12.2 $\pm$ 1.3 hr and 75.8 $\pm$ 2.0 yr, and 10.5 $\pm$ 1.9 hr and 54.6 $\pm$ 3.3 yr, respectively. (B) Putamen: means $\pm$ SEM of PMI and age were 10.6 $\pm$ 2.9 hr and 68.5 $\pm$ 1.9 yr, 14.8 $\pm$ 1.4 hr and 71.8 $\pm$ 2.3 yr, and 14.1 $\pm$ 2.0 hr and 60.1 $\pm$ 2.6 yr in control, AD, or HD patients. *, P < 0.05. (C) Cerebellum: means $\pm$ SEM of PMI and age were 7.6 $\pm$ 1.6 hr and 67.8 $\pm$ 4.2 yr, 13.1 $\pm$ 1.7 hr and 76.0 $\pm$ 1.9 yr, and 10.6 $\pm$ 2.1 hr and 54.6 $\pm$ 3.1 yr in control, AD, or HD patients, respectively. *, P < 0.05. ***, P < 0.005.

of μ CANP in glia accompanying gliosis, however, are not excluded by these data.

Additional evidence indicates that μ CANP activation in Alzheimer brain is not simply a consequence of end-stage neuronal degeneration but reflects a more widespread metabolic alteration that precedes as well as contributes to neuronal cell death. μ CANP activation was abnormally increased in the cerebellum and putamen—brain regions where few neurons have advanced to a stage of overt degeneration in Alzheimer disease, but where some cells display abnormal lysosome accumulations resembling those in “at-risk” populations of neurons in neocortex from the Alzheimer brain (39, 44). Moreover, the 3-fold increase in μ CANP activation in the prefrontal cortex of Alzheimer brains greatly exceeds the change that could be expected from the small proportion of neocortical neurons that exhibit the chromatolytic changes of end-stage degeneration. Finally, alteration of the CANP system within a broad population of neocortical neurons is suggested by preliminary immunocytochemical studies in which calpastatin, the endogenous inhibitor of CANPs, was found to be reduced in the majority of pyramidal neurons in the prefrontal cortex of Alzheimer brain (45). The latter finding suggests that the differential vulnerability of neuronal populations to increased μ CANP activation may depend on the cell's ability to maintain adequate levels of endogenous protease inhibitors or, possibly, to mount other protective responses.

Widespread μ CANP activation in Alzheimer disease is consistent with evidence for increased degradation of spectrin and diminished content of protein kinase C in fibroblasts from Alzheimer patients (9, 46), since both proteins are preferred substrates of CANPs (15–18, 20). Brain spectrin breakdown is also increased in cortex and hippocampus of the Alzheimer brain (47). Our results on total μ CANP levels are in accord with previous reports that CANP activity measured *in vitro* is not significantly altered in Alzheimer brain (48–50) but show further that measurements of the total CANP pool may not sensitively reflect the functional activity of the CANP system. The cause of the abnormal μ CANP activation is not known. μ CANP is activated directly by calcium, a process modulated by acidic phospholipids (51, 52) and possibly other cellular messengers (52). Evidence for altered calcium homeostasis in aging (4, 53) and in Alzheimer disease (4, 7–11) suggests increased intracellular calcium as one possible basis for μ CANP activation.

Apart from promoting the loss of synapses and eventual death of some neurons, abnormal μ CANP activation may have additional consequences in these cells and other neurons that are more resistant to degeneration. CANPs regulate events at the interface between the membrane and cytoskeleton by modifying specific membrane skeleton proteins and the cytoplasmic domains of certain transmembrane proteins by limited proteolysis (15, 16, 54–56). CANPs have been shown to selectively cleave regulatory domains from protein kinase C and calcium- and calmodulin-dependent protein kinase, thereby influencing protein phosphorylation (12–15). These actions regulate the assembly state and other dynamics of the proteins (15, 54) and may underlie the suspected involvement of CANPs in membrane fusion (57, 58) and trafficking (59). Abnormalities of the endosomal–lysosomal system are, in fact, an early marker of affected neurons in Alzheimer brain, and these abnormalities may alter APP processing (37, 44). A further link between CANP activity and APP metabolism is suggested by the observation that phorbol esters, which activate a signal transduction cascade involving CANP and protein kinase C (12–15), induce a phosphorylation-dependent increase in the secretion of APP derivatives in PC12 cells (19). In this regard, the degree of abnormal μ CANP activation found in the Alzheimer neocortex inversely correlates ($r = 0.87$; $P < 0.001$) with levels of

soluble APP in the brain (69). We speculate that decreased secretion of APP (60) and altered lysosomal/endosomal processing in Alzheimer brain (37, 44) could both be partial consequences of μ CANP activation. Finally, by altering the phosphorylation and proteolytic modification of cytoskeletal proteins, protracted low-level μ CANP activation might contribute to neurofibrillary tangle formation and altered synaptic efficacy (61).

Our findings are compatible with hypotheses of Alzheimer etiology that propose either primary disturbances of neuronal membranes or metabolic function (4, 5, 62) or a primary role of β -amyloid or amyloidogenic fragments (63). Metabolic and compositional changes in membranes of Alzheimer brain (62, 64–66) may reflect membrane dysfunction that could contribute to μ CANP abnormalities, given that μ CANP is activated principally on membranes (36). Also, the toxicity of β -amyloid or APP fragments may be exerted partly through altered calcium homeostasis (6, 67), which would be expected to lead to CANP activation.

Pharmacological modulation of the CANP system, which has been effective *in vivo* in limiting neural damage in ischemia and vasospasm (16–18, 68), may merit further consideration as a potential therapeutic strategy in Alzheimer disease.

We thank R. Neve, K. Kosik, and J. Coyle for manuscript review; L. Kanaley-Andrews and T. Wheelock for technical assistance; and J. Khan and D. McCarthy for assistance with manuscript preparation. This work was supported in part by National Institutes of Health Grants 5 R01 AG08278 and 2 P50 AG05134 and by the Mitsubishi Kasei Corporation.

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