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Aluminum inhibits neurofilament protein degradation by multiple cytoskeleton-associated proteases

Thomas B. Shea[a,b], Philip Balikian[a] and Mary Lou Beermann[a]

[a]*Laboratory for Cellular and Developmental Neurobiology, Laboratories for Molecular Neuroscience, Mailman Research Center, McLean Hospital, Belmont, MA 02178, USA* and [b]*Department of Psychiatry, Harvard Medical School, Boston, MA 02115, USA*

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The environmental neurotoxin aluminum exerts several distinct biochemical effects on neurofilament proteins, including subunit aggregation, disruption of the normal segregation of phosphorylated subunits within axons leading to abnormal perikaryal accumulation, and inhibition of in vitro degradation by the calcium-dependent neutral protease, calpain. In the present study, we demonstrate that exposure of mouse CNS cytoskeletal preparations to aluminum chloride inhibits the degradation of neurofilament proteins by both calcium-dependent and -independent proteases that co-purify with cytoskeletons. Aluminum inhibited both calcium-dependent and calcium-independent proteolysis of the high and middle molecular weight neurofilament subunits, but inhibited only calcium-dependent, and not calcium-independent proteolysis of the low molecular weight neurofilament subunit. These findings demonstrate that aluminum interferes with multiple aspects of neurofilament protein metabolism.

Aluminum; Neurofilament protein; Calpain; Proteolysis; Cytoskeleton; Neurotoxicity

1. INTRODUCTION

The environmental neurotoxin aluminum causes disordered whorls of neurofilaments to accumulate in perikarya in situ and in culture [1–9]. Since the expression of mRNA coding for neurofilament subunits is reduced during aluminum intoxication [10,11], these studies suggest that aluminum may disrupt postsynthetic events such as neurofilament protein turnover, posttranslational processing and axonal transport. This line of reasoning is supported by the ability of aluminum salts to induce the formation of aggregates of neurofilament proteins in vitro [12–14] which are relatively protease-resistant [13], and to induce the in vitro aggregation of homopolymers of the low (NF-L) molecular weight neurofilament subunit [15]. Aluminum-induced aggregation of the high (NF-H) and middle (NF-M) molecular weight subunits is dependent upon their phosphorylation state [14]. In this regard, increased addition of phosphate groups to neurofilament proteins have been observed in vivo following the administration of aluminum in drinking water [16]. Aluminum toxicity also results in the abnormal appearance within perikarya of phosphorylated neurofilament epitopes [2,17–20] in vivo. A selective impairment in the axonal transport of neurofilaments accompanies aluminum intoxication [20–22], which may reflect abnormalities of neurofilament subunit phosphorylation and neurofilament interactions, and may contribute to perikaryal neurofilament accumulation. These effects are likely to be exacerbated by the relative resistance of neurofilament subunit aggregates to degradation by calpains [13], calcium-activated neutral proteinases that are particularly active towards neurofilaments [13,23,24] and other cytoskeletal proteins [24–28] and mediate aspects of neurofilament metabolism in vivo [29–32].

In the present study, we demonstrate that aluminum interferes with neurofilament degradation not only by inhibiting an endogenous calcium-dependent protease (presumably calpain, as described above) but also by inhibiting a calcium-independent protease, both of which co-sediment with Triton-insoluble cytoskeletons.

Correspondence address: B. Shea, Mailman Research Center, McLean Hospital, Belmont, MA 02178, USA. Fax: (1) (617) 855 3299.

2. EXPERIMENTAL

2.1. *Incubation of cytoskeletons with aluminum*

Mouse CNS cytoskeletons isolated as described previously [13] were resuspended in 50 mM Tris-HCl (pH 7.4) containing either 5 mM ethylenediaminetetraacetic acid (EDTA) 5 mM ethyleneglycol-bis-(β-amino-ethyl ether) (EGTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml leupetin and 0.1% aprotinin (to inhibit proteolysis and monitor aluminum-induced aggregation of neurofilament proteins); 2 mM dithiothreitol and 5 mM $CaCl_2$ (to monitor calcium-dependent protease activity) or 2 mM dithiothreitol and 5 mM EDTA (to monitor calcium-independent protease activity), and incubated at 30°C for 0–60 min in the presence and absence of 1 mM $AlCl_3$. Incubations were terminated by adding ice-cold Laemmli treatment buffer [33] and immediate freezing (−20°C). Isolation and incubation of cytoskeletons was carried out on two separate occasions.

2.2. *Gel electrophoresis and immunoblotting*

The incubation mixture was boiled for 5 min and 10 μg aliquot of total cytoskeletal protein (determined before incubation) were electrophoresed on 6% polyacrylamide SDS gels [33]. Gels were stained with

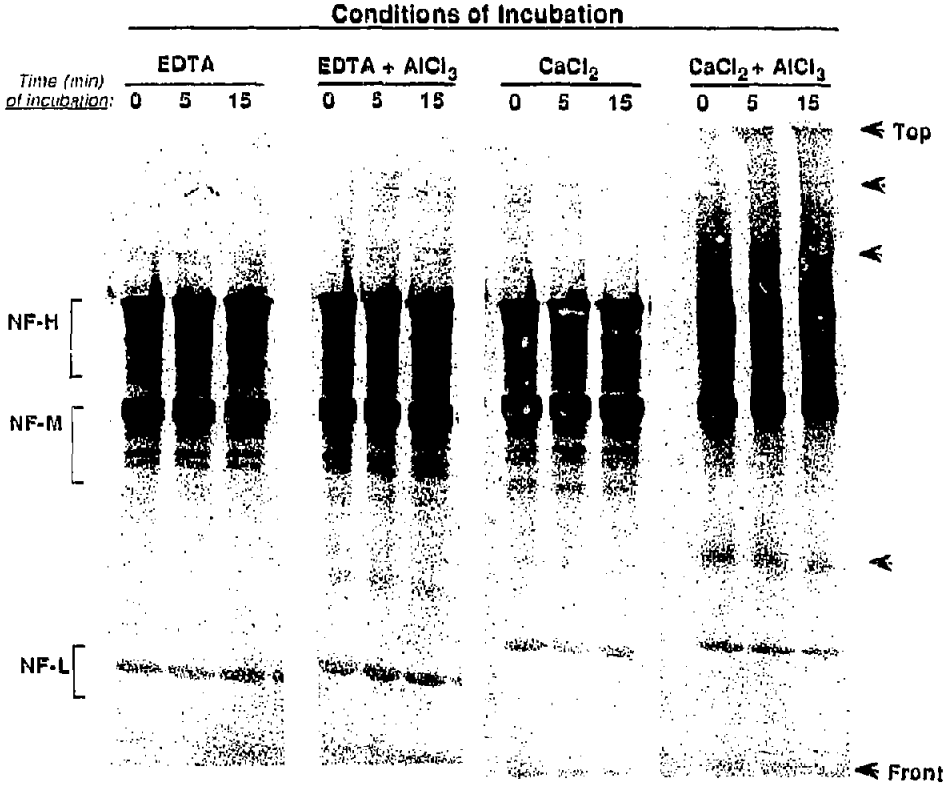

Fig. 1. Alteration in the electrophoretical properties of neurofilament proteins following exposure of cytoskeletal preparations to aluminum. Mouse CNS cytoskeletons were incubated in the presence of protease inhibitors under the indicated conditions for 0–60 min at 30°C, electrophoresed, transfered to nitrocellulose and immunostained with a cocktail of anti-neurofilament antisera as described in section 2. The migratory positions of the neurofilament subunits are indicated along the left side of the figure. The top and front of the electrophoretegram are indicated. The first 6 lanes are from the same gel and the second 6 are from a co-run gel, resulting in a slight difference in apparent electrophoretic mobility of neurofilament proteins between the first and last 6 lanes. Addition of aluminum to the incubation mixture in the presence of calcium results in a smear of immunoreactivity from the top of the replica to, and including, the NF-M region, as well as significant increases in immunoreactivity of several protein species (unlabeled arrows). These protein species were also detected, albeit in lesser amounts in samples incubated with aluminum and EDTA, but smearing of immunoreactivity in the high molecular weight region was not observed. Note also that smearing was induced immediately upon addition of aluminum to cytoskeletons, increased during the first 5 min of incubation, then did not increase following continued incubation.

either Coomassie brilliant blue or the separated proteins transferred to nitrocellulose [34]. Nitrocellulose replicas were immunostained with a polyclonal antiserum (H3) directed specifically against extensively phosphorylated (200 kDa) NF-H, a polyclonal antiserum (M2) directed against NF-M, a polyclonal antiserum directed against NF-L (L3) or a cocktail comprised of all three antisera; the specificity of these antisera has been previously demonstrated [14]. The replicas were then sequentially reacted with peroxidase-conjugated goat anti-mouse antibody and diaminobenzidine in the presence of H_2O_2 as described [35]. The stacking gel was routinely left attached to the separating gel during transfer to nitrocellulose. At least 3 gels and corresponding replicas were generated for each subunit.

2.3. *Densitometric analysis of neurofilament proteolysis*

Nitrocellulose replicas were scanned with a Bio-Rad Model 620 Video Densitometer and Computer system equipped with the appropriate analytical software, and the total peak area [defined as optical density (O.D.) × mm − background O.D. levels] determined. Incubation of cytoskeletons [12,13] and purified neurofilament subunits [14] in vitro results in the rapid formation of high-molecular-weight complexes of neurofilament subunits. This effect is most pronounced during the initial 5 min of in vitro incubation of cytoskeletons with aluminum, and, as shown in the present study (see Fig. 1) is far more substantial in the presence than in the absence of calcium. Therefore, to reduce the potential interference of this aluminum-induced depletion of neurofilament protein immunoreactivity from interfering with analyses of subunit proteolysis in the presence and absence of aluminum and calcium, the first 5 min of incubation under all conditions were excluded from quantative analyses. The time course of subunit proteolysis was then determined by the following formula: (mean peak area obtained following 15, 30, or 60 min incubations / mean peak area obtained following 5 min incubation) × 100. All time points for individual conditions were always derived from the same replica, and multiple replicas were analyzed for each neurofilament subunit.

3. RESULTS AND DISCUSSION

Aluminum induces aggregation of neurofilament subunits [12–14] and inhibits their degradation by exogenously-added calpain [13], both of which reduce the amount of normally-migrating neurofilament subunit immunoreactivity. Therefore, in order to distinguish between aggregation and proteolysis during aluminum treatment, we incubated cytoskeletal preparations with and without protease inhibitors. Mouse CNS cytoskeletons were incubated with aluminum chloride in the presence of protease inhibitors under the indicated condi-

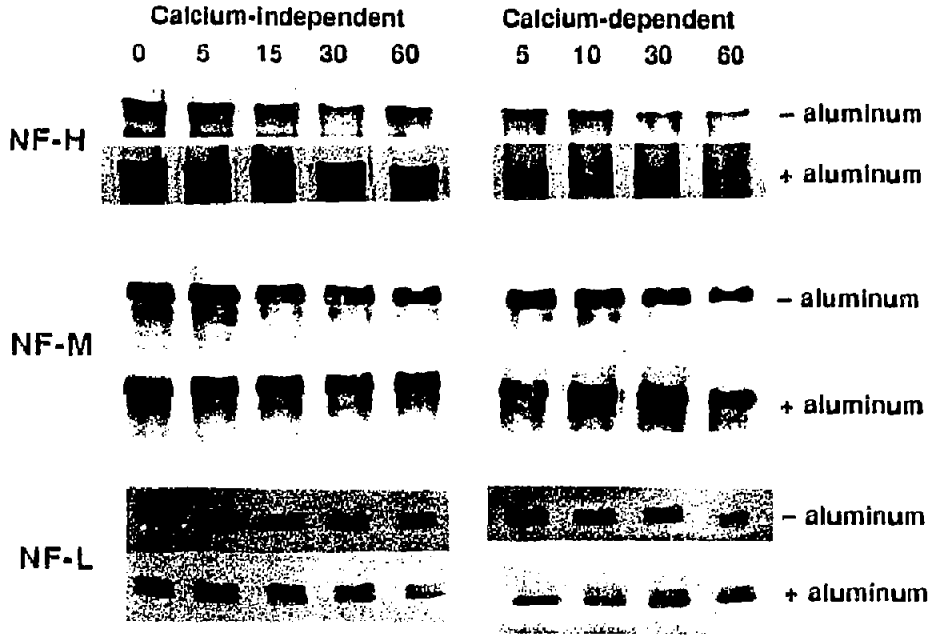

Fig. 2. Influence of aluminum on calcium-dependent and -independent proteolysis of neurofilaments by cytoskeleton-associated proteases. Mouse CNS cytoskeletons were subjected to degradation by endogenous calcium-dependent and -independent proteases in the presence and absence of aluminum as described in the Experimental section, after which samples were subjected to SDS gel electrophoresis and transfer to nitrocellulose. Relevent portions of nitrocellulose replicas are shown: replicas labeled 'NF-H' depict the 200 kDa region following reaction with H3 antibody, those labeled 'NF-M' depict the 145 kDa region following reaction with M2 antibody, and those labeled 'NF-L' depict the 70 kDa region following reaction with L3 antibody. Note that exposure to aluminum inhibits calcium-dependent and independent proteolysis of NF-H and NF-M, but only inhibits calcium-dependent proteolysis of NF-L.

tions for 0–60 min at 30°C, electrophoresed, transfered to nitrocellulose and immunostained with a cocktail of anti-neurofilament antisera as described in section 2. As previously demonstrated [12–14], addition of aluminum to the incubation mixture results in a smear of immunoreactivity from the top of the replica to, and including, the NF-M region, as well as significant increases in immunoreactivity of several protein species (unlabeled arrows). These protein species were also detected, albeit in substantially lesser amounts in samples, incubated with aluminum and EDTA; however, smearing of immunoreactivity in the high molecular weight region was not observed. Note also that smearing was induced immediately upon addition of aluminum to cytoskeletons, increased during the first 5 min of incubation, then did not exhibit a further increase by 15 min (Fig. 1) to 60 min (not shown). Whether the differential effect of aluminum on neurofilament protein electrophoretic migration in the presence of calcium or EDTA is a function of the presence of calcium, or a result of chelation of aluminum by EDTA is beyond the scope of this study and was therefore not examined.

Immunoblot analysis of neurofilament proteins in Triton-insoluble cytoskeletons incubated in the absence of protease inhibitors revealed that cytoskeletons contained both calcium-dependent and -independent protease activities active against all three neurofilament proteins (Fig. 2 and 3), and that these activities were differentially affected by exposure to aluminum. Both calcium-dependent and -independent proteolysis of NF-H and NF-M were substantially inhibited by aluminum; by contrast, only calcium-dependent, and not calcium-independent proteotysis of NF-L was inhibited (Figs. 2 and 3).

The calcium-dependent protease activity is likely to include, but may not be limited to, calpain [13]. The identity of the calcium-independent protease is unknown at this time; however, a PMSF-sensitive neuronal protease, active at physiological pH in the absence of calcium (termed calcium-independent neutral protease, or CINP) has been previously noted [36]. Also undetermined is the nature of association of these proteases with the cytoskeleton; the recovery of these protease activities in Triton-insoluble pellets should not be interpreted to indicate their exclusive or even enriched localization within cytoskeletons. Indeed, co-sedimentation of these activites with cytoskeletons may only reflect a transient metabolic association of protease with their cytoskeletal substrates.

The relative activity of these classes of proteases in these in vitro assays cannot be interpreted as an accurate index of their relative activity in situ. Incubation of cytoskeletons in the presence of calcium should result in neurofilament degradation by both calcium-dependent and -independent proteases; however, despite the degradation of NF-L by calcium-independent protease

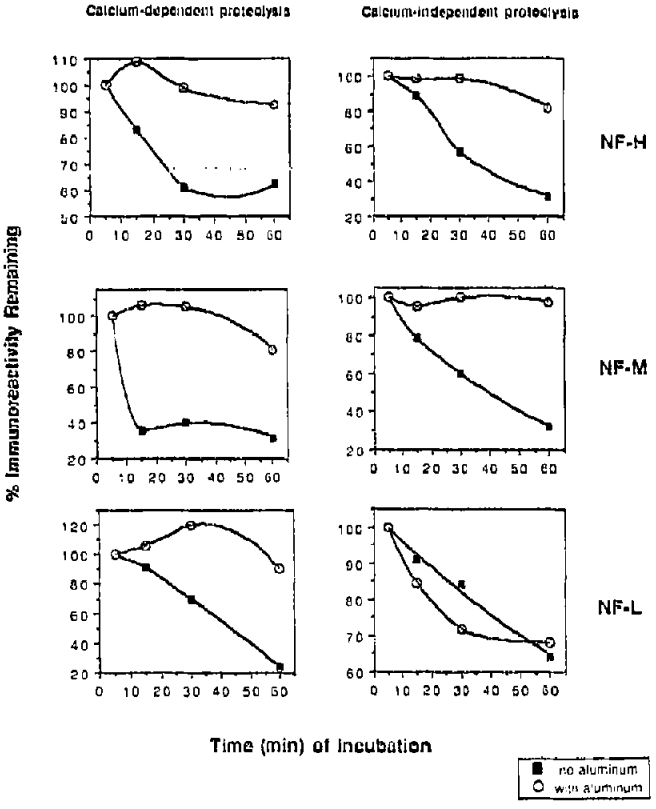

Fig. 3. Quantitation of neurofilament protein degradation by cytoskeleton-associated calcium-dependent and -independent proteolysis of neurofilaments by proteases in the presence and absence of aluminum. Nitrocellulose replicas were scanned with a Bio-Rad Model 620 Video Densitometer and analysed as described in section 2. Values represent the percentage of each subunit remaining from 15–60 min as compared with that present at 5 min under each condition of incubation determined as described in section 2. Note that exposure to aluminum inhibits calcium-dependent and independent proteolysis of NF-H and NF-M, but only inhibits calcium-dependent proteolysis of NF-L.

activity, this activity was not observed during incubation with calcium. One possible explanation for this phenomenon is that binding of calcium by neurofilaments [37], induces a conformational change [38] which may render NF-L less susceptible to degradation by the calcium-independent protease. Nevertheless, these findings indicate that aluminum interferes with neurofilament protein degradation by multiple protease systems, and lend additional support to the hypothesis [13] that deficiencies in proteolysis contribute to the abnormal accumulation of neurofilament epitopes in aluminum neurotoxicity [1–9].

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