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Anticonvulsants Attenuate Amyloid β-Peptide Neurotoxicity, Ca^{2+} Deregulation, and Cytoskeletal Pathology

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MARK, R. J., J. W. ASHFORD, Y. GOODMAN AND M. P. MATTSON. *Anticonvulsants attenuate amyloid β-peptide neurotoxicity, Ca^{2+} deregulation, and cytoskeletal alterations.* NEUROBIOL AGING **16**(2) 187–198, 1995.—Increasing evidence supports the involvement of amyloid β-peptide (Aβ) and an excitotoxic mechanism of neuronal injury in the pathogenesis of Alzheimer's disease. However, approaches aimed at preventing Aβ toxicity and neurofibrillary degeneration are undeveloped. We now report that anticonvulsants (carbamazepine, phenytoin, and valproic acid) can protect cultured rat hippocampal neurons against Aβ- and glutamate-induced injury. Each of the anticonvulsants attenuated the elevation of intracellular free calcium levels [$(Ca^{2+})_i$] elicited by Aβ or glutamate suggesting that their neuroprotective mechanism of action involved stabilization of $[Ca^{2+}]_i$. These compounds were effective at clinically relevant concentrations (carbamazepine, 100 nM–10 μM; phenytoin, 100 nM–1 μM; valproic acid, 100 nM–100 μM). The anticonvulsants suppressed glutamate-induced alterations in tau and ubiquitin immunoreactivities. Compounds that stabilize $[Ca^{2+}]_i$ may afford protection against the kinds of insults believed to underlie neuronal injury in Alzheimer's disease.

Alzheimer's disease Anticonvulsant Calcium Carbamazepine Fura-2 Glutamate Hippocampus
Microtubule-associated protein Neurofibrillary tangle Phenytoin Phosphorylation Tau Valproic acid

ALZHEIMER'S disease (AD), which is the leading cause of dementia in the elderly, is characterized histopathologically by the accumulation of amyloid plaques and neurofibrillary tangles in vulnerable brain regions such as the hippocampus. Neurofibrillary tangles (NFTs) consist of abnormal accumulations of cytoskeletal and other proteins, and plaques contain amyloid β-peptide (Aβ) (see refs. 60 & 73 for reviews). Major components of NFTs are the microtubule-associated protein tau, and ubiquitin, a "heat-shock" protein involved in targeting proteins for proteolytic degradation (28,39,59,64). Post-translational alterations in tau such as phosphorylation (28) and dissociation of tau from microtubules (50) may promote the assembly of tau into the abnormal straight and paired helical filaments that characterize NFTs. Although the mechanism leading to neurofibrillary degeneration is not clear, several observations suggest a role for dysregulation of neuronal calcium homeostasis with resultant elevations of $[Ca^{2+}]_i$ (see ref. 54 for review). Among the data supporting this hypothesis are: experimentally induced elevations of $[Ca^{2+}]_i$ in hippocampal neurons (in vitro and in vivo) can elicit antigenic, biochemical, and ultrastructural changes in cytoskeletal proteins (tau, MAP2, and spectrin) similar to those seen in NFTs (15,19,22,48,49,71); neurons vulnerable to neurofibrillary degeneration bear high levels of glutamate receptors (27); Aβ can be neurotoxic (53,65,66,87) and can render neurons vulnerable to excitotoxicity (38,52,53) by a mechanism that involves free radical accumulation and destabilization of $[Ca^{2+}]_i$ homeostasis (33,52,53). In addition, recent findings indicate that mitogen-activated protein (MAP) kinases (21) and cell cycle-dependent kinases (3) can phosphorylate tau in a manner very similar to that observed in the paired helical filaments of AD, although it is not clear that these particular kinases are causally involved in the formation of the abnormal filaments. Interestingly, MAP kinases are known to be activated glutamate and elevation of $[Ca^{2+}]_i$ (2).

The Aβ that accumulates in selectively vulnerable brain regions in AD is a 40–42 amino acid peptide derived from a larger (695–770 amino acid) β-amyloid precursor protein (βAPP) which is believed to be a transmembrane glycoprotein (see refs.

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60 & 73 for review). The major metabolic pathway for βAPP metabolism involves an enzymatic cleavage within the Aβ sequence and obviates deposition of amyloidogenic Aβ. On the other hand, Aβ is released from brain cells at low levels and is present in the cerebrospinal fluid at nanomolar concentrations (30,74,77) indicating an alternative processing pathway of βAPP. A cleavage of βAPP at the *N*-terminus of Aβ leaves behind a *C*-terminal fragment of βAPP that contains potentially amyloidogenic Aβ (75). Some cases of inherited AD have been linked to mutations in βAPP (14,25,60,61) that may alter processing of βAPP in a way that leads to increased production of Aβ (13,17). The link between altered metabolism of βAPP and neuronal injury in AD is supported by studies showing that synthetic Aβ peptides can be directly neurotoxic to primary cultures of hippocampal and cortical neurons (53,65,66,87), and can render neurons vulnerable to glutamate excitotoxicity (38,52), glucose deprivation (18), and oxidative injury (26). Antioxidants can protect cultured PC12 cells (4) and hippocampal neurons (26) against Aβ toxicity indicating a role for free radicals in the mechanism of Aβ toxicity. Interestingly, recent data indicate that Aβ itself may form free radical peptides (33) which may induce membrane lipid peroxidation (11,26). Data indicate that the neurotoxicity of Aβ is related to its ability to form aggregates (53,66), which accumulate at plasma membranes and disrupt cellular calcium homeostasis (53). Aβ toxicity is apparently mediated, in part, by calcium influx because removal of extracellular calcium (53) and blockers of voltage-dependent calcium channels (85) can protect cultured neurons against Aβ toxicity. The mechanism whereby Aβ disrupts calcium regulation at the plasma membrane may involve the peptide forming calcium-conducting pores (1), alterations in K^+ channels (23), activation of tachykinin receptors (36), inhibition of redox activity (76), or free radical-mediated damage to calcium-regulating proteins in the plasma membrane (43).

If loss of neuronal calcium homeostasis is involved in the pathogenesis of AD (35,51), then strategies to prevent neuronal degeneration in AD should include agents that prevent or attenuate elevations of $[Ca^{2+}]_i$. The latter might be accomplished using compounds which are known to reduce neuronal excitability. In the present study, we examined the neuroprotective potential of anticonvulsant compounds that are in clinical use for the treatment of epileptic seizures. The general mode of action of these compounds is to reduce neuronal excitability and calcium influx (20,42). We determined whether the anticonvulsants would protect cultured rat hippocampal neurons against Aβ toxicity and excitotoxicity. In addition, the effects of these compounds on Aβ- and glutamate-induced elevation of $[Ca^{2+}]_i$ was established using the $[Ca^{2+}]_i$ indicator dye fura-2. These in vitro data suggest that anticonvulsants can protect neurons against insults believed relevant to the pathogenesis of AD.

METHOD

Cell Culture

Primary hippocampal cell cultures were established from embryonic (Day 18 of gestation; GD) rats using established procedures (44,55). Cells were plated into polyethyleneimine-coated plastic and glass bottom 35 mm^2 culture dishes at a density of 70–120/mm^2 of culture surface. The culture maintenance medium (0.8 ml/culture dish) consisted of Eagle's Minimum Essential Medium supplemented with heat-inactivated fetal bovine serum (10%, v/v), 20 mM KCl, and 1 mM pyruvate. The atmosphere consisted of 6% CO_2/94% room air and was maintained near saturation with H_2O. Experiments were performed in cultures that had been maintained for 6–10 days.

Experimental Treatments and Analysis of Cell Survival

Cell survival studies were conducted without changing the culture maintenance medium. Glutamate (Sigma) was prepared as ×100–500 stocks in culture medium (pH 7.2). Aβ1–40 (lot #ZK600; a generous gift from Athena Neurosciences, Inc.) and Aβ25–35 (Bachem) were prepared as 1 mM stocks in H_2O immediately prior to addition to cultures. Valproic acid, phenytoin, and carbamazepine (Sigma), were each prepared as ×1000 stocks in dimethylsulfoxide (vehicle was added to control cultures). D-Glutamylglycine (Sigma) was prepared as a ×100 stock in saline. Cell survival was quantified by counting viable neurons in premarked microscope fields prior to, and at indicated time points following exposure to experimental treatments as described previously (46,55). This method correlates well with independent measures of cell viability including trypan blue staining, reduction of MTT, and Alamar blue (55). Statistical comparisons utilized ANOVA with Sheffé's post hoc test for pairwise comparisons.

Measurement of Intracellular Free Calcium Levels

Ratiometric imaging of the calcium indicator dye fura-2 was performed by methods detailed in our previous reports (46,53,55). Cells were incubated for 30 min in the presence of 6–8 μM of the acetyoxymethylester form of fura-2, followed by 2 washes in fresh culture medium, and a subsequent incubation of at least 30 min prior to imaging. Immediately prior to imaging the culture medium was replaced with Hank's Balanced Saline Solution supplemented with 10 mM glucose and buffered with 10 mM Hepes (pH 7.2). Cells were imaged on the stage of a Nikon Diaphot inverted microscope using a Nikon Fluoro 40×, N.A. 1.3 objective. Images were acquired and processed using an intensified CCD camera and a Quantex Imaging system with QFM software (Quantex Corp., Sunnyvale, CA). The ratio of the fluorescence emission using two different excitation wavelengths (350 nm and 380 nm) was used (following background subtraction of the individual wavelength images) to calculate $[Ca^{2+}]_i$ using the method of Grynkiewicz et al. (29). Calibration parameters for our system were: Rmin = 0.80; Rmax = 10.40; Fo/Fs = 13.99; K_d = 224.0. Values represent the average level of $[Ca^{2+}]_i$ in the neuronal cell body. Statistical comparisons used analysis of variance (ANOVA) with Sheffé's post hoc test for pairwise comparisons.

Immunocytochemistry

The procedures for immunostaining cultured hippocampal neurons with tau and ubiquitin antibodies were the same as in our previous studies (48). Cells were fixed for 30 min in cold 4% paraformaldehyde dissolved in PBS. Cells were processed using the appropriate mouse or rabbit vectastain AβC kit (Vector Laboratories) as described (48). The primary antibodies included: Alz-50 (1:10 dilution), a mouse monoclonal antibody raised against AD brain homogenate (86) that recognizes an altered form of tau (41,83); MAP-2 (1:1000 dilution) a mouse monoclonal antibody (clone AP-20, Sigma; ref. 7); and polyclonal ubiquitin antiserum (1:1000) raised in rabbit (Sigma). Photographic negatives and prints were prepared using identical exposure conditions to allow comparisons of staining among the different experimental treatment groups.

RESULTS

Anticonvulsants Protect Against Aβ Neurotoxicity and Attenuate Aβ-Induced Elevation of $[Ca^{2+}]_i$

We previously characterized the neurotoxic properties of Aβ1-40 in hippocampal cell cultures (53), and in preliminary studies characterized the neurotoxicity of the particular lot of Aβ1-40 used in the present study. This peptide aggregated rapidly and therefore did not require preaggregation to elicit toxicity during exposure periods of several days (cf. 53,65). Exposure of hippocampal cultures to 10–40 μM Aβ resulted in a dose-dependent reduction in neuronal survival during a 4-day exposure period (Fig. 1A). Co-treatment with 1 μM valproic acid significantly enhanced neuronal survival in cultures exposed to Aβ. Phenytoin and carbamazepine also protected against Aβ neurotoxicity (Fig. 1B). A window of concentrations of each anticonvulsant afforded protection against Aβ toxicity: carbamazepine, 100 nM to 1 μM; phenytoin, 100 nM to 1 μM; valproic acid, 100 nM to 10 μM. Higher concentrations of each anticonvulsant were neurotoxic (Fig. 2A).

In accordance with previous findings (31,52,53), exposure of hippocampal cultures to Aβ resulted in elevated levels of $[Ca^{2+}]_i$. After 20 h of exposure to Aβ the rest $[Ca^{2+}]_i$ was approximately 486 ± 129 nM compared to 152 ± 21 nM in control cultures not exposed to Aβ (Fig. 3A). This Aβ-induced elevation of $[Ca^{2+}]_i$ occurred prior to cell loss that was first evident 48 h following exposure to Aβ. Exposure of cultures to 1 μM valproic acid for 24 h resulted in a modest but significant 15%–20% reduction in rest $[Ca^{2+}]_i$ (Fig. 2B). The rest neuronal $[Ca^{2+}]_i$ in cultures co-treated with 1 μM valproic acid plus Aβ was significantly less than in cultures exposed to Aβ alone (Fig. 3A). The $[Ca^{2+}]_i$ response to glutamate was also attenuated in cultures co-treated with valproic acid plus Aβ (Fig. 3A). Phenytoin (10 μM) and carbamazepine (1 μM) also caused a modest reduction of rest $[Ca^{2+}]_i$ during a 24-h exposure period (Fig. 2B) and attenuated the elevation of $[Ca^{2+}]_i$ induced by Aβ (Fig. 3B).

Anticonvulsants Protect Hippocampal Neurons Against Glutamate Toxicity and Attenuate $[Ca^{2+}]_i$ Responses to Glutamate

Exposure of hippocampal cultures to glutamate resulted in a progressive reduction in neuronal survival during a 24-h exposure period (Fig. 4A). Excitotoxic neuronal injury was characterized by neurite fragmentation, and swelling and vacuolation of the cell body (Fig. 5). Valproic acid (100 nM) protected against excitotoxicity across a range of glutamate concentrations (10–100 μM) (Fig. 4B). A significant enhancement of neuronal survival in cultures exposed to glutamate was observed with valproic acid concentrations from 10 nM to 10 μM with 1 μM valproic acid providing maximum protection. As previously reported (45), phenytoin (1–100 μM) and carbamazepine (100 nM to 10 μM) also afforded significant protection against glutamate neurotoxicity in hippocampal cell cultures (data not shown).

It was previously reported that excitotoxic neuronal injury is exacerbated in cultured rat neocortical (38), human neocortical (52), and rat hippocampal (53) neurons pretreated with Aβ. Because valproic acid attenuated the toxicities of both Aβ and glutamate, we determined whether blockade of glutamate receptors would afford similar protection against Aβ toxicity. The broad-spectrum glutamate receptor antagonist D-glutamylglycine (DGG) significantly increased neuronal survival in cultures exposed to Aβ (Fig. 6). Whereas approximately 50% of the neurons were killed in cultures exposed to Aβ alone, less than 30% were killed in cultures exposed to DGG plus Aβ, and less than 20% were killed in cultures exposed to valproic acid plus Aβ. Taken together, the data indicate that a significant component of Aβ toxicity involves glutamate receptor activation which can be blocked by anticonvulsants.

Previous studies indicated that glutamate toxicity is mediated by Ca^{2+} influx through NMDA receptor channels and voltage-

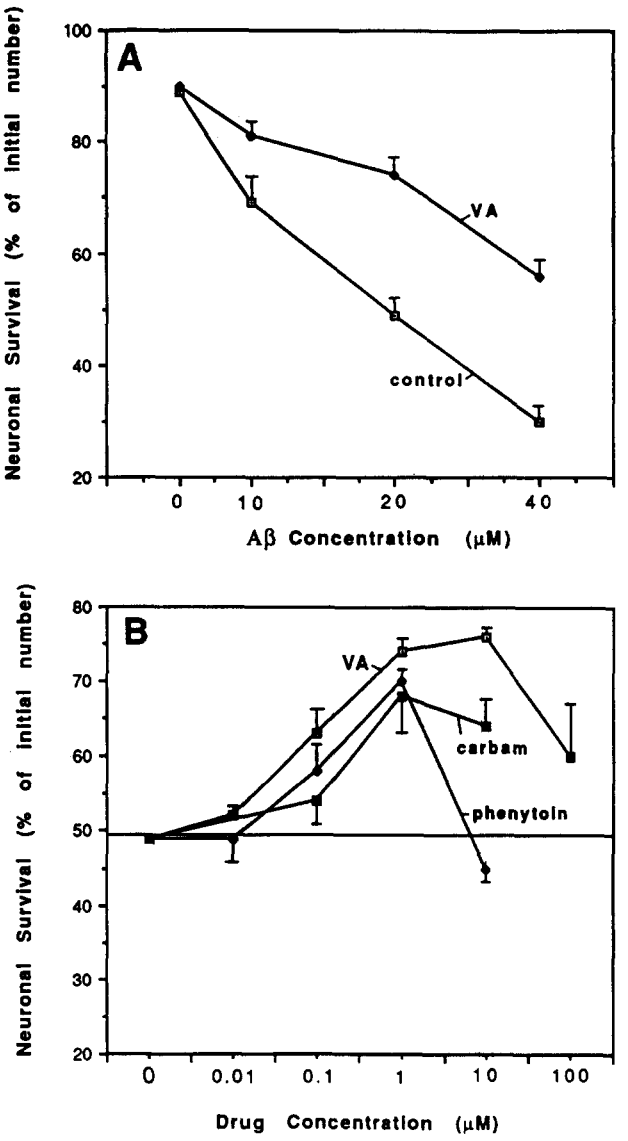

FIG. 1. Anticonvulsants protect neurons against Aβ toxicity. (A) Cultures were exposed to the indicated concentrations of Aβ1–40 alone (control) or in combination with 1 μM valproic acid (VA), and neuronal survival was assessed 4 d later. Values represent the mean and SEM of determinations made in four separate cultures. Values for valproic acid-treated cultures exposed to 20 and 40 μM Aβ were significantly greater than corresponding values in control Aβ-treated cultures ($p < 0.01$). (B) Cultures were exposed the indicated concentrations of anticonvulsant plus Aβ (20 μM) and neuronal survival was assessed 4 d later. Value for 0 drug concentration is survival in cultures exposed to Aβ alone (approximately 50% survival); survival in untreated control cultures was 92 ± 4.1%. Values represent the mean and SEM of determinations made in four separate cultures.

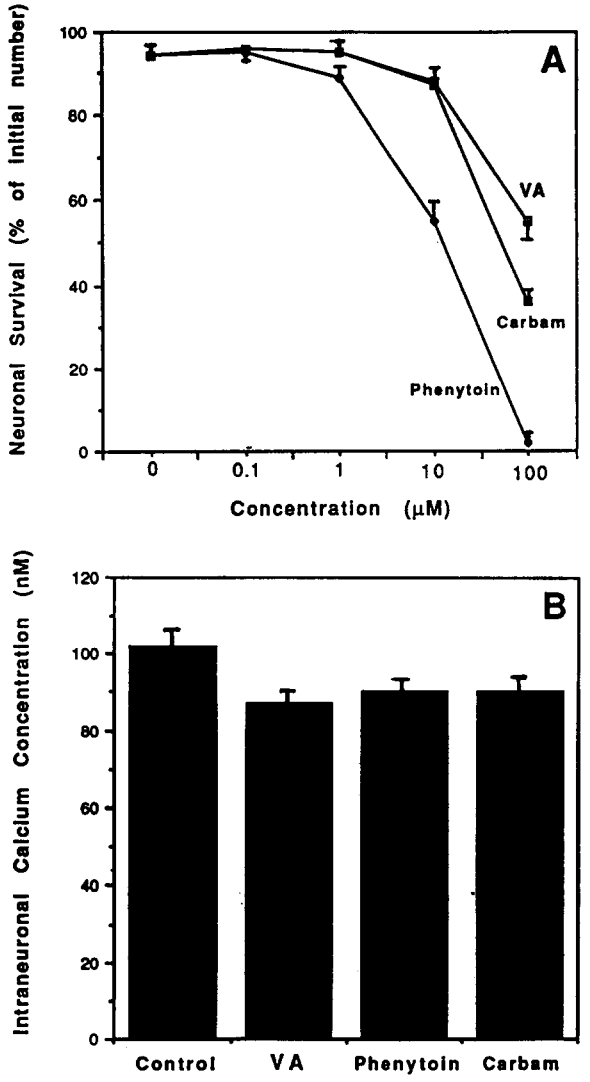

FIG. 2. Effects of anticonvulsants on neuronal survival and rest $[Ca^{2+}]_i$ in hippocampal cell cultures. (A) Cultures were exposed to the indicated concentrations of valproic acid (VA), phenytoin, or carbamazepine (Carbam) and neuronal survival was assessed 48 h later. Values represent the mean and SEM of determinations made in four separate cultures. (B) Cultures were left untreated (Control) or were exposed to 1 μM valproic acid (VA), phenytoin, or carbamazepine (Carbam) for 24 h. Rest $[Ca^{2+}]_i$ was then determined. Values represent the mean and SEM of determinations made in 16–21 neurons.

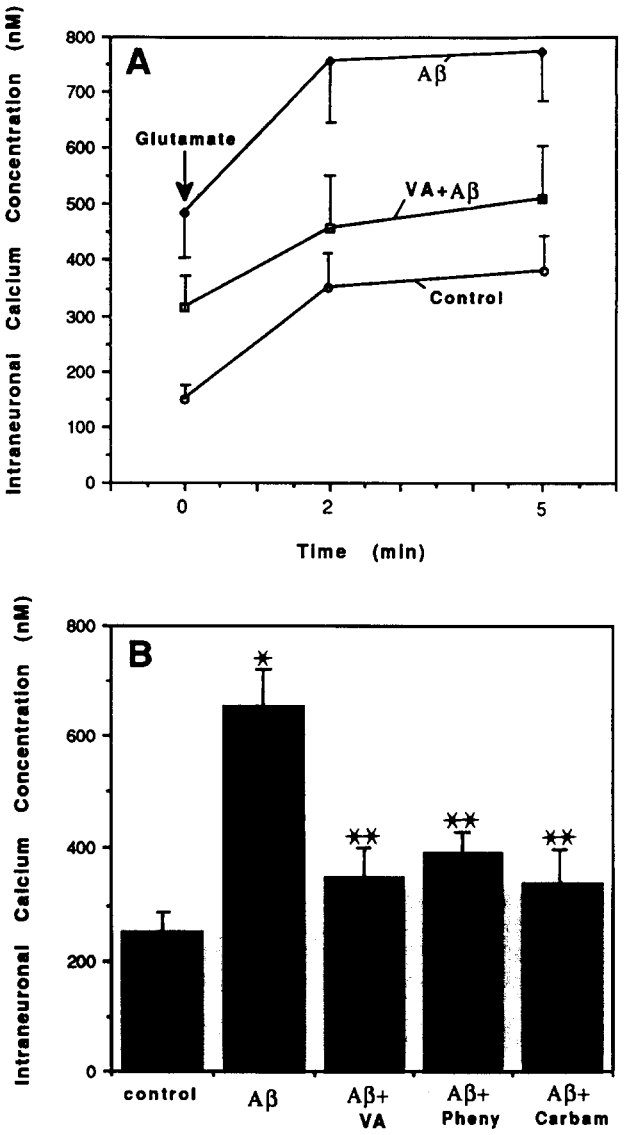

FIG. 3. Anticonvulsants attenuate Aβ-induced elevation of $[Ca^{2+}]_i$. (A) Cultures were exposed for 20 h to Aβ1-40 (20 μM) or valproic acid (1 μM) plus Aβ. The $[Ca^{2+}]_i$ was then determined prior to, and 2 and 5 min following exposure to 100 μM glutamate. Values represent the mean and SEM ($n = 12$). (B) Neuronal $[Ca^{2+}]_i$ was determined 24 h following exposure to 0.1% dimethylsulfoxide (control), 20 μM Aβ1-40, or Aβ plus valproic acid (1 μM), phenytoin (1 μM) or carbamazepine (1 μM). Values represent the mean and SEM ($n = 12$–16). *$p < 0.01$ compared to control. **$p < 0.01$ compared to Aβ value.

dependent calcium channels resulting in a prolonged elevation of $[Ca^{2+}]_i$ (16,44,45,46). It was therefore of interest to determine whether anticonvulsants would modify $[Ca^{2+}]_i$ responses to glutamate. The rest $[Ca^{2+}]_i$ in cultured hippocampal neurons was approximately 140 nM. Exposure of the neurons to 200 μM glutamate resulted in a rapid and sustained elevation of $[Ca^{2+}]_i$, with the $[Ca^{2+}]_i$ reaching 380 nM at 7 min following addition of glutamate (Fig. 7A). Pretreatment with 1 μM valproic acid caused a small (10–20 nM) reduction in rest $[Ca^{2+}]_i$, and markedly attenuated the $[Ca^{2+}]_i$ response to glutamate. The $[Ca^{2+}]_i$ rose only 30–50 nM following exposure to 200 μM glutamate in valproic acid-treated cultures. Glutamate-induced elevation of $[Ca^{2+}]_i$ was also significantly attenuated in neurons pretreated with 1 μM phenytoin or carbamazepine (Fig. 7B).

Anticonvulsants Attenuate Glutamate-Induced Alterations in Tau and Ubiquitin Immunoreactivities

Prolonged elevations of $[Ca^{2+}]_i$, such as those that result from exposure to glutamate or Aβ, have been shown to result in antigenic changes in the microtubule associated protein tau and increased levels of the heat shock protein ubiquitin (48,52).

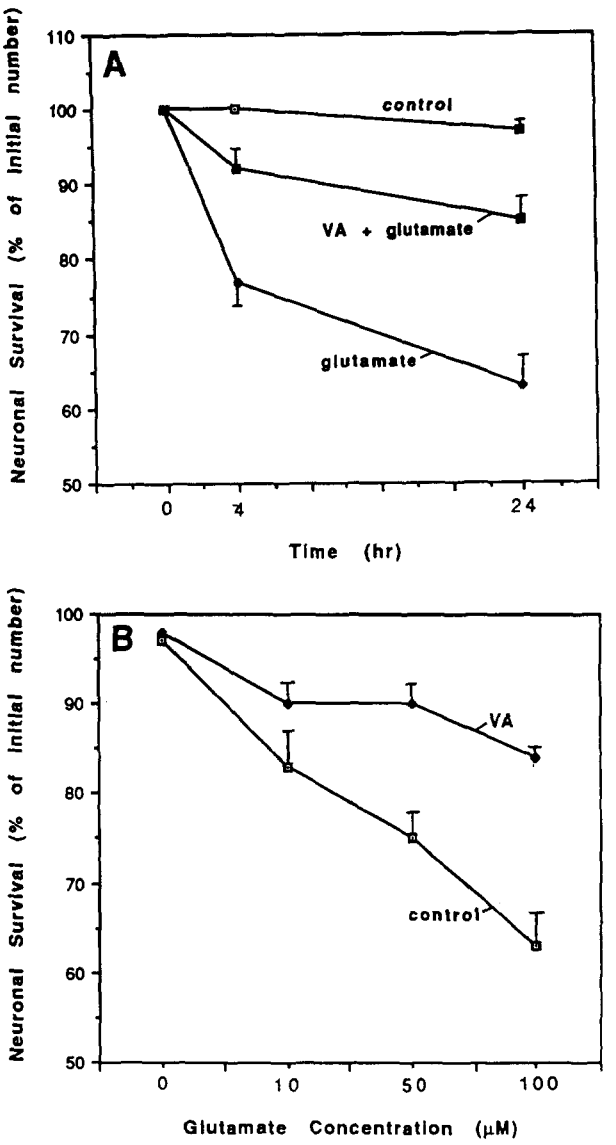

FIG. 4. Valproic acid protects hippocampal neurons against glutamate-induced injury. (A) Time course of glutamate toxicity and protection by valproic acid (VA). Cultures were exposed to saline (control), glutamate (100 μM), or valproic acid (100 nM) plus glutamate (100 μM). Neuronal survival was assessed at 4 and 24 h post-treatment. Values represent the mean and SEM of determinations made in four separate cultures Values for cultures exposed to valproic acid plus glutamate were significantly greater than values for cultures exposed to glutamate alone at the 4 h ($p < 0.01$) and 24 h ($p < 0.01$) time points. (B) Cultures were exposed to the indicated concentrations of glutamate in the presence or absence of valproic acid (100 nM), and neuronal survival was assessed 24 h later. Values represent the mean and SEM of determinations made in four separate cultures. Values for cultures exposed to valproic acid were significantly greater than control cultures at concentrations of glutamate of 50 μM ($p < 0.01$) and 100 μM ($p < 0.005$).

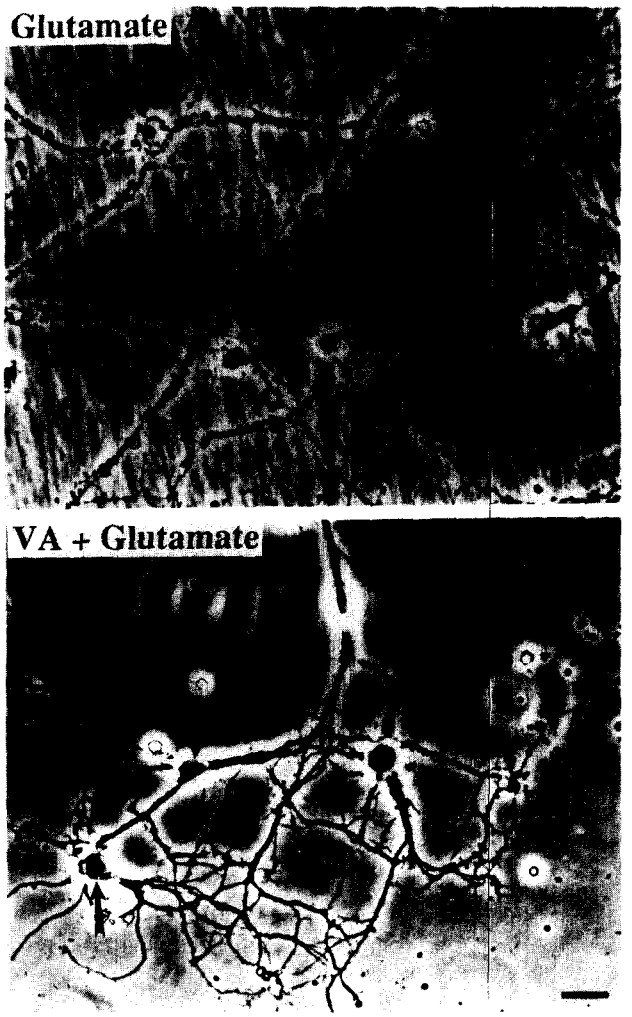

FIG. 5. Glutamate-induced neuronal injury is attenuated by valproic acid. Phase-contrast micrographs of cultured hippocampal cells 20 h following exposure to 100 μM glutamate alone or to the combination of 1 μM valproic acid (VA) plus 100 μM glutamate (valproic acid was added 15 min prior to addition of glutamate). Note marked degeneration of cell bodies (arrows) and neurites in glutamate-treated cultures. Scale bar, 10 μm.

In the present study, glutamate caused a marked increase in neuronal immunoreactivity with antibody Alz-50 which recognizes an altered form of tau in NFTs (32). The increased Alz-50 staining was particularly obvious in the cell body and axons (Fig. 8). The glutamate-induced increase in Alz-50 immunoreactivity was suppressed by anticonvulsants (e.g., Fig. 8). Glutamate caused a marked increase in ubiquitin immunoreactivity in neurons, which was suppressed in cultures cotreated with valproic acid (Fig. 9), or phenytoin and carbamazepine (not shown). MAP-2 is a microtubule-associated protein that is particularly sensitive to calcium-mediated proteolysis (34,78). In the present study, glutamate caused a reduction in MAP-2 immunoreactivity in the dendrites and cell body of cultured hippocampal neurons (Fig. 10). Valproic acid (Fig. 10) and the other anticonvulsants (data not shown) largely prevented the glutamate-induced reduction of MAP-2 immunoreactivity.

DISCUSSION

The present data demonstrate that anticonvulsants can protect hippocampal neurons against insults believed to be involved

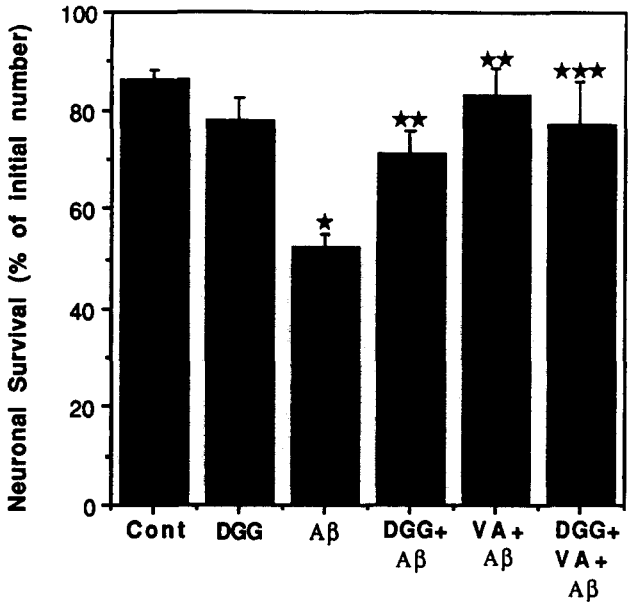

FIG. 6. A glutamate receptor antagonist and valproic acid protect against Aβ toxicity. Cultures were exposed to vehicle (control), 1 mM D-glutamylglycine (DGG), 50 μM Aβ25-35 (Aβ), 1 mM DGG plus Aβ, 1 μ M valproic acid (VA) plus Aβ, or DGG plus VA plus Aβ. Neuronal survival was quantified 3 d following treatment. Values are the mean and SEM ($n = 4$). *$p < 0.001$ compared to control value. **$p < 0.01$ compared to value for Aβ alone. ***$p < 0.05$ compared to value for Aβ alone.

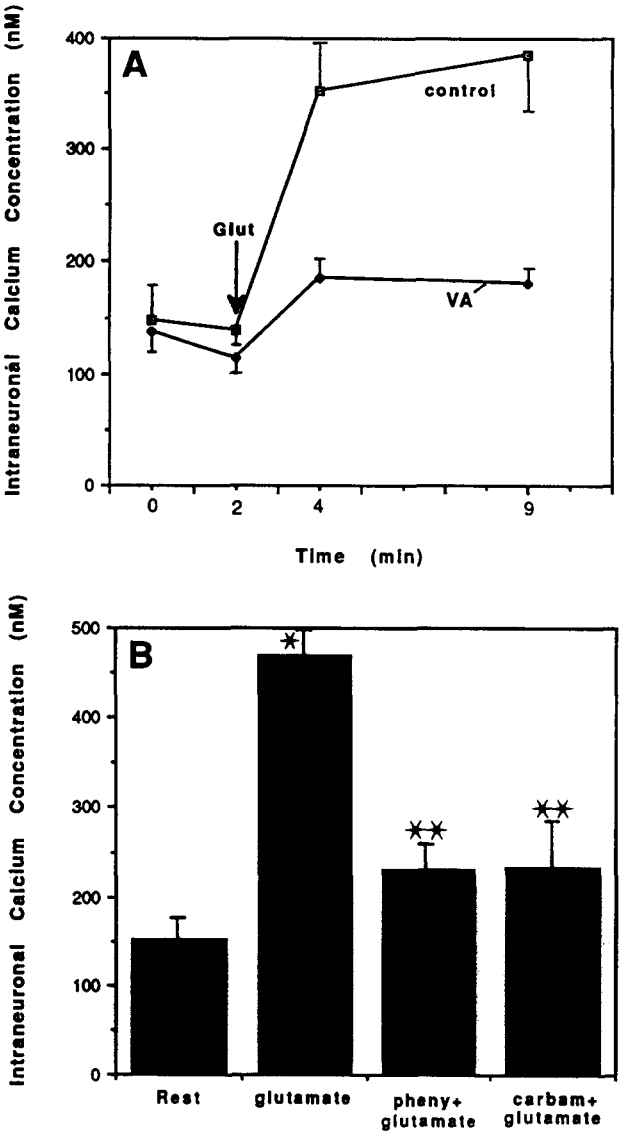

FIG. 7. Anticonvulsants attenuate glutamate-induced elevation of $[Ca^{2+}]_i$. (A) Neurons were exposed to 0.1% dimethylsulfoxide (control) or 1 μM valproic acid (VA) for 2 min followed by exposure to 200 μM glutamate. The $[Ca^{2+}]_i$ was measured at the indicated times. Values represent the mean and SEM of determinations made in 11-12 neurons. Values for valproic acid-treated cultures were significantly less than corresponding values for control cultures at the 4- and 9-min time points ($p < 0.01$). (B) The $[Ca^{2+}]_i$ was measured in unstimulated neurons (Rest) and in neurons subjected to a 5-min exposure to 100 μM glutamate alone or in combination with phenytoin (10 μM) or carbamazepine (10 μM). Values represent the mean and SEM of determinations made in 10-56 neurons. *$p < 0.01$ compared to Rest. **$p < 0.01$ compared to glutamate alone.

in the pathogenesis of AD and related disorders. Carbamazepine, phenytoin, and valproic acid protected neurons against Aβ toxicity and glutamate toxicity, and suppressed glutamate-induced alterations in tau and ubiquitin immunoreactivity. The mechanisms of excitotoxic and Aβ-induced neuronal injuries involve sustained elevations of $[Ca^{2+}]_i$ (53), and our data indicate that anticonvulsants protect against glutamate- and Aβ-induced toxicity by suppressing elevations of $[Ca^{2+}]_i$.

Reduction of neuronal excitability is a property of anticonvulsants that makes them effective in reducing epileptic seizure activity (see ref. 42 for review). By hyperpolarizing membranes, compounds such as carbamazepine, phenytoin, and valproic acid reduce calcium influx through voltage-dependent channels and supress transmitter release. Previous data and the present findings demonstrate that several different anticonvulsants can reduce excitotoxic neuronal injury. We previously reported that phenytoin and carbamazepine can protect cultured hippocampal neurons against glutamate toxicity (45). Subsequently, Boxer et al. (8) showed that phenytoin can reduce brain injury in a model of focal brain ischemia in the rat, McDonald and Johnston (56) reported that carbamazepine significantly reduced NMDA-mediated brain injury in neonatal rats, Mehler et al. (57) showed that phenytoin can protect neurons in the mdx mouse against hypoxia-induced damage, and Taft et al. (81) reported that phenytoin can reduce ischemic neuronal injury. We previously reported that phenytoin and carbamazepine can reduce dendritic atrophy in cultured hippocampal neurons exposed to glutamate (45). A similar action of phenytoin was recently reported by Watanabe et al. (84) who found that phenytoin can reduce dendritic damage induced by glucocorticoids in rat hippocampal neurons in vivo. Interestingly, glucocorticoids apparently contribute to hippocampal damage by inhibiting glucose uptake into neurons and increasing their vulnerability to excitotoxicity (70).

We found that both the broad-spectrum glutamate receptor antagonist DGG, and valproic acid afforded significant protection against Aβ toxicity. These findings suggest that activation of glutamate receptors contributes to neuronal death initiated

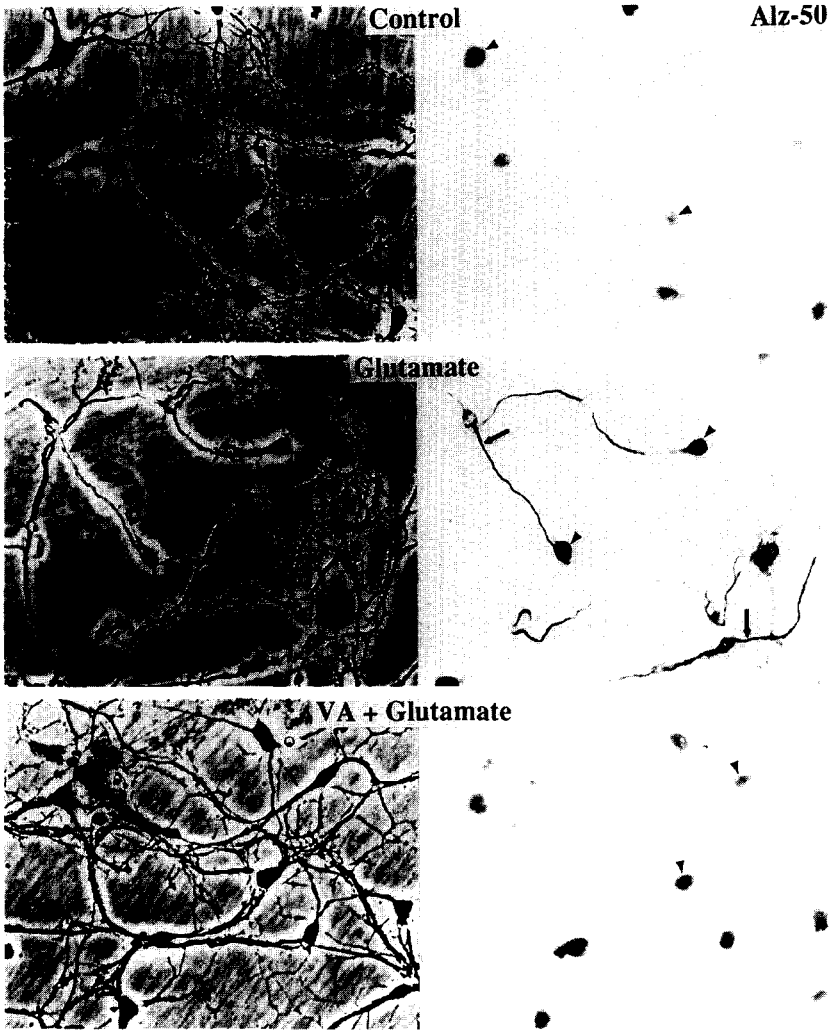

FIG. 8. Valproic acid suppresses glutamate-induced increase in Alz-50 immunoreactivity. Phase-contrast (left) and bright-field (right) micrographs of cells immunostained with Alz-50. Upper, untreated control culture; middle, culture exposed for 2 h to 200 μM glutamate; lower, culture exposed for 2 h to 10 μM valproic acid (VA) plus 200 μM glutamate. Note that glutamate induced a marked increase in axonal immunoreactivity with Alz-50 (e.g., arrows in middle panels), and valproic acid suppressed the increase in Alz-50 immunoreactivity. Alz-50 immunoreactivity was also increased in the cell bodies of glutamate-treated neurons (e.g., arrowheads). Similar results were obtained in 3 separate experiments.

by Aβ. The data are consistent with the possibility that anticonvulsants protect against Aβ neurotoxicity, in part, by suppressing the excitotoxic component of the toxicity. The latter possibility is consistent with previous studies showing that anticonvulsants protect hippocampal neurons against excitotoxic insults (8,40,42,45). A variety of neurotoxic insults can be reduced by glutamate receptor antagonists including glucose deprivation (16) and oxidative insults (89). Moreover, elevation of $[Ca^{2+}]_i$ in neurons results in release of glutamate which can then, by activating receptors, contribute to further elevation of $[Ca^{2+}]_i$. Aβ has been shown to elevate $[Ca^{2+}]_i$ (31,52,53). It therefore seems reasonable to consider that activation of glutamate receptors, by glutamate released from the cultured cells, contributes to the neurotoxicity of Aβ. On the other hand, Busciglio et al. (10) reported that the broad-spectrum glutamate receptor antagonist kynurenate did not protect cultured fetal human cortical neurons against Aβ toxicity, a result suggesting that Aβ can kill neurons independently of glutamate receptor activation.

By directly monitoring $[Ca^{2+}]_i$ we found that valproic acid, phenytoin, and carbamazepine suppressed the elevation of $[Ca^{2+}]_i$ induced by Aβ and glutamate. Electrophysiological and biochemical studies have provided insight into the mechanism of action of these anticonvulsants (see ref. 42 for review). Each of these compounds can suppress Na^+-dependent action potentials and Ca^{2+}-conductances. In agreement with our findings in hippocampal neurons, others previously reported that anticonvulsants attenuate glutamate- and depolarization-induced elevations of $[Ca^{2+}]_i$ in other types of cells. For example, Lampe and Bigalke (40) showed that carbamazepine blocks NMDA-induced currents in cultured spinal cord neurons, Zeise et al. (88)

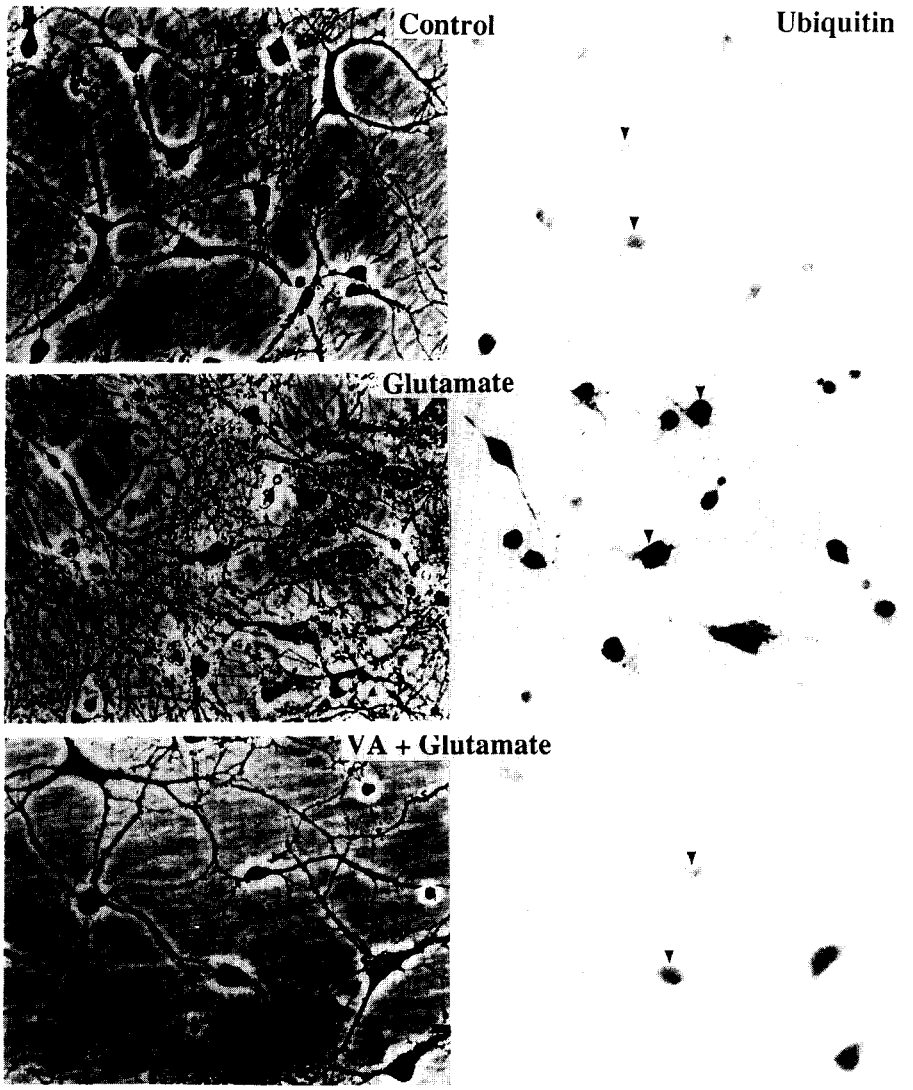

FIG. 9. Valproic acid suppresses glutamate-induced increase in ubiquitin immunoreactivity. Phase-contrast (left) and bright-field (right) micrographs of cells immunostained with ubiquitin antiserum. (Upper) untreated control culture. (Middle) culture exposed for 2 h to 200 μM glutamate. (Lower) culture exposed for 2 h to 10 μM valproic acid plus 200 μM glutamate. Note that glutamate induced a marked increase in ubiquitin immunoreactivity in neuronal cell bodies and valproic acid largely prevented this effect of glutamate (e.g., arrowheads). Similar results were obtained in 4 separate experiments.

reported that valproic acid suppresses NMDA-induced depolarizations in cultured rat neocortical neurons, and Nilsson et al. (62) found that valproic acid blocks glutamate-induced elevation of $[Ca^{2+}]_i$ in cultured astrocytes. Steppuhn and Turski (80) reported that several anticonvulsants raised the seizure threshold for excitatory amino acids in mice. In addition, anticonvulsants including valproic acid reduced hypoxia-induced glutamate release from hippocampal slices (68) which would be expected to reduce excitotoxic injury in vivo. A study in which $[Ca^{2+}]_i$ was directly measured in cultured cerebellar neurons showed that carbamazepine suppressed NMDA- and KCl-induced elevation of $[Ca^{2+}]_i$ (12). Finally, valproic acid was recently shown to reduce mitochondrial oxidation (67) suggesting that this compound may reduce free radical-mediated injury. In agreement with the latter possibility, we found that valproic acid reduces neuronal injury induced by iron in cultured hippocampal neurons (M.P.M. and Y.G., unpublished data). Iron damages cultured hippocampal neurons by inducing hydroxyl radical production with subsequent loss of calcium homeostasis which involves NMDA receptor activation (89).

Previous studies in several laboratories have shown that glutamate and elevation of $[Ca^{2+}]_i$ can elicit some antigenic and biochemical alterations in tau in cultured rat and human neurons similar to those seen in NFTs (48,49,71). In addition, kainic acid induced the accumulation of tau immunoreactivity and caused spectrin proteolysis in CA3 neurons in vivo, and these actions of kainic acid were potentiated by glucocorticoids and physiological stress (22,79). The alterations in tau induced by glutamate and exacerbated by Aβ include a shift in localization from the axon to the soma and dendrites (22,48). These changes

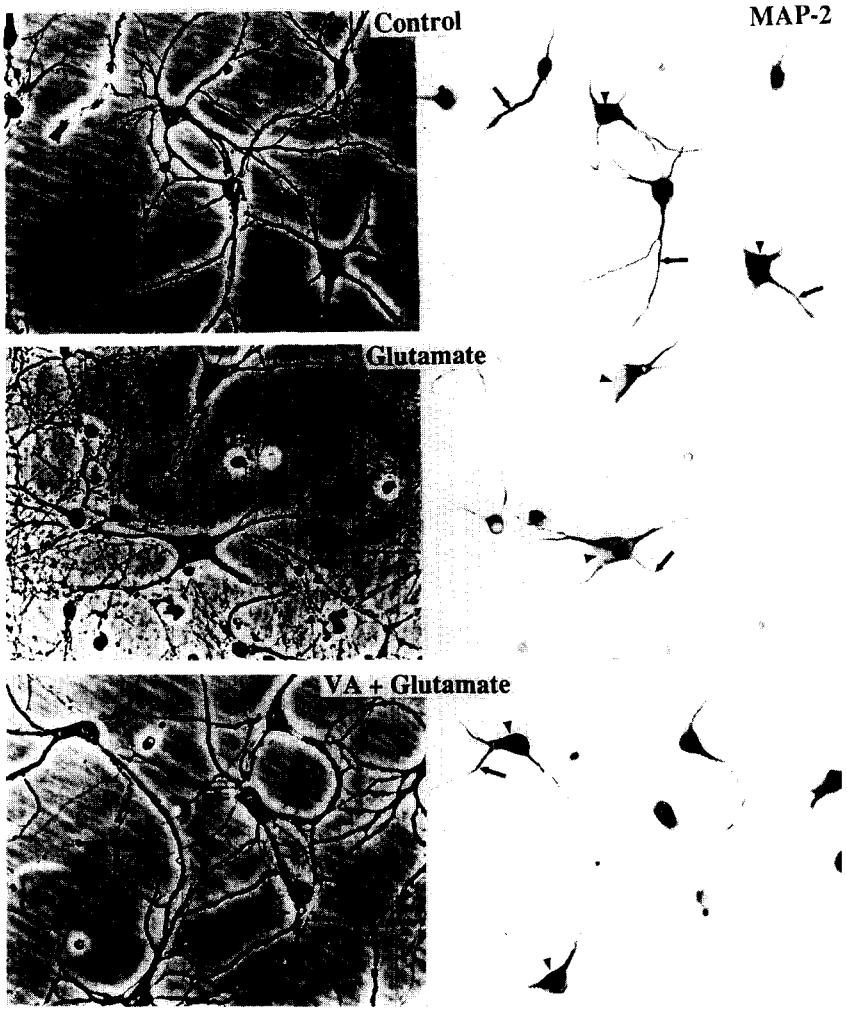

FIG. 10. Valproic acid suppresses glutamate-induced alterations in MAP-2 immunoreactivity. Phase-contrast (left) and bright-field (right) micrographs of cells immunostained with MAP-2 antibody. (Upper) untreated control culture. (Middle) culture exposed for 2 h to 200 μM glutamate. (Lower) culture exposed for 2 h to 10 μM valproic acid plus 200 μM glutamate. Note that glutamate induced a reduction in MAP-2 immunoreactivity in dendrites (e.g., arrows) and cell bodies (e.g., arrowheads), and valproic acid largely prevented this effect of glutamate. Similar results were obtained in 2 separate experiments.

are prevented by agents that suppress elevation of intracellular calcium levels, including neurotrophic factors such as basic fibroblast growth factor (15,46,47,48). The shift in tau localization is characteristic of AD (39) and is also observed in cerebral ischemia (24) which is know to involve an excitotoxic mechanism of neuronal injury. However, the changes in tau are clearly not identical to those observed in PHF tau of AD where tau is hyperphosphorylated at the tau-1 site (5). Loss of MAP2 is a characteristic of the neurodegenerative process in a variety of disorders including ischemia (37) and AD (39). Ubiquitin is considered a "heat-shock" protein, and excitotoxic insults in vivo have been shown to increase levels of heat-shock proteins in neurons (63). We found that anticonvulsants largely prevented glutamate-induced accumulation of tau and ubiquitin immunoreactivity in hippocampal neurons. Moreover, the anticonvulsants prevented glutamate-induced loss of MAP-2 immunoreactivity. MAP-2 is a microtubule-associated protein that is very sensitive to calcium-mediated proteolysis (34,78). Taken together with previous findings (48,79), the present data suggest that the mechanism whereby anticonvulsants suppress cytoskeletal alterations induced by glutamate is by attenuating the elevation of $[Ca^{2+}]_i$ induced by these insults.

Alterations in βAPP are implicated in the pathogenesis of AD (see refs. 54,60,73 for review). It is becoming clearer that Aβ can be directly neurotoxic (87) and can increase neuronal vulnerability to excitoxic and metabolic insults (18,38,52). The neurotoxic activity of the peptide is correlated with its secondary structure (10,53,65), and may involve formation of free radical peptides which damage cell membranes (11,26,33,53). Prolonged exposure to Aβ causes an elevation of rest $[Ca^{2+}]_i$, and enhances $[Ca^{2+}]_i$ responses to glutamate and membrane depolarization (31,53,54). The present data indicate that anticonvulsants can attenuate Aβ-induced elevation of $[Ca^{2+}]_i$ and attendant neuronal injury. To date, compounds that have proven effective in

reducing neuronal vulnerability to glutamate and Aβ toxicities act by stabilizing $[Ca^{2+}]_i$ or suppressing free radicals. Reducing the concentration of extracellular Ca^{2+} protected against both glutamate (44) and Aβ toxicities (53) in hippocampal cell cultures. Fibroblast growth factor attenuated $[Ca^{2+}]_i$ responses to, and protected against the toxicity of, both glutamate and Aβ (46,53). The antioxidant vitamin E protected against excitotoxicity (72) and Aβ toxicity (4,26).

The anticonvulsants were effective in protecting against Aβ toxicity at concentrations that are at or below the concentrations observed during treatment of epilepsy patients. For example, plasma levels of valproic acid required to control seizures are typically in the 0.1–0.6 mM range (32), and the levels of valproic acid in the CSF relative to plasma levels are approximately 5- to 10-fold lower than in plasma. Phenytoin and carbamazepine were also effective in protecting against glutamate and Aβ toxicities at concentrations (100 nM to 10 μM) below those required to prevent seizures in epilepsy patients (10–100 μM) (20). Although it is difficult to extrapolate dose–response relationships observed in embryonic rat neurons in vitro, to the adult human brain, the present data are consistent with the possibility that the levels of anticonvulsants that afford neuroprotection would be tolerated by AD patients. In addition to possibly reducing neuronal injury, valproic acid may be useful in treating the aggressive/combative stage that some Alzheimer's patients experience, because valproic acid was reported to have an anti-conflict effect (69). Treatment of AD patients with anticonvulsants is not a novel idea. Mohr et al. (58) conducted a study using the GABA agonist THIP [4,5,6,7-tetrahydroisoxazolo (5,4-c) pyridin-3-ol]. They reported that THIP did not restore lost cognitive ability as determined by mental aptitude tests. However, as in most clinical trials to date, the latter study did not determine whether the drug affected the rate of progression of the disease. It seems likely that approaches aimed at reducing neuronal degeneration in AD will reduce the rate of progression of the disease rather than restore lost functions.

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