

Activation of the L Voltage-sensitive Calcium Channel by Mitogen-activated Protein (MAP) Kinase following Exposure of Neuronal Cells to β -Amyloid

MAP KINASE MEDIATES β -AMYLOID-INDUCED NEURODEGENERATION*

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Neuronal degeneration in Alzheimer's disease (AD) has been variously attributed to increases in cytosolic calcium, reactive oxygen species, and phosphorylated forms of the microtubule-associated protein tau. β -Amyloid (β A), which accumulates extracellularly in AD brain, induces calcium influx in culture via the L voltage-sensitive calcium channel. Since this channel is normally activated by protein kinase A-mediated phosphorylation, we examined kinase activities recruited following β A treatment of cortical neurons and SH-SY-5Y neuroblastoma. β A increased channel phosphorylation; this increase was unaffected by the protein kinase A inhibitor H89 but was reduced by the mitogen-activated protein (MAP) kinase inhibitor PD98059. Pharmacological and antisense oligonucleotide-mediated reduction of MAP kinase activity also reduced β A-induced accumulation of calcium, reactive oxygen species, phospho-tau immunoreactivity, and apoptosis. These findings indicate that MAP kinase mediates multiple aspects of β A-induced neurotoxicity and indicates that calcium influx initiates neurodegeneration in AD. β A increased MAP kinase-mediated phosphorylation of membrane-associated proteins and reduced phosphorylation of cytosolic proteins without increasing overall MAP kinase activity. Increasing MAP kinase activity with epidermal growth factor did not increase channel phosphorylation. These findings indicate that redirection, rather than increased activation, of MAP kinase activity mediates β A-induced neurotoxicity.

This 42-amino acid peptide is generated by alternate cleavage from the larger "amyloid precursor protein" (4, 5). β A is either directly toxic to neurons in culture or potentiates neuronal vulnerability to excitatory neurotoxins (e.g. Refs. 6 and 7). These effects may derive from the formation of ion channels within the cell membrane by β A, fostering direct leakage of calcium into cells (8). Additional studies indicate that, rather than forming *de novo* calcium channels, β A induces calcium influx via existing channels (9), including the L voltage-sensitive calcium channel (10). Increased calcium influx, with resultant disruption of calcium homeostasis, may be the underlying cause of β A neurotoxicity and ultimate neurodegeneration (7, 11–15).

A consequence of disruption in calcium homeostasis is the induction of oxidative stress and accumulation of free radicals, collectively referred to as reactive oxygen species (ROS; Ref. 15). One target of ROS is the phospholipid membrane (16–18). Cumulative ROS-induced membrane damage compromises membrane integrity and increases the permeability of several ions, including calcium; resultant calcium influx is a crucial factor in neurodegeneration (19). Calcium influx, moreover, promotes recruitment of other ion channels and generation of additional ROS, resulting in neuronal excitotoxicity (20–22). Importantly, ROS accumulation fosters calcium influx, which, in turn, fosters yet further ROS accumulation (23). β A-induced aberrant calcium influx may therefore initiate a cascade of cytosolic calcium and ROS accumulation. In this regard, additional studies have attributed β A toxicity to ROS accumulation (24–26). Since the L voltage-sensitive calcium channel (activated by β A; Ref. 10) is activated by phosphorylation (27), elucidation of kinase pathways activated by β A represents one potential approach toward understanding β A-induced neurodegeneration.

In the present study, we demonstrate that β A induces calcium influx by MAP kinase-mediated phosphorylation of the L voltage-sensitive calcium channel and that inhibition of MAP kinase activity prevents multiple known aspects of β A toxicity.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—SH-SY-5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (Cellgro) containing 10% fetal bovine serum in 5% CO₂. Cultures were differentiated for 7 days with 10 μ M retinoic acid, during which time they elaborate extensive neurites that exhibit characteristics of axons (28). Cortical neurons were harvested from day 16 embryonic mice and cultured on poly-L-lysine in Dulbecco's modified Eagle's medium/F-12 containing B27 supplements (Life Technologies, Inc.) in the absence of serum according to Ref. 29. Cells were utilized 3 days after plating, during which time they had established polarity and elaborated axons (e.g. Fig. 1). SH-SY-5Y cells and cortical neurons were deprived of serum and treated for 2 h with one or more of the following: β A_{25–35} and β A_{1–40}

Alzheimer's disease (AD)¹ is a neurodegenerative disorder that affects the cognitive function of the brain. Pathological changes in AD are punctuated by the formation of amyloid plaques and neurofibrillary tangles as well as extensive neuronal loss (1). Amyloid plaques, which accumulate extracellularly, are composed of aggregated β -amyloid (β A; Refs. 2 and 3).

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¹ The abbreviations used are: AD, Alzheimer's disease; β A, β -amyloid; ROS, reactive oxygen species; MAP, mitogen-activated protein; BAPTA, bis(o-aminophenoxy)ethane-N,N',N',N'-tetraacetic acid.

(Sigma; 10–25 μ M for cortical neurons and 22–40 μ M for SH-SY-5Y cells (9, 28)), the antioxidant vitamin E (0.15 mg/ml; Ref. 24), the calcium chelator BAPTA (1 μ M; Molecular Probes, Inc., Eugene, OR), the MAP kinase inhibitor PD98059 (10–20 μ M; RBI, Natick, MA; Ref. 30), the PKA inhibitor H-89 (200 nM; Calbiochem; Ref. 31), or 10–12 M epidermal growth factor to activate MAP kinase (32). β A_{25–35} and β A_{1–40} were initially dissolved in a small volume of Me₂SO, and diluted to a final stock concentration of 200 μ M in serum-free medium. β A_{25–35} and β A_{1–40} were then aggregated overnight at 37 °C before use. Cultures of SH-SY-5Y cells were radiolabeled by inclusion of 100 μ Ci of [³²P]orthophosphate in culture medium during these incubations as described previously (33). Additional cultures were transfected with oligonucleotides corresponding to the sequence of MAP kinase in sense (ATG GCG GCG GCG GCG GCG GCT) or antisense (AGC CGC CGC CGC CGC CGC CAT) orientation; these antisense oligonucleotides have previously been demonstrated effective at down-regulation of MAP kinase steady-state levels (34). Oligonucleotides (32 μ g) were incubated for 15 min at room temperature with 32 μ l of Superfect (Qiagen, Santa Clarita, CA) that was diluted to a total of 150 μ l with serum-free medium. Cultures in serum-free medium were incubated for 6 h with a final concentration of 4 μ g/ml oligonucleotide/Superfect mixture, after which the medium was replaced with fresh medium containing 10% serum and 4 μ g/ml oligonucleotides (without Superfect). Cultures were maintained for 6 days in the presence of oligonucleotides (shown in preliminary studies to be required for reduction of MAP kinase steady-state levels), during which time they received fresh medium containing serum and oligonucleotides every 2 days. All supplies were from Sigma unless otherwise specified.

Monitoring of Intracellular Calcium Concentrations—Intracellular calcium concentration was monitored as described previously (28) by incubation with Fluo-3 (acetoxymethyl ester; Molecular Probes) for 30 min. Images were captured using a DAGE CCL-72 cooled CCD camera via a Scion LG-3 frame grabber operated by NIH Image analysis software and stored in a Macintosh Power PC 7100AV (e.g. Refs. 35 and 36). Analysis was carried out with NIH Image software (28).

Monitoring of Intracellular Peroxide Concentrations—Cultures were treated with 2',7'-dichlorofluorescein diacetate (Eastman Kodak Co.) for 20 min. The cultures were rinsed with serum-free medium. Intracellular peroxide levels were measured in individual cells in multiple cultures under a fluorescein optic (24). The antioxidant vitamin E (0.15 mg/ml) was included in some cultures to curtail intracellular ROS accumulation, and additional cultures received 10 nM H₂O₂ to induce ROS (24).

Immunofluorescence—Cells fixed for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer and immunostained by sequential reaction with a mouse monoclonal antibody (PHF-1; generous gift of Dr. Peter Davies) raised against tau from paired helical filaments from AD brains, followed by rhodamine-conjugated goat anti-mouse IgG and visualization by standard methods (37). Identical results were obtained following substitution of methanol for paraformaldehyde. Additional controls, which yielded only background fluorescence, included substitution of nonimmune murine IgG for PHF-1 or omission of primary antibody.

Analysis of Membrane and Cytosolic Protein Phosphorylation—Cells were harvested by scraping with a rubber policeman, and membrane preparations were generated by centrifugation of the homogenate for 15 min at 13,000 \times g (38). Aliquots of resultant membranes (pellet) and cytosolic (supernatant) proteins were subjected to SDS-gel electrophoresis and autoradiography. The L voltage calcium channel was immunoprecipitated from membrane preparations using a mouse anti-dihydropyridine antibody specific for this channel (Upstate Biotechnology Inc., Lake Placid, NY; Ref. 38) followed by protein G-Sepharose via standard procedures (36, 38). Immunoprecipitated material was subjected to SDS-gel electrophoresis. Dried gels were placed against X-Omat film (Kodak) to generate autoradiographs.

Analysis of MAP Kinase Activity— β A-treated and untreated SH-SY-5Y cultures were rinsed, scraped from the plate, and homogenized in 1% Triton X-100 in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 0.5 mM Na₃VO₄, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β -glycerol phosphate, and 0.1% 2-mercaptoethanol. Aliquots (200 μ g) were subjected to immunoblot analysis with an antibody specific for phosphorylated p42 and p44 MAP kinase (NE BioLabs, Beverly, MA), which provided an index of relative levels of phosphorylated (active) kinase. MAP kinase activity was assayed via the "MAP kinase immunoprecipitation kinase cascade assay kit" according to the manufacturer's instructions (catalog no. 17-184; Upstate Biotechnology). Briefly, MAP kinase was immunoprecipitated from additional aliquots (500 μ g) by incubation for 2 h with anti-rat MAP

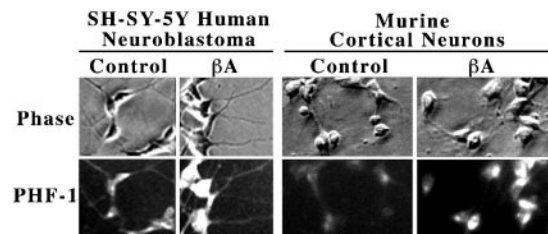


FIG. 1. β A induces accumulation of phospho-tau immunoreactivity in cultured neurons and neuroblastoma. Panels present representative phase contrast and corresponding UV images of SH-SY-5Y human neuroblastoma cells 7 days after differentiation with RA and murine cortical cultures 3 days after plating, after which they were treated for 2 h with 22 or 10 μ M β A, respectively, and subjected to immunofluorescent analyses for phospho-tau with PHF-1. Note the increase in PHF-1 immunoreactivity following β A treatment.

kinase antibody R2 complexed with protein A-agarose. Controls included nonspecific IgG instead of anti-MAP IgG. Immunoprecipitated samples were incubated for 20 min at 30 °C along with myelin basic protein as substrate and [³²P]ATP (3000 Ci/mmol; NEN Life Science Products) and analyzed by scintillation counting.

Densitometric Analyses—Fifty to one hundred cells in at least five randomly selected microscopic fields processed as above for cytosolic calcium, ROS, or PHF-1 immunoreactivity were scored for fluorescent intensity using NIH Image analysis software (35). Representative background areas devoid of cells were similarly analyzed and subtracted from cell values to yield net densitometric values. All fields for each individual assay were illuminated, captured, and processed at the identical intensity. Cell aggregates were excluded from the assays; only individual cells were encircled. Immunoblots and autoradiographs were digitized with a UMAX flat bed scanner and subjected to whole band densitometric analyses using NIH Image as described (35). Values were exported to Excel for statistical analyses via Student's *t* test.

Toxicity Assay—SH-SY-5Y cells in duplicate cultures in the absence (Control) or presence of 10 or 20 μ M PD98059, 22 μ M β A_{25–35}, or both, were trypsinized 24 h after incubation, and viable cells were counted in a hemacytometer using trypan blue.

All experiments were carried out at least twice with comparable results. Presented results are representative of all experiments.

RESULTS

β A Treatment Alters Intracellular Kinase Activities—Our prior studies demonstrated that treatment of SH-SY-5Y human neuroblastoma cells and mouse cortical neurons with 40 and 25 μ M β A, respectively, induced widespread neurodegeneration within 2 h (9). However, treatment of SH-SY-5Y cells and cortical neurons for 2 h with lower β A concentrations (22 and 10 μ M, respectively, did not invoke significant overt cell loss for 24 h, which provided an experimental window to monitor upstream events prior to degeneration. Treatment with these lower concentrations for 30 min to 2 h induced accumulation of phosphorylated tau epitopes common to those that accumulate in affected neurons in AD (Fig. 1). In addition, a 2-h treatment of SH-SY-5Y human neuroblastoma cells with β A induced a >6-fold increase in phosphorylation of membrane-associated proteins (Fig. 2). These findings indicated these culture systems could serve as models for probing the intracellular consequences of β A toxicity.

Phosphorylation of the L Voltage-sensitive Calcium Channel by β A—Since calcium influx following β A exposure has been attributed to activation of the L voltage-sensitive calcium channel (10), we examined potential kinases affected by β A treatment. Immunoprecipitation of the channel followed by autoradiography confirmed that β A increased channel phosphorylation by >2-fold (Fig. 3). Although this channel is normally regulated during synaptic transmission by PKA, inclusion of the PKA inhibitor, H89, at up to twice the concentration reported to inhibit PKA (31) did not diminish β A-induced channel phosphorylation (Fig. 3; Table I), suggesting that β A invoked an alternate kinase to phosphorylate this channel.

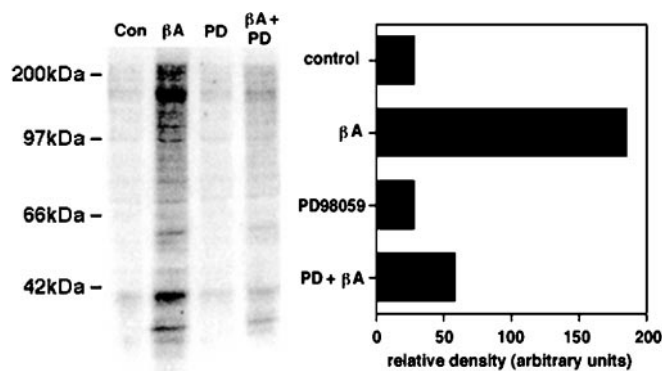


FIG. 2. β A induces an increase in phosphorylation of membrane proteins. Representative autoradiograph of membrane preparations derived from SH-SY-5Y cells incubated with [32 P]orthophosphate for 2 h in the presence and absence of 22 μ M β A, 10 μ M PD98059, or both. The accompanying graph presents densitometric analysis of these samples. Note that β A induces a >6-fold increase in phosphorylation of membrane proteins and that co-treatment with the MAP kinase inhibitor P998059 attenuated this increase.

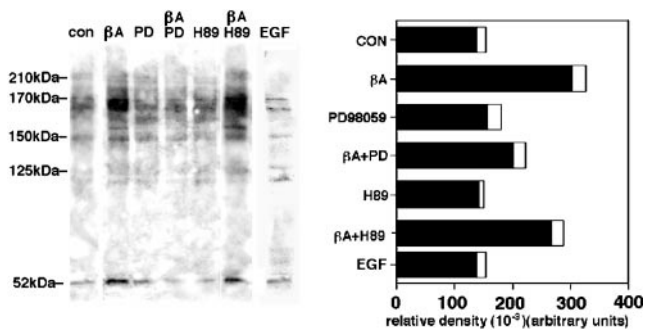


FIG. 3. β A induces phosphorylation of the L voltage calcium channel. Representative autoradiograph of material immunoprecipitated by an antibody directed against the L voltage-sensitive calcium channel from SH-SY-5Y cells incubated with [32 P]orthophosphate for 2 h in the presence and absence of 22 μ M β A, 10 μ M PD98059, or both. The accompanying graph presents densitometric analysis of 3–5 such samples. Note that β A includes an approximately 2-fold increase in channel phosphorylation. The MAP kinase inhibitor PD98059 attenuated this increase, but the PKA inhibitor H89 did not. Treatment of cells for 2 h with 10^{-12} M epidermal growth factor to activate MAP kinase did not result in increased channel phosphorylation.

TABLE I
Phosphorylation of the L voltage-sensitive calcium channel by β A

Culture conditions	Incorporation of 32 P into channel ^a	-Fold change vs. control ^b	p value vs. control ^c	p value vs. β A ^c
Control	137,317 \pm 16,705			
β A	302,312 \pm 23,266	2.2 \pm 0.08	0.002	
PD98059	156,849 \pm 22,559	1.14 \pm 0.14	0.512	0.008
β A + PD	200,448 \pm 21,862	1.46 \pm 0.11	0.080	0.050
H89	142,203 \pm 8178	1.04 \pm 0.06	0.827	0.004
H89 + β A	265,463 \pm 22,373	1.93 \pm 0.08	0.007	0.378

^a Values present density of radiolabel associated with the L voltage-sensitive calcium channel immunoprecipitated from cultures treated with [32 P]orthophosphate. Values represent the mean \pm S.E., expressed in arbitrary units, derived from at least three experiments.

^b Calculated by dividing the autoradiographic density of treated samples by that of control samples.

^c Student's *t* test.

Inhibition of MAP Kinase Prevented β A-induced Phosphorylation of the L Voltage-sensitive Calcium Channel and of Membrane Proteins—Unlike the PKA inhibitor H89, PD98059, which inhibits MAP kinase kinase (the immediate upstream activator of MAP kinase; Fig. 4; see also Ref. 30), diminished β A-induced channel phosphorylation (Fig. 3., Table I). Moreover, PD98059 reduced overall phosphorylation of membrane-associated proteins following β A treatment (Fig. 2). As ex-

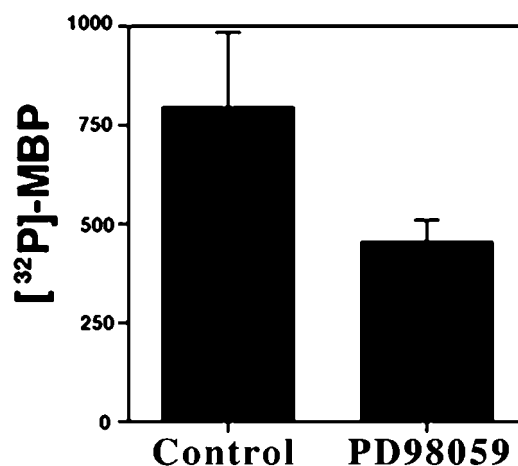


FIG. 4. MAP kinase activity is decreased by short term treatment with the MAP kinase kinase inhibitor, PD98059. Graphical representation of levels of 32 P incorporated into myelin basic protein (MBP) in a commercial MAP kinase activity assay of cells with and without 2-h prior treatment with 10 μ M PD98059 as described under "Experimental Procedures." In the presence of PD98059, myelin basic protein phosphorylation was reduced by 43%.

pected, cell-free assays of MAP kinase activity revealed considerable constitutive MAP kinase activity in non- β A-treated SH-SY-5Y cells; no further increase was observed in cells treated for 2 h with β A (Fig. 5; but see also Ref. 39). These findings suggested, at least under conditions of prior activation of MAP kinase, that β A can redirect MAP kinase to phosphorylate the L voltage-sensitive calcium channel without necessarily invoking a significant increase in overall MAP kinase activity. In support of this conclusion, we observed that, along with increasing phosphorylation of membrane-associated proteins, β A induced a compensatory decrease in phosphorylation of cytosolic proteins (Fig. 6). Moreover, PD98059 diminished this decrease in cytosolic protein phosphorylation along with inhibiting the increase in membrane-associated protein phosphorylation.

Inhibition of MAP Kinase Prevented β A-induced Degenerative Changes—We next examined whether or not MAP kinase mediated additional aspects of β A toxicity by pharmacological and antisense oligonucleotide-mediated inhibition of MAP kinase activity and steady-state levels. SH-SY-5Y human neuroblastoma cells and mouse cortical neurons were treated for 30 min to 2 h with and without 22 μ M (for neuroblastoma) or 10 μ M (for neurons) β A_{25–35} or β A_{1–40} in the presence and absence of vitamin E, BAPTA, and 10 μ M PD98059 and assayed for calcium influx, ROS and PHF-1 immunoreactivity (Figs. 7 and 8). Both β A peptides induced an increase in intracellular calcium, ROS, and phospho-tau immunoreactivity in both culture types (Figs. 7 and 8). Maximal cytosolic calcium levels were obtained prior to maximal levels of ROS and PHF-1 in both cell types (Fig. 7). The intracellular calcium chelator, BAPTA, inhibited the β A-induced increase in intracellular calcium, ROS, and PHF-1 ($p < 0.001$; Fig. 8). The antioxidant, vitamin E, blocked accumulation of ROS and PHF-1 ($p < 0.001$) but did not significantly lower the level of the β A-induced increase in intracellular calcium (Fig. 8). These latter findings suggested that accumulation of ROS and phospho-tau immunoreactivity may be secondary effects of the β A-induced increase in intracellular calcium. Antisense oligonucleotide-mediated reduction in SH-SY-5Y MAP kinase levels (Fig. 9) also diminished β A-induced cytosolic accumulation of calcium, ROS, and PHF-1 (Fig. 10).

While short term (*i.e.* 2-h) incubation with β A at this relatively low concentration does not induce overt neuronal degeneration (10), continued treatment for 24 h results in approxi-

Phospho-MAP kinase



MAP kinase activity

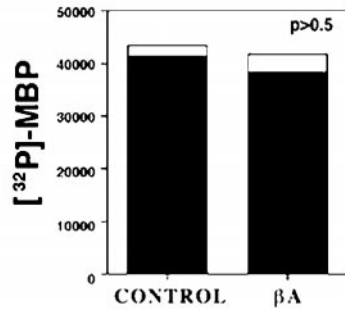


FIG. 5. MAP kinase is constitutively active in differentiated SH-SY-5Y cells and its activity is not increased by short term β A treatment. Homogenates of untreated and β A-treated SH-SY-5Y cells were subjected to immunoblot analysis with an antibody that recognizes only phosphorylated MAP kinase (top panel) and a MAP activity assay using myelin basic protein as a substrate (lower panel). Note that phosphorylated MAP kinase is present in untreated SH-SY-5Y cells as evidenced by immunoblot analysis; the activity of this kinase was further confirmed by its ability to phosphorylate myelin basic protein (see also Fig. 4). Note further that no additional increase in MAP kinase activity (as indicated by lack of increase in phosphoisoforms and activity) was induced following β A treatment.

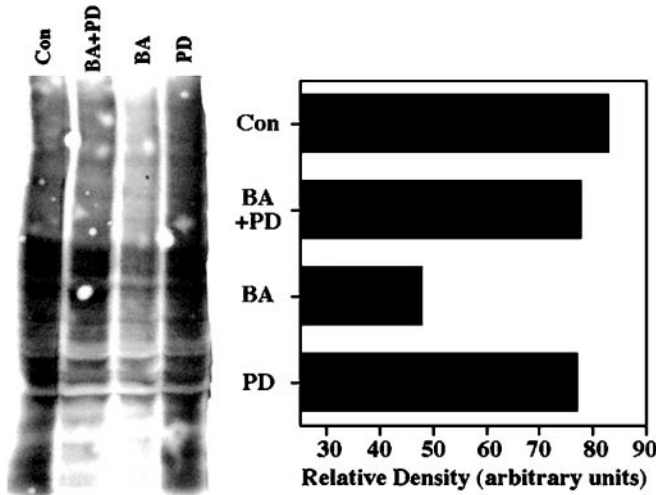


FIG. 6. β A reduces MAP kinase-mediated phosphorylation of cytosolic proteins. Representative autoradiograph of cytosolic proteins derived from SH-SY-5Y cells incubated with [³²P]orthophosphate for 2 h in the presence and absence of 22 μ M β A, 10 μ M PD98059, or both. The accompanying graph presents densitometric analysis of these samples. Note that β A induces a decrease in phosphorylation of cytosolic proteins and that co-treatment with the MAP kinase inhibitor PD98059 attenuated this decrease.

mately 50% loss of both SH-SY-5Y cells (Fig. 7) and cortical neurons (not shown; see also Refs. 9 and 28). However, inclusion of 10 μ M PD98059 during this time significantly reduced the extent of neuronal loss (Fig. 11). These findings further indicate that alterations in MAP kinase activity represent a pivotal event in β A neurotoxicity. Higher levels of this inhibitor were toxic, in accordance with the pivotal role of MAP kinase in signal transduction; as anticipated, toxic levels of PD98059 did not provide protection against β A neurotoxicity (Fig. 11).

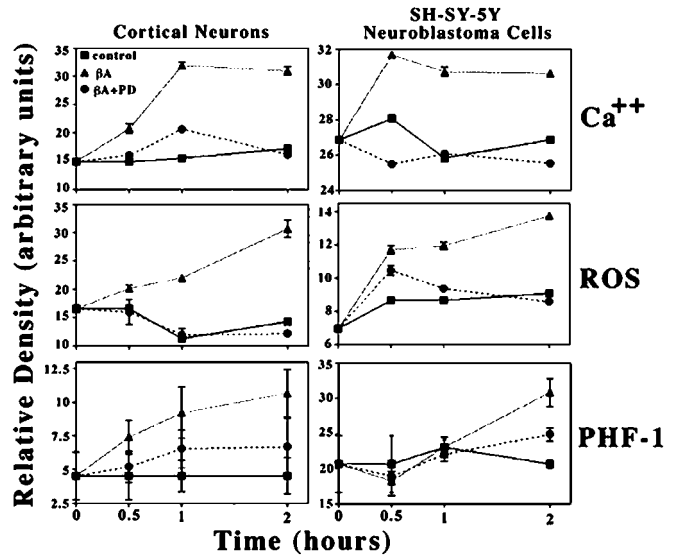


FIG. 7. Pharmacological inhibition of MAP kinase diminishes β A-induced increases in cytosolic calcium, ROS, and phospho-tau immunoreactivity. Densitometric data derived from multiple fields of SH-SY-5Y cells and cortical neurons following treatment for 30 min to 2 h with β A₂₅₋₂₅ (10 μ M for cortical cultures, 22 μ M for SH-SY-5Y cells) in the presence and absence of 10 μ M PD98059. Cells were processed to visualize cytosolic calcium, ROS, and PHF-1, and the relative fluorescent intensity of 50–100 cells in multiple fields was quantified via NIH Image software as described under “Experimental Procedures.” Values are presented as the mean \pm S.E. of the mean. Note the increase in cytosolic calcium, ROS, and phospho-tau immunoreactivity following β A treatment and the attenuation of these increases by PD98059. Note further that maximum levels of cytosolic calcium were attained before those of ROS and PHF-1 following β A treatment of both cell types.

DISCUSSION

The findings of the present study confirm and extend the demonstration by Ueda and co-workers (10) that β A induces calcium influx via the L voltage-sensitive calcium channel. Our demonstration that β A-induced channel phosphorylation does not involve PKA, which is involved in synaptic channel activation under normal conditions (27), but rather is dependent upon MAP kinase, underscores that β A alters signal transduction pathways.

Altered signal transduction and aberrant kinase activities have been extensively studied with regard to tau phosphorylation. Several candidate intracellular tau kinases have been identified, including glycogen synthase kinase 3 β (40–44) and cyclin-dependent kinase 5 (45, 44), calcium-calmodulin kinase (47), and protein kinase C (35, 36, 48, 49). Although MAP kinase clearly phosphorylates tau in cell-free analyses and, in doing so, increases phospho-tau immunoreactivity (49–51), the role of MAP kinase in intracellular tau phosphorylation remains controversial, since it has been reported to phosphorylate tau within cells in some (52–55) but not all studies (43, 44). The findings of the present study potentially shed light upon this controversy in that MAP kinase may not directly phosphorylate tau but instead may, by activating the L voltage sensitive calcium channel, foster tau phosphorylation by other calcium-dependent kinases. Notably, the intracellular tau kinases, calcium-calmodulin kinase and protein kinase C, are both calcium-dependent. In addition, prior phosphorylation of tau by CaM or protein kinase C facilitates subsequent phosphorylation by glycogen synthase kinase 3 β (57, 58). MAP kinase-mediated calcium influx following β A treatment may therefore induce downstream activation of a succession of tau kinases. Similarly, β A-induced phosphorylation of the L voltage-sensitive calcium channel may involve additional kinases upstream and/or downstream of MAP kinase. In support of the

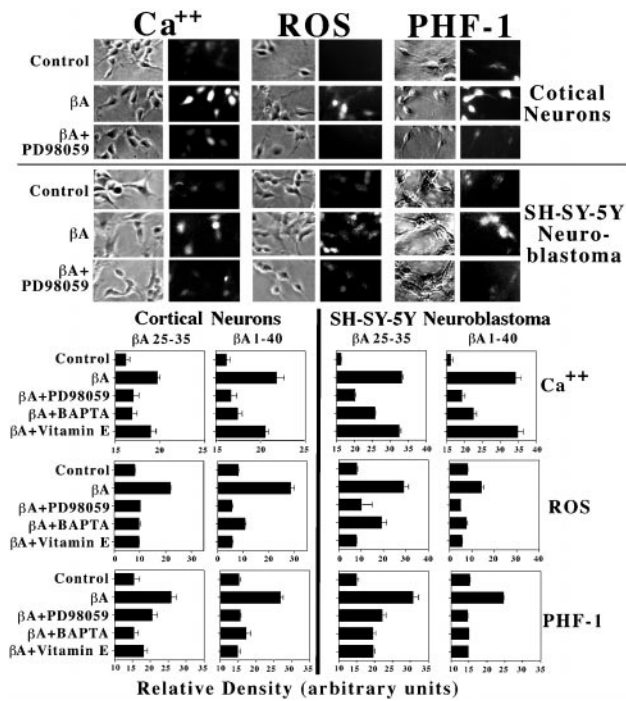


FIG. 8. Pharmacological inhibition of MAP kinase diminishes the increase in cytosolic calcium, ROS, and phospho-tau immunoreactivity induced by β A₂₅₋₃₅ and β A₁₋₄₀. Panels present phase and corresponding UV images of representative fields of cortical and SH-SY-5Y cultures processed to visualize calcium, ROS, and PHF-1 immunoreactivity as described under "Experimental Procedures." Cultures were treated for 2 h as indicated with β A (10 μ M for cortical cultures, 22 μ M for SH-SY-5Y cells) in the presence and absence of 10 μ M PD98059, BAPTA, and vitamin E. For clarity of images, only cells treated with β A₁₋₄₀ in the presence and absence of PD98059 are presented. The accompanying graphs present densitometric data derived from multiple fields of cultures treated as described above. Both β A peptides induced a significant ($p < 0.005$ or less) increase in cytosolic calcium, ROS, and PHF-1 in both cell types. Note that BAPTA and PD98059 prevented the β A-induced accumulation of calcium, ROS, and PHF-1 immunoreactivity ($p < 0.05$ or less). Vitamin E inhibited the β A-induced increase in ROS and PHF-1 ($p < 0.05$ or less) but did not block calcium accumulation.

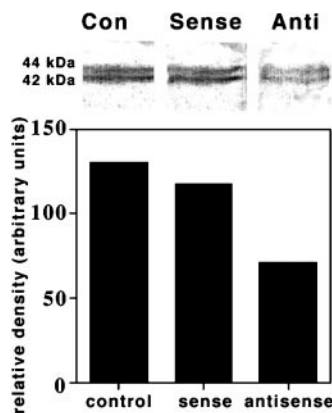


FIG. 9. Antisense oligonucleotides reduce steady-state levels of MAP kinase. Immunoblot analysis was visualized with an anti-phospho-MAP kinase antibody of homogenates of SH-SY-5Y cultures following treatment for 6 days with sense- and antisense-oriented oligonucleotides corresponding to the sequence of MAP kinase as described under "Experimental Procedures." Note the 45% reduction in kinase steady-state levels by antisense oligonucleotides versus those treated with sense-oriented or no oligonucleotides.

potential involvement of MAP kinase in AD, MAP kinase activity is detected within affected regions susceptible to AD neurodegeneration (59) and has been reported to be activated

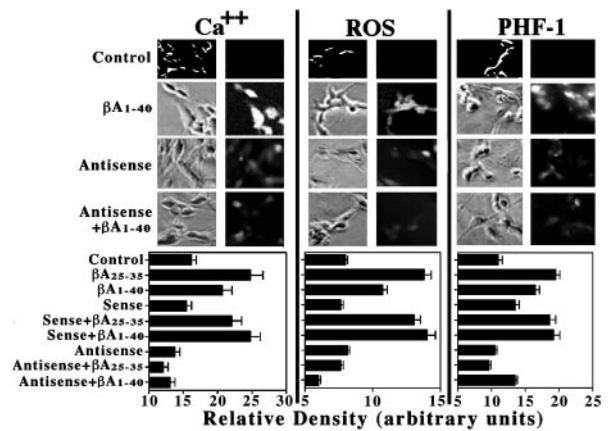


FIG. 10. Reduction in MAP kinase steady-state levels diminishes the increase in cytosolic calcium, ROS, and phospho-tau immunoreactivity induced by β A₂₅₋₃₅ and β A₁₋₄₀. SH-SY-5Y cells were treated for 6 days with sense and antisense oligonucleotides corresponding to the sequence of MAP kinase as described under "Experimental Procedures" to down-regulate steady-state MAP kinase levels. Cultures were then treated for 2 h with 22 μ M β A₂₅₋₃₅ or β A₁₋₄₀ and processed to visualize cytosolic calcium, ROS, and PHF-1. For clarity of images, only cells treated with β A₁₋₄₀ in the presence and absence of antisense oligonucleotides are presented. The accompanying graphs present densitometric analyses (mean \pm S.E.) of 50–100 individual cells in multiple cultures from duplicate experiments. Note that antisense-treated cultures did not demonstrate the characteristic increase in calcium, ROS, or PHF-1 following β A treatment.

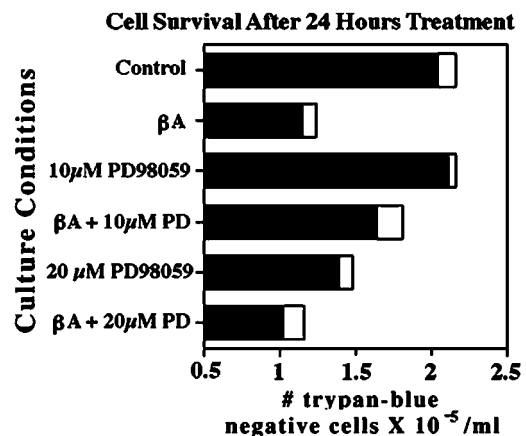


FIG. 11. Pharmacological inhibition of MAP kinase prevents β A-induced neuronal death. SH-SY-5Y cells in duplicate cultures were trypsinized, and trypan blue-negative cells were counted in a hemacytometer 24 h after incubation in the absence (Control) or presence of 10 or 20 μ M PD98059, 22 μ M β A₂₅₋₃₅, or both. Note that β A induced extensive loss of cells by 24 h and that 10 μ M PD98059 attenuated the extent of β A-induced cell death. Higher concentrations of PD98059 (e.g. 20 μ M) were themselves toxic and did not confer protection against β A-induced cell death (see text).

in AD (60), and at least one novel MAP kinase is detected within neurons (61). While β A is capable of increasing MAP kinase activity (39), it is of interest that, under conditions of constitutive MAP kinase activity, β A is capable of inducing this kinase to phosphorylate a distinct set of substrates without necessarily invoking a significant increase in kinase activity. β A also did not increase MAP kinase activity in a prior study (62). These findings suggest that β A can induce neurodegenerative effects by relatively subtle alteration of signal transduction pathways. Subtle alteration of signal transduction is consistent with the protracted degeneration of neurons *in situ* in AD. Moreover, depending upon the mode of analysis (e.g. canvassing exclusively for increased activity of one or more kinases), such subtle consequences may escape detection.

β A-induced neurotoxicity has been separately attributed to

calcium influx, ROS accumulation, and increased phosphorylation of tau (7–9, 11–15, 19, 24–28). In addition to inhibition of tau phosphorylation, inhibition of MAP kinase prevented multiple consequences of β A toxicity, including accumulation of cytosolic calcium ROS, and ultimate neuronal death. These findings indicate that MAP kinase plays a pivotal role in β A neurotoxicity and highlight MAP kinase-mediated pathways as potential therapeutic targets to alleviate the progression of AD (63). In this regard, calcium chelation by BAPTA suppressed both ROS phospho-tau accumulation following β A treatment, demonstrating that calcium influx alone can provoke the additional aspects of β A neurotoxicity. This interpretation is further supported by the observation of maximal calcium accumulation prior to that of ROS or PHF-1 during β A treatment of both cortical neurons and SH-SY-5Y cells. Our data also demonstrate that prevention of accumulation of ROS attenuates β A neurotoxicity despite cytosolic calcium accumulation, further suggesting that ROS accumulation is downstream of calcium accumulation in β A toxicity. These findings collectively suggest that aberrant activation of the L voltage-sensitive calcium channel by MAP kinase may represent the initial toxic event resulting from β A treatment. This latter conclusion should be viewed with caution, however, since β A induced MAP kinase-mediated phosphorylation of many membrane-associated proteins, one or more of which may also contribute to neurodegeneration.

An important unexpected aspect of the present study is that redirection, rather than increased activity, of MAP kinase by β A is what promotes the above intracellular cascade leading to neurodegeneration. Epidermal growth factor, which increases MAP kinase activity (32), did not alter channel phosphorylation, yet β A, which does not increase MAP kinase activity as shown herein, did alter channel phosphorylation. These data suggest that redirection of, rather than an overall increase in, MAP kinase activity is a unique and essential event in β A-induced neurotoxicity.

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