Phosphorylation of 69-kDa Choline Acetyltransferase at Threonine 456 in Response to Amyloid-β Peptide 1-42*

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Choline acetyltransferase synthesizes acetylcholine in cholinergic neurons. In the brain, these neurons are especially vulnerable to effects of β-amyloid (Aβ) peptides. Choline acetyltransferase is a substrate for several protein kinases. In the present study, we demonstrate that short term exposure of IMR32 neuroblastoma cells expressing human choline acetyltransferase to Aβ-(1–42) changes phosphorylation of the enzyme, resulting in increased activity and alterations in its interaction with other cellular proteins. Using mass spectrometry, we identified threonine 456 as a new phosphorylation site in choline acetyltransferase from Aβ-(1–42)-treated cells and in purified recombinant ChAT phosphorylated in vitro by calcium/calmodulin-dependent protein kinase II (CaM kinase II). Whereas phosphorylation of choline acetyltransferase by protein kinase C (PKC) alone caused a 2-fold increase in enzyme activity, phosphorylation by CaM kinase II alone did not alter enzyme activity. A 3-fold increase in choline acetyltransferase activity was found with coordinate phosphorylation of threonine 456 by CaM kinase II and phosphorylation of serine 440 by protein kinase C. This phosphorylation combination was observed in choline acetyltransferase from Aβ-(1–42)-treated cells. Treatment of cells with Aβ-(1–42) resulted in two phases of activation of choline acetyltransferase, the first within 30 min and associated with phosphorylation by protein kinase C and the second by 10 h and associated with phosphorylation by both CaM kinase II and protein kinase C. We also show that choline acetyltransferase from Aβ-(1–42)-treated cells co-immunoprecipitates with valosin-containing protein, and mutation of threonine 456 to alanine abolished the Aβ-(1–42)-induced effects. These studies demonstrate that Aβ-(1–42) can acutely regulate the function of choline acetyltransferase, thus potentially altering cholinergic neurotransmission.

Cholinergic neurons in brain are especially vulnerable to effects of both soluble/oligomeric and deposited/fibrillar forms of β-amyloid (Aβ) peptides released from amyloid precursor protein (APP). Shifts in production of soluble APPα by α-secretase to production of Aβ-(1–40) and Aβ-(1–42) with activation of β- and γ-secretase in Alzheimer’s disease and following traumatic head injury are associated with decreased function and communication by cholinergic neurons (1–3). A complex relationship exists between cholinergic neuron function and APP processing and Aβ peptide production (4–6). Short term exposure to low (picomolar or nanomolar) concentrations of soluble/oligomeric Aβ peptide leads to presynaptic cholinergic dysfunction with a reduction in the availability of acetylcholine (ACh) precursors choline (7) and acetyl-coenzyme A (8, 9) coupled to decreased ACh synthesis and release from hippocampal slices or neuronal cultures (9–13). These acute effects of Aβ peptides on neurotransmission and synaptic efficacy probably differ from the neurotoxicity produced by long term exposure and high (micromolar) concentrations of the peptides that cause death of cholinergic neurons. Mechanisms underlying acute and long term effects of Aβ peptides on cholinergic function have not been resolved.

Choline acetyltransferase (ChAT; EC 2.3.1.6) produces the neurotransmitter ACh in cholinergic neurons. ChAT undergoes covalent modification post-translationally by protein kinase-mediated phosphorylation (14–18), and we showed previously that it is a substrate for a number of protein kinases (17). Catalytic activity of this enzyme, its subcellular distribution, and potentially its interaction with other cellular proteins can be regulated in a phosphorylation-dependent manner. For example, phosphorylation of ChAT by protein kinase C (PKC) on Serine 440 led to a significant increase in its activity and ionic binding to plasma membrane in cells (18).

Phosphorylation of ChAT could be altered by changes in activity or subcellular redistribution of protein kinases brought about by neuronal perturbations or pathology such as Alzheimer’s disease and traumatic brain injury. This could alter ACh biosynthesis and cholinergic neurotransmission and cause dysfunction of cholinergic neurons. Aβ peptides modulate a range of cellular signal transduction pathways and protein kinases (19–21). Whereas a number of potential cell surface receptors for Aβ peptides have been identified (20–23), it is unclear how these peptides mediate their cellular actions either acutely or in the longer term. It is known, however, that Aβ peptides can alter cell calcium homeostasis, leading to increased cytosolic free calcium levels (24, 25). Within a certain concentration range, this could activate a number of calcium-dependent processes, including calcium-dependent protein kinases such as APP, amyloid precursor protein; ChAT, choline acetyltransferase; CTab, anti-ChAT carboxyl-terminal peptide antibody; CaM kinase II, calcium/calmodulin-dependent protein kinase II; ESI, electrospray ionization; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; PKC, protein kinase C; VCP, valosin-containing protein.
PKC and α-calcium/calmodulin-dependent protein kinase II (CaM kinase II). Since ChAT is known to be a substrate for both of these protein kinases (17), it is likely that Aβ peptides could affect cholinergic neurotransmission through regulation of function of this enzyme.

In the present study, we tested the hypothesis that short-term exposure of IMR32 neuroblastoma cells stably expressing human ChAT to Aβ peptides would lead to altered function of the enzyme. Interestingly, we observed that treatment of cells with Aβ1–42, but not Aβ1–40, changed the state of phosphorylation of ChAT, revealing a new putative CaM kinase II phosphorylation site. Furthermore, phosphorylation at this site, when coordinated with phosphorylation of Ser440 by PKC, leads to a hierarchical activation of ChAT and phosphorylation-dependent association of the enzyme with other cellular proteins, including valosin-containing protein (VCP; p97, Cdc48).

EXPERIMENTAL PROCEDURES

Preparation of ChAT Constructs—The cDNA for human 69-kDa ChAT (NH1-ChAT) in pcDNA3 was kindly provided by Dr. H. Misawa (Tokyo Metropolitan Institute for Neuroscience). The mutant ChAT-T456A gene (containing the site-directed mutation of Thr456→Aila) in wild-type 69-kDa ChAT was used to construct ChAT-T456A (20) and ChAT-S440A in pcDNA3.1 using LipofectAMINE 2000 (Invitrogen). G418-resistant stable cell lines were transfected and tested for ChAT enzyme activity by radiolabeled assay and ChAT protein by immunoblot. Cells were maintained in modified Eagle's medium containing 10% fetal calf serum, 50 μg/ml gentamicin, and 0.5% G418 in humidified 5% CO2 at 37°C. For experiments, monolayers of cells were treated at ~70% confluence. At 4 h before treatment, fresh medium was added to cells, and then Aβ peptides (1–40 or 1–42) or reverse peptides (40–1 or 42–1) were used as negative controls were diluted in culture medium to final concentrations of 100 nM from 100 μM stocks and added to cells for varying times up to 18 h. For protein kinase inhibition studies, cell-permeable inhibitors (Calbiochem) of PKC (H7; 50 μM), CaM kinase II (RN-93; 5 μM), p38 mitogen-activated protein kinase (SB202190; 10 μM), or ERK-1/ERK-2 (U0126; 50 μM) were added to the cells 2 h before the addition of Aβ peptides. All peptides (Bachem) were dissolved in double-distilled H2O (1–40 and 40–1) or 1 mM ammonium hydroxide (1–42 and 42–1) at 100 μM and incubated at 37°C for 4 days (26); aliquots were stored at –80°C until use. For positive phosphorylation studies in cells, culture medium was changed to phosphate-free modified Eagle's medium (Sigma) containing [32P]orthophosphate (200 μCi/ml) at 3 h before the end of the treatment with Aβ peptides; in the case of treatment times shorter than 3 h, phosphate-free modified Eagle's medium and [32P]orthophosphate were added with Aβ peptides at the beginning of the incubation interval. Following treatment, lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin/protaminit/peptatin at 10 μg/ml) (50 μM sodium vanadate, 100 μM sodium fluoride, and 700 units/ml DNase I) was added to cells and incubated for 30 min on ice. Lysates were centrifuged (15,000 × g for 10 min), and supernatants were used for analysis of activity or phosphorylation of ChAT or for analysis of proteins that co-immunoprecipitate with ChAT. ChAT activity was measured radioenzymatically using a modification of the method of Fonnum (27), as published previously (18).

Analysis of β-Amyloid Peptides—CD spectra of Aβ(1–40), Aβ(1–42), and the corresponding reverse peptides were recorded on a Jasco spectropolarimeter, model J-810, at 25°C in a 0.1-cm path length cell at 0.2-nm intervals over the wavelength range 190–260 nm. Peptides were analyzed at a concentration of 50 μM in double-distilled H2O (1–40 and 40–1) or 0.1% TFA in 25 mM phosphate buffer (Whatman filter paper, and 700 units/ml DNase I) was added to cells and incubated for 30 min on ice. Lysates were centrifuged (15,000 × g for 10 min), and supernatants were used for analysis of activity or phosphorylation of ChAT or for analysis of proteins that co-immunoprecipitate with ChAT. ChAT activity was measured radioenzymatically using a modification of the method of Fonnum (27), as published previously (18).

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bicarbonate. After two changes of acetonitrile, gel pieces were dried by vacuum centrifuge and rehydrated in trypsin digestion buffer (50 mM ammonium bicarbonate, 5 mM CaCl₂) containing 12.5 ng/μl trypsin (Roche Molecular Biochemicals) (34). After a 45-min incubation on ice, excess trypsin solution was removed, 15 μl of digestion buffer without trypsin was added, and samples were incubated for 18 h at 37 °C. Tryptic peptides were extracted from the gel pieces with two changes of 100 μl of ammonium carbonate buffer by shaking in an orbital shaker for 45 min. After a brief centrifugation, the supernatants with eluted peptides were pooled and concentrated by vacuum centrifugation to a final volume of 15 μl. Pooled extracts were acidified with glacial acetic acid. This solution of peptides was used directly for MALDI-TOF mass spectrometric analysis. For ESI-MS/MS sequencing, peptides were purified on ZipTipC₁₈ (Millipore Corp.) and eluted from the tip resin with 65% acetonitrile and 1% acetic acid.

Phosphopeptides required for further analysis were eluted from TLC plates with water/acetonitrile (4:1, v/v) (36) and then reduced to dryness in a vacuum centrifuge and reconstituted in 2% acetonitrile and 1% acetic acid. This solution of peptides was used directly for MALDI-TOF mass spectrometric analysis. For ESI-MS/MS sequencing, peptides were purified on ZipTipC₁₈ according to the manufacturer’s instructions (Millipore Corp.) and eluted from the tip resin with 65% acetonitrile and 1% acetic acid.

Phosphoamino acid analysis was also performed on phosphopeptides eluted from cellulose plates or directly on mixtures of phosphopeptides recovered after in-gel tryptic digestion. Tryptic peptides were lyophilized, resuspended in 70 μl of 6 M HCl, and boiled at 110 °C for 1 h. One-
or two-dimensional phosphoamino acid mapping were performed as described by Boyle et al. (37).

**In Vitro Parent Ion Scanning—**Experiments were performed on a Q-TOF2 mass spectrometer (Micromass), equipped with a nanoflow source. The instrument was calibrated with [Glu1]Fibrinopeptide B (Sigma), and following desalting on a ZipTipC18™ (4 µl), the concentrated protein digest sample was loaded into a borosilicate capillary (type P; Micromass). A voltage between 600 and 1000 V was applied to the capillary in order to produce nanomolar flow. Parent ion scanning was performed over the m/z range of 300–1500 monitoring neutral loss of 97.9769 and 49.9885 with a collision energy of 32 V. Masses that corresponded incompletely hydrolyzed phosphopeptides. Data illustrated are representative of between three and five independent experiments.

**Peptide Sequencing by Mass Spectrometry—**Amino acid sequences of tryptic phosphopeptides of ChAT isolated from control and Aβ-treated cells or for identification of unknown proteins that co-immunoprecipitated with ChAT were obtained by mass spectrometry performed on a Micromass Q-TOF2 mass spectrometer equipped with a nanospray source and an online Waters CapLC (Waters). In all cases, 1 µl of sample was injected from the autosampler. The instrument was calibrated with [Glu1]Fibrinopeptide B (Sigma). A gradient consisting of 5–65% B in 15.5 min (A = 0.1% formic acid, B = acetonitrile with 0.1% formic acid) flowing at 1 µl/min was used to elute peptides from a 300-µm inner diameter reversed-phase precolumn (LC-packings, San Francisco, CA) to the mass spectrometer. Survey spectra were acquired in the m/z range of 400–2000. Doubly or triply charged precursor ions were automatically selected for fragmentation by the quadrupole mass filter. In some cases, specific masses were identified for fragmentation. Fragmentation was achieved by collision with argon gas in the collision cell. The collision energy was automatically varied depending on the charge state of the parent peptide. Resultant spectra were background-subtracted and deconvoluted using MaxEnt3 software provided in the Micromass MassLynx 3.5 software package. The sequence of the peptides was determined using PepSeq version 3.3 software also provided in the MassLynx 3.5 software.

**MALDI-TOF MS—**Proteins co-immunoprecipitating with ChAT were identified initially by MALDI-TOF mass spectrometry. Samples were mixed 1:1 (v/v) with matrix solution containing 1:1 ethanol/acetonitrile saturated with α-cyano-4-hydroxycinnamic acid. Each sample (1 µl) was spotted onto the MALDI target plate in triplicate. MALDI-TOF MS was performed on a MALDI-R mass spectrometer (Micromass). Calibration was performed externally using angiotensin I (Sigma), renin substrate (Sigma), adrenocorticotropic hormone clip 18–39 (Sigma) for a three-point calibration. In addition, for each sample, the lockmass method was used as additional calibration with the standard adrenocorticotropic hormone clip 18–39. The peptide mass fingerprint spectra were matched to the NCBI nonredundant data base entries by using the following programs, available on the World Wide Web: Profound (www.proteometrics.com) and Mascot (www.matrixscience.com). The mass tolerance was set to 60 ppm, and two missed cleavage sites were tolerated with the search restricted to human proteins.

**RESULTS**

**Characterization of Aβ Peptides—**Stock solutions of Aβ peptide used to treat cells in the present studies were analyzed by CD and electron microscopy to obtain information that would allow relative comparisons to be made about peptide conformations. As shown in Fig. 1A, CD analysis revealed that the secondary structure of Aβ(1–40) and Aβ(1–42) were qualitatively identical in that they were both composed predominantly of β-sheet conformation indicated by minimum molar ellipticity at 215 nm. Based on the shape of the CD spectra, negligible random coil or α-helical content was present in these two peptides. To obtain quantitatively similar signal strength on CD spectra, the concentration of Aβ(1–40) and Aβ(1–42) used were 50 and 30 µM, respectively. By comparison, the reverse

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**Fig. 3.** Treatment of IMR32 cells with Aβ-(1–42) results in phosphorylation of ChAT at a new site. **A**, ChAT was immunoprecipitated from lysates of untreated cells (C) and cells treated for 10 h with Aβ-(1–40), Aβ-(40–1), Aβ-(42–1), and Aβ-(1–42) and separated by SDS-PAGE. Following in-gel tryptic digestion, tryptic peptides of ChAT were analyzed by two-dimensional phosphopeptide mapping. Under all conditions tested, a phosphopeptide(s) with phosphorylation on serine residue(s) was observed. By comparison, an additional phosphopeptide corresponding to phosphorylation on threonine residue(s) was observed only in cells treated with Aβ-(1–42). **B**, tryptic peptides were also subjected to acid hydrolysis, and the phosphoamino acids produced were separated by two-dimensional electrophoresis. Circles indicate the migration of ninhydrin-stained phosphoamino acid standards, with closed circles corresponding to phosphoserine and broken circles corresponding to phosphothreonine. Positive spots remaining near sample origins represent incompletely hydrolyzed phosphopeptides. Data illustrated are representative of between three and five independent experiments.
peptides Aβ-(40–1) and Aβ-(42–1) had a predominantly random coil conformation. In support of the CD data, as illustrated in Fig. 1B, electron microscopic analysis confirmed that solutions of both Aβ-(1–40) and Aβ-(1–42), respectively. A doubly charged peak at 621.6 that corresponds to the phosphorylation of peptide 454–464 of human 69-kDa ChAT is seen in the Aβ-(1–42) sample but not in the sample from cells treated with Aβ-(1–40). Samples from untreated control samples and cells treated with reverse peptide Aβ-(42–1) yielded spectra similar to A, indicating lack of phosphorylation of this ChAT peptide. All samples were prepared in duplicate, and multiple injections were examined with identical results. C and D, two-dimensional patterns for phosphoamino acids and phosphopeptides of ChAT-T456A, respectively, from cells treated with Aβ-(1–42). This demonstrates the lack of threonine phosphorylation as compared with that observed in wild-type ChAT (shown in Fig. 3).

Identification of a New Phosphorylation Site in ChAT in Cells Treated with Aβ-(1–42)—IMR32 cells expressing ChAT were treated with Aβ-(1–42) peptidase for 10 h, and then ChAT was recovered by immunoprecipitation and subjected to in-gel tryptic cleavage. In control cells and cells treated with Aβ-(1–40) and the reverse sequence control peptides Aβ-(40–1) and Aβ-(42–1), a single phosphopeptide was observed, as illustrated in Fig. 3A. In contrast, treatment of cells with Aβ-(1–42) resulted in the appearance of a second phosphorylated ChAT peptide.

Short Term Exposure to Aβ-(1–42) Enhances Activity and Phosphorylation of ChAT—IMR32 cells expressing 69-kDa human ChAT were incubated with Aβ-(1–40) for varying times, and then incorporation of [32P]phosphate and enzyme activity were monitored. As illustrated in Fig. 2A, catalytic activity of ChAT was increased within 30 min of the addition of Aβ-(1–42) to cultures, with this effect being maximal at 10 h (2-fold increase in ChAT-specific activity). The effect of Aβ-(1–42) on ChAT activity followed a biphasic time course, with the response diminishing beyond 10 h. ImmunobLOTS shown in Fig. 2C (upper panel) demonstrate that cellular ChAT concentration was unchanged over the treatment interval. Phosphorylation of ChAT was also increased up to 3-fold by treatment of cells with Aβ-(1–42) in a manner that paralleled the time course for change in activity of the enzyme, as shown in Fig. 2B; the corresponding autoradiography data are provided in Fig. 2C (lower panel). Control cells were treated with inactive peptide Aβ-(42–1); ChAT activity measured in cells with the addition of Aβ-(42–1) did not differ from cells with no Aβ peptide added. ChAT activity was not altered in cells treated for up to 18 h with Aβ-(1–40) or its reverse peptide Aβ-(40–1) (data not shown). However, a 2-fold increase in [32P]phosphate incorporation into ChAT was found in IMR32 cells treated with Aβ-(1–40) between 2 and 6 h (data not shown).

Identification of a New Phosphorylation Site in ChAT in Cells Treated with Aβ-(1–42)—IMR32 cells expressing ChAT were treated with Aβ-(1–42) for 10 h, and then ChAT was recovered by immunoprecipitation and subjected to in-gel tryptic cleavage. In control cells and cells treated with Aβ-(1–40) and the reverse sequence control peptides Aβ-(40–1) and Aβ-(42–1), a single phosphopeptide was observed, as illustrated in Fig. 3A. In contrast, treatment of cells with Aβ-(1–42) resulted in the appearance of a second phosphorylated ChAT peptide.

Fig. 4. ESI-MS analysis of phosphorylated ChAT in IMR32 cells treated with Aβ-(1–42). ESI-MS spectra in the range of 620–625 m/z for tryptically digested ChAT from control and Aβ peptide-treated IMR32 cells are shown. A and B, spectra obtained for ChAT from cells treated with Aβ-(1–40) and Aβ-(1–42), respectively. A doubly charged peak at 621.6 that corresponds to the phosphorylation of peptide 454–464 of human 69-kDa ChAT is seen in the Aβ-(1–42) sample but not in the sample from cells treated with Aβ-(1–40). Samples from untreated control samples and cells treated with reverse peptide Aβ-(42–1) yielded spectra similar to A, indicating lack of phosphorylation of this ChAT peptide. All samples were prepared in duplicate, and multiple injections were examined with identical results. C and D, two-dimensional patterns for phosphoamino acids and phosphopeptides of ChAT-T456A, respectively, from cells treated with Aβ-(1–42). This demonstrates the lack of threonine phosphorylation as compared with that observed in wild-type ChAT (shown in Fig. 3).
Further analysis of these phosphopeptides following acid hydrolysis showed that all samples contained phosphorylated serine residue(s). Interestingly, phosphorylated ChAT obtained from cells treated with Aβ-(1–42) contained phosphothreonine as well as phosphoserine residues (Fig. 3B).

To identify the threonine residue phosphorylated in ChAT with Aβ-(1–42) treatment, ChAT was immunoprecipitated from IMR32 cells after 10 h of treatment with Aβ peptides. Following isolation by SDS-PAGE, ChAT-containing bands were subjected to in-gel tryptic digestion, and samples were prepared for mass spectrometric analysis by ESI-MS/MS. Mass spectra revealed a doubly charged peak at 621.6 m/z present in ChAT recovered from cells treated with Aβ-(1–42) (Fig. 4B) but not in cells treated with Aβ-(1–40) (Fig. 4A) or Aβ-(42–1) or in ChAT from untreated control cells (data not shown). Although other peaks were detected in tryptic digests of samples treated with Aβ-(1–42), none of these corresponded to ChAT peptides with potential phosphorylation sites. The amplitude of the signal detected for this tryptic phosphopeptide from ChAT from Aβ-(1–42)-treated cells was very low when compared with purified ChAT phosphorylated by incubation with protein kinases in vitro (see below). This suggests that a relatively low proportion of the enzyme is phosphorylated in situ. Consequently, sequence information was obtained by fragmenting at this m/z value for extended periods. Subsequent analysis of the fragmentation pattern revealed the C-terminal sequence VR as well as the partial sequence EAL. This allowed identification of this peptide as amino acid residues 454–464 of 69-kDa human ChAT with the sequence SATPEALAFVR and a mass of 1160.626. The immonium ion region also revealed the presence of the correct amino acids for the peptide encoding residues 454–464 of 69-kDa human ChAT (Pro, Val, Thr, Leu, Glu, Phe, and Arg). This sequence contains a putative consensus sequence for CaM kinase II involving phosphorylation of Thr456; the canonical consensus sequence for CaM kinase II is RX**S/T** (38–40), with the corresponding ChAT sequence of **RSA**T** (43). To confirm this assignment, Thr456 was changed to an alanine residue (ChAT-T456A) by site-directed mutagenesis to provide a plasmid for use as an investigative tool; the presence of the mutation was verified by DNA sequencing of the plasmid and ESI-MS/MS sequencing of the tryptic peptide encoding amino acid residues 454–464 in T456A-ChAT and absence of the tryptic peptide encoding the wild-type sequence. As illustrated in Fig. 4, C and D, analysis of tryptic peptides of ChAT-T456A obtained from IMR32 cells expressing the mutant enzyme and treated with Aβ-(1–42) by phosphopeptide and phosphoamino acid analysis revealed a single phosphopeptide, with serine being the residue phosphorylated. This finding is in sharp contrast to the results obtained with cells expressing wild-type ChAT shown in Fig. 3, A and B.

A doubly charged peak at 658.3 m/z (M = 1314.6) was also found in samples. This corresponds to the phosphorylated form of the tryptic peptide 432–442 of 69-kDa human ChAT (LVP-TYESASIR). This peptide contains a serine residue (Ser440) that was found previously to be phosphorylated by PKC (18).

ESI-MS/MS Identification of Threonine 456 as a Putative CaM Kinase II Phosphorylation Site in ChAT in Vitro—Immunoadfinity-purified 69-kDa ChAT was incubated under phosphorylating conditions with CaM kinase II and then resolved by SDS-PAGE and digested with trypsin for analysis by ESI-MS to identify phosphorylated peptide(s) and amino acid residue(s). Using parent ion scanning, a doubly charged peak at 621.6 m/z was found to produce a neutral loss of 98 that is indicative of loss of a phosphate group under moderate fragmentation conditions (41). This m/z value was subjected to full fragmentation in order to obtain sequence information. This
peptide sequenced to SApTPEALAFVR (where pT represents phosphothreonine), as can be seen in Fig. 5, indicating the presence of a phosphorylated threonine residue. This sequence corresponds to tryptic fragment 454–465 of 69-kDa ChAT that contains a putative consensus sequence for CaM kinase II; this is the same threonine residue (Thr456) found to be phosphorylated in IMR32 cells treated with Aβ(1–42).

**Assay of CaM Kinase II Activity in IMR-32 Cells with Aβ Peptide Treatment**—We tested whether CaM kinase II was activated in IMR32 cells treated with Aβ peptides. Cells expressing wild-type ChAT were grown with Aβ(1–42), Aβ(1–40), or reverse peptides Aβ(42–1) or Aβ(40–1) and then digi-
ton-permeabilized and incubated with substrate peptide encoding a CaM kinase II phosphorylation consensus sequence. As illustrated in Fig. 6A, 10-h treatment with Aβ(1–42) selectively increased phosphorylation of the CaM kinase II substrate peptide by more than 2-fold, indicating activation of this protein kinase. Phosphorylation of the substrate peptide in cells treated with the other three Aβ peptides did not differ from untreated control cells. This corresponds to the time point when Aβ(1–42)-mediated increases in activity and phosphorylation of ChAT are maximal. Time course experiments revealed that phosphorylation of CaM kinase II substrate peptide was not different from control at 30 min or 2 h, significantly increased (160% compared with control) by 6 h, and returned to control levels at 14 and 18 h after the addition of Aβ(1–42) (data not shown). To further confirm that phosphorylation of the substrate peptide in Aβ(1–42)-treated cells was related to activation of CaM kinase II, we tested the effects of the CaM kinase II inhibitor KN-93 and its inactive analogue KN-92. The addition of 5 μM KN-93, but not KN-92, to cells during treatment with Aβ(1–42) markedly reduced subsequent phosphorylation of the CaM kinase II substrate peptide (Fig. 6B).

**Hierarchical Activation of ChAT with Phosphorylation by PKC and CaM Kinase II**—We investigated the relationship between phosphorylation of ChAT by PKC and CaM kinase II and activation of the enzyme using both purified recombinant ChAT and IMR32 cells treated with Aβ(1–42). As demonstrated in Fig. 7A, purified ChAT activity was increased about 3-fold by CaM kinase II-mediated phosphorylation only when the enzyme was also phosphorylated by PKC. Phosphorylation of ChAT by PKC alone led to a 2-fold increase in enzyme activity, whereas phosphorylation by CaM kinase II alone did not alter ChAT activity. Moreover, inhibition of PKC by H7 (10 μM) blocked activation of ChAT observed when the enzyme was sequentially incubated with PKC and CaM kinase II under phosphorylating conditions. Incubation of purified ChAT with PKC and CaM kinase II in the presence of KN-93 resulted in a 2-fold increase in ChAT activity similar to that observed for phosphorylation of ChAT by PKC alone.

As shown in Fig. 7B, H7 also inhibited activation of ChAT in IMR32 cells treated with Aβ(1–42) for either 30 min or 10 h. On the other hand, the CaM kinase II inhibitor KN-93 partially attenuated activation of ChAT by 10 h of Aβ treatment and had no effect on ChAT activation at 30 min of treatment. Whereas Thr456 is situated in a consensus sequence that could be recognized by CaM kinase II, it is also positioned at −1 from a proline residue, creating the possibility that this proline-directed threonine residue could be phosphorylated by other protein kinases such as mitogen-activated protein kinase. To test this, we used inhibitors of MEK-1/MEK-2 (U0126) and p38 mitogen-activated protein kinase (SB202190) to probe their involvement in phosphorylation of Thr456 in Aβ(1–42)-treated IMR32 cells. The addition of U0126 or SB202190 to IMR32 cells during Aβ treatment also had no effect on Aβ-mediated activation of ChAT at either time point.

To address this further, we investigated the temporal relationship of serine and threonine phosphorylation of ChAT in IMR32 cells treated with Aβ(1–42) and the effect of kinase inhibitors on phosphorylation of ChAT in Aβ-treated cells. As shown in Fig. 7C, H7 reduced serine phosphorylation to control levels at both 30 min and 10 h, whereas KN-93 eliminated threonine phosphorylation in cells treated with Aβ(1–42) for 10 h. U0126 or SB202190 had no effect on phosphorylation at either 30 min or 10-h time points. We also tested the effect of Aβ(1–42) treatment on activity of ChAT-S440A and ChAT-T456A mutants in comparison with that of wild-type enzyme. As shown in Fig. 8, ChAT-specific activity does not differ in the
Fig. 7. Hierarchical activation of purified ChAT and ChAT in Aβ-(1–42)-treated IMR32 cells related to phosphorylation by PKC and CaM kinase II. A, purified recombinant ChAT (1 μg/sample) was phosphorylated by either PKC or CaM kinase II or by both kinases. Phosphorylation by CaM kinase II alone did not alter ChAT activity compared with control, whereas phosphorylation by PKC alone led to a 2-fold increase in ChAT activity that was blocked by H7. When ChAT was phosphorylated by both PKC and CaM kinase II, activity of the enzyme increased 3-fold. Importantly, inhibition of CaM kinase II by KN-93 attenuated enhancement of ChAT activity to the level obtained with phosphorylation by PKC alone. Moreover, inhibition of PKC by 10 μM H7 blocked activation of ChAT when it was incubated under phosphorylating conditions with PKC and CaM kinase II. B, IMR32 cells were incubated with Aβ-(1–42) for 30 min or 10 h in the absence or presence of protein kinase inhibitors to test involvement of these kinases in the two phases of activation of ChAT observed in these studies. None of the inhibitors altered basal ChAT activity. At 30 min of treatment with Aβ-(1–42), ChAT activity was increased about 1.6-fold, with this effect blocked by H7. At 10 h of treatment with Aβ-(1–42), ChAT activity was increased about 2-fold. This effect was attenuated to a 1.6-fold increase by KN-93 and completely blocked by H7. The MEK-1/MEK-2 and p38-kinase inhibitors U0126 and SB202190 did not alter Aβ-(1–42)-mediated changes in ChAT activity. C, phosphoamino acid analysis revealed basal phosphorylation of ChAT on serine but not threonine residue(s) under control conditions. This was not altered by any of the kinase inhibitors. Treatment of cells with Aβ-(1–42) for 30 min increased phosphoserine levels, but phosphothreonine was not observed. H7 decreased phosphoserine levels. At 10 h of Aβ-(1–42) treatment, there was an increase in phosphoserine levels and phosphorylation on threonine residue(s). KN-93 blocked threonine phosphorylation, whereas H7 blocked serine phosphorylation. For A and B, results are expressed as mean ± S.E. of four or five independent experiments with duplicate or triplicate determinations. Statistical differences at the level of p < 0.05 were determined by one-way analysis of variance with post hoc Tukey’s multiple comparison test. *, differences relative to controls; #, differences relative to PKC alone (A) or between treatment of cells for 30 min and 10 h (B). Data shown in C are representative of at least three separate experiments.
proteins co-immunoprecipitated with both wild-type ChAT and mutant ChAT-S440A from cells treated with Aβ(1–42). These were not observed in lanes corresponding to treatment of cells with Aβ-(1–40) or Aβ-(42–1) or from cells expressing mutant ChAT-T456A treated with Aβ(1–42).

A combined mass spectrometry and immunoblot approach was taken to begin to identify proteins co-immunoprecipitating with ChAT. For mass spectrometric analysis, bands were excised from SDS-PAGE gels and digested with trypsin. Based on MALDI-TOF peptide mass fingerprint data of the trypically digested protein, we identified one co-immunoprecipitating protein with an apparent molecular mass of about 90 kDa to be human VCP. Sequence coverage of 40% was obtained, and 38 peptides were matched to VCP in two independent analysis. Partial sequence data of tryptic peptides obtained by ESI-MS/MS also matched to VCP sequences. Fig. 9B confirms the identity of VCP by immunoblot with anti-VCP antibody in ChAT-immunoprecipitates only from cells expressing wild-type 69-kDa ChAT that were treated with Aβ(1–42) but not other Aβ peptides; this finding was obtained in three independent experiments. To further verify this interaction, VCP was immunoprecipitated from lysates of IMR32 cells expressing wild-type ChAT treated with Aβ peptides using an antibody to VCP. As illustrated in Fig. 9C, a ChAT immunopositive band was observed in the anti-VCP immunoprecipitate from cells treated with Aβ(1–42).

**DISCUSSION**

Increased concentrations of Aβ peptides are released into the brain by cleavage of APP in neurodegenerative diseases such as Alzheimer’s disease and following traumatic head injury. In the present study, we tested the hypothesis that exposure of IMR32 neuroblastoma cells stably expressing ChAT to Aβ peptides would alter function of the enzyme. We demonstrate for the first time that 1) phosphorylation patterns and activity of ChAT are changed by short-term exposure of cells to Aβ(1–42), 2) exposure of cells to Aβ(1–42) leads to activation of CaM kinase II and phosphorylation on a threonine residue in ChAT, 3) Thr^{456} in ChAT is phosphorylated by CaM kinase II in *vitro* and in cells following treatment with Aβ(1–42), 4) the increase in ChAT activity observed with Aβ(1–42)-treatment is hierarchically organized with phosphorylation of Ser^{440} by PKC being required, and this effect is amplified by CaM kinase II-mediated phosphorylation of Thr^{456} at 10 h; 5) treatment of cells with Aβ(1–42) leads to Thr^{456} phosphorylation-dependent protein-protein interactions between ChAT and other cellular proteins, with one of these identified as VCP.

Although mechanisms by which Aβ peptides mediate either acute or long term actions on neurons are not resolved, it is known that they interact with several cell surface receptors including α-7 nicotinic ACh receptor (21, 42), p75 nerve growth factor receptor (43), G-protein-linked formyl-peptide receptor (44), and advanced glycosylation end product receptor (45). Signal transduction pathways and cellular responses recruited by binding of Aβ peptides to these receptors have not been worked out, but some responses are mediated by pertussis toxin-sensitive G-protein-coupled mechanisms (22). Interaction of Aβ peptides with some receptors alters cellular calcium homeostasis with enhanced influx of extracellular calcium or release from intracellular stores (24, 48). Also, Aβ peptides can form calcium ionophores in plasma membrane, leading to calcium conductances across plasma membrane (48, 49). In addition, intracellular accumulation Aβ(1–42) but not other Aβ peptides, possibly mediated by interaction with the α-7 nicotinic ACh receptor (50), led to neuronal toxicity through a p53-Bax cell death pathway (51). In regard to the present studies, it is known that IMR32 cells express α-7 nicotinic ACh
Altered Phosphorylation of ChAT with β-Amyloid Treatment

receptors (50) and may express low levels of p75 nerve growth factor receptors (51); it is not known whether other putative binding sites for Aβ peptides are expressed by these cells. Increased cytosolic free calcium levels caused by Aβ peptide treatment could activate Ca\(^{2+}\)-dependent protein kinases such as PKC and CaM kinase II. ChAT is a substrate for both of these kinases (17). The present study extends these findings by demonstrating a link between activation of CaM kinase II and PKC and regulation of ChAT activity and phosphorylation of ChAT and its interaction with other proteins. We also showed that Aβ-(1–42) increased CaM kinase II activity in permeabilized IMR32 cells, and, although we did not measure PKC activation in permeabilized cells, we found increased PKC activity in cell lysates (data not shown). Activation of PKC isoforms by Aβ peptides was reported previously (52–54). A key observation in the present study is that only Aβ-(1–42) caused changes in ChAT phosphorylation, activity, and protein interactions. Mechanisms underlying this selectivity are unclear, but other studies have demonstrated actions mediated specifically by Aβ-(1–42) and not other Aβ peptides. To confirm that this did not relate to differences in conformation between Aβ-(1–42) and Aβ-(1–40), we analyzed samples of peptides by CD and electron microscopy and found both preparations to be comprised of Aβ-fibrils and to have similar β-sheet structure. It is likely that the differences in cellular responses to Aβ-(1–42) and Aβ-(1–40) observed in the present study relate to differences in ability of the peptides to stimulate receptors that initiate the cellular events.

Using phosphopeptide mapping, phosphoamino acid analysis, and mass spectrometry, we identified Thr\(^{456}\) as a novel phosphorylation site in 69-kDa ChAT following short treatment of IMR32 cells with Aβ-(1–42). Residue Thr\(^{456}\) in ChAT is phosphorylated in Aβ-(1–42)-treated cells over a time course coinciding with increased activity of CaM kinase II. Since other protein kinases including members of the mitogen-activated protein kinase family could be activated by Aβ-treatment (21), we used specific inhibitors to determine that these are probably not mediating phosphorylation of Thr\(^{456}\) in this situation. Also, kinase inhibitors and site-directed mutagenesis of critical residues in ChAT were used to establish a relationship between phosphorylation of Ser\(^{440}\) by PKC and phosphorylation of Thr\(^{456}\) by CaM kinase II in enhanced catalytic activity of ChAT. Phosphorylation of ChAT on Ser\(^{440}\) by PKC alone increased ChAT activity by 2-fold, with this increased to about 3-fold when Thr\(^{456}\) is also phosphorylated by CaM kinase II (Fig. 2B). Importantly, phosphorylation by CaM kinase II alone did not alter ChAT activity. This suggests that phosphorylation of ChAT on Ser\(^{440}\) leads to the initial activation of ChAT by 30 min. This increased ChAT activity is not maintained over the next few hours, but a second phase of activation occurs by 10–14 h after the addition of Aβ-(1–42) paralleling increased CaM kinase II activity, phosphorylation of Thr\(^{456}\), and increased serine phosphorylation. Mechanisms underlying this delayed increase in ChAT activity and activation of CaM kinase II are unclear. One possibility, however, is that cellular events initiated with acute addition of Aβ-(1–42) result in production/release of cellular mediators producing effects several hours later. These delayed effects include activation of CaM kinase II, enhanced activity of ChAT, and interaction of ChAT with VCP and other cellular proteins.

To date, there have been no reports identifying proteins that ChAT interacts with in the cell. Previously, it was suggested that neurons contain endogenous modulator(s) of ChAT that may be proteins (55, 56). Other compounds that may regulate ChAT appear to be small molecules or lipid products, such as phosphomonoesters (57) and dihydrolipoic acid (58). In the present study, a number of proteins immunoprecipitated with ChAT following treatment of cells expressing ChAT with Aβ-(1–42) and associated with phosphorylation of Thr\(^{456}\). We identified one of these by mass spectrometry, with confirmation by immunoblotting, to be VCP. VCP, a member of the AAA-ATPase family of proteins, is a multifunctional protein in yeast and mammalian cells (59–61) with roles in diverse cellular functions including cell cycle regulation, clathrin-mediated receptor endocytosis, protein ubiquitination, and proteasome function. Other cellular proteins with which VCP is known to interact include BRCA1 (62) and histone deacetylase-6 (46).
Interestingly, it appears that association of VCP with ChAT is phosphorylation-dependent, occurring under conditions where both Thr^466 and Ser^460 are phosphorylated. VCP does not co-immunoprecipitate with ChAT in βA-(1–42)-treated cells expressing mutant ChAT-S440A. The functional significance of interaction of ChAT with VCP is unclear. A predominant cellular function of VCP is its role in the link between acetylation/deacetylation/ubiquitination and proteasomal degradation of proteins (46, 61). One function for an acetyltransferase is to add acetyl-residues to proteins to protect them from ubiquitination and targeting for degradation (46, 47). Although a function for the interaction between ChAT and VCP remains to be determined, two hypothesis could be tested. In the first, ubiquitinated-ChAT binds to VCP, which then serves as a chaperone to target the enzyme to the proteosome. In the second, ChAT may serve as an acetylase, transferring acetate to other proteins to alter their function or delay their degradation. As an acetylase, ChAT catalyzes the transfer of acetyl groups to proteins (46, 61). One function for an acetyltransferase is to add acetyl-residues to proteins to protect them from ubiquitination and targeting for degradation (46, 47). Although a function for the interaction between ChAT and VCP remains to be determined, two hypothesis could be tested. In the first, ubiquitinated-ChAT binds to VCP, which then serves as a chaperone to target the enzyme to the proteosome. In the second, ChAT may serve as an acetylase, transferring acetate to other proteins to alter their function or delay their degradation. As an acetylase, ChAT catalyzes the transfer of acetyl groups to proteins (46, 61). One function for an acetyltransferase is to add acetyl-residues to proteins to protect them from ubiquitination and targeting for degradation (46, 47). Although a function for the interaction between ChAT and VCP remains to be determined, two hypothesis could be tested. In the first, ubiquitinated-ChAT binds to VCP, which then serves as a chaperone to target the enzyme to the proteosome. In the second, ChAT may serve as an acetylase, transferring acetate to other proteins to alter their function or delay their degradation. As an acetylase, ChAT catalyzes the transfer of acetyl groups to proteins (46, 61). One function for an acetyltransferase is to add acetyl-residues to proteins to protect them from ubiquitination and targeting for degradation (46, 47).

The findings reported in the present study identify a new action of βA-(1–42) on cholinergic neuron function. Although loss of ChAT activity as a consequence of the toxic effects of Aβ has been examined. Exposure of primary cultures of rat septal neurons to Aβ-(1–42), but not Aβ-(1–40), at concentrations used in the present study for 12–24 h suppressed ACh synthesis but did not alter ChAT activity (9). Also, exposure of SN56 cells to 100 nM Aβ-(1–42) for 48 h significantly reduced ChAT activity (10). As demonstrated in the present study, activation of cell signaling pathways and alteration of the intracellular milieu in response to exposure to Aβ-(1–42) leads to short term changes in the phosphorylation state of ChAT that could result in acute changes in cholinergic neurotransmission.

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Phosphorylation of 69-kDa Choline Acetyltransferase at Threonine 456 in Response to Amyloid-β Peptide 1–42
Tomas Dobransky, Dyanne Brewer, Gilles Lajoie and R. Jane Rylett

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