Functional Characterization of Phosphorylation of 69-kDa Human Choline Acetyltransferase at Serine 440 by Protein Kinase C*

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Received for publication, December 26, 2000, and in revised form, February 28, 2001
Published, JBC Papers in Press, April 12, 2001, DOI 10.1074/jbc.M011702200

Choline acetyltransferase, the enzyme that synthesizes the transmitter acetylcholine in cholinergic neurons, is a substrate for protein kinase C. In the present study, we used mass spectrometry to identify serine 440 in recombinant human 69-kDa choline acetyltransferase as a protein kinase C phosphorylation site, and site-directed mutagenesis to determine that phosphorylation of this residue is involved in regulation of the enzyme’s catalytic activity and binding to subcellular membranes. Incubation of HEK293 cells stably expressing wild-type 69-kDa choline acetyltransferase with the protein kinase C activator phorbol 12-myristate 13-acetate showed time- and dose-related increases in specific activity of the enzyme; in control and phorbol ester-treated cells, the enzyme was distributed predominantly in cytoplasm (about 88%) with the remainder (about 12%) bound to cellular membranes. Mutation of serine 440 to alanine resulted in localization of the enzyme entirely in cytoplasm, and this was unchanged by phorbol ester treatment. Furthermore, activation of mutant enzyme in phorbol ester-treated HEK293 cells was about 50% that observed for wild-type enzyme. Incubation of immunoaffinity purified wild-type and mutant choline acetyltransferase with protein kinase C under phosphorylating conditions led to incorporation of [32P]phosphate, with radiolabeling of mutant enzyme being about one-half that of wild-type, indicating that another residue is phosphorylated by protein kinase C. Acetycholine synthesis in HEK293 cells expressing wild-type choline acetyltransferase, but not mutant enzyme, was increased by about 17% by phorbol ester treatment.

Choline acetyltransferase (Chat, EC 2.3.1.6) catalyzes synthesis of the neurotransmitter acetylcholine (ACh) in cholinergic neurons in peripheral and central nervous systems. These neurons control a wide range of physiological and biochemical processes in most organ systems, including regulation of cardiovascular and motor functions, and cognitive functions such as learning, attention, and memory. Diminished Chat activity signals degeneration of cholinergic neurons in a number of neurodegenerative disorders. For example, a consistent finding in necropsy brain of subjects with Alzheimer disease is profound loss of Chat that correlates with diminished cognitive function early in the course of the disease. Decreased Chat activity can be accounted for, at least in part, by loss of cholinergic neurons, but may also be related to decreased expression of cholinergic phenotypic genes and/or altered regulation of the enzymes catalytic activity leading to decreased function.

There is polymorphism in expression of mRNA for Chat and, in human only, one of these transcripts, denoted the M isoform, has two translation initiation sites yielding proteins with apparent molecular masses of 69 and 82 kDa; all other transcript isoforms encode the 69-kDa form of enzyme only (1, 2). We demonstrated recently that the 82-kDa form of the enzyme is targeted to nucleus of cells, whereas 69-kDa Chat is localized to non-nuclear cellular compartments such as cytoplasm and plasma membrane (3). Whereas cytosolic-membrane-associated Chat is clearly involved in catalyzing ACh biosynthesis, the functional role of the nuclear form of the enzyme remains to be elucidated.

A critical issue in production of the neurotransmitter ACh is subcellular distribution and regulation of catalytic activity of its biosynthetic enzyme Chat. Factors controlling Chat enzyme activity, and the role that post-translational modifications play in this in healthy neurons and during pathological processes such as Alzheimer disease is poorly understood. It has been demonstrated previously that Chat undergoes phosphorylation both in vitro and in nerve terminals by calcium-dependent protein kinases (4–6). Results obtained recently in our laboratory showed that Chat serves as a substrate for a number of protein kinases, but that its enzymatic activity is regulated by phosphorylation by only some of the kinases. The highest activities induced by phosphorylation were observed following phosphorylation of Chat by protein kinase C (PKC) (7). In terms of subcellular compartmentalization, it appears that phosphorylation may regulate association of Chat with plasma membrane or membranes of subcellular organelles (4), and partitioning of enzyme between cytosol and membrane fractions.

The current studies are aimed at identification of phosphorylation sites of 69-kDa human Chat by PKC, and characterization of their functional role in regulation of enzymatic activity and/or subcellular compartmentalization of the enzyme within the cell. Using matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) analysis and MALDI-TOF (time-of-flight) in linear and reflectron mode, we identified serine 440 as a PKC phosphorylation site, and determined...
that phosphorylation of this amino acid plays a role in membrane-association of the enzyme and participates in regulation of its catalytic activity.

**Experimental Procedures**

**Preparation of ChAT Constructs**—The cDNA for human 69-kDa ChAT (N1-ChAT) in pcDNA3 was kindly provided by Dr. H. Misawa (Tokyo Metropolitan Institute for Neuroscience, Tokyo). The mutant S440A-ChAT was prepared by site-directed mutagenesis of Ser440→Ala in wild-type 69-kDa human ChAT by polymerase chain reaction using the forward primer 5'-GGAGACCAGGCATTGCGGCA-3' and the reverse primer 5'-TCGCGGTACCGGCGGCTTCTC-3' coupled with forward primer 5'-AAGGGTGTGCGACCTAGGCACAGAACT-3' and reverse primer 5'-TGTGGGTACCGTGAAGGGTGTT-3' to give the full-length mutant cDNA with KpnI and BamHI restriction sites at the 5'- and 3'-ends, respectively. Following restriction enzyme nuclelease digestion of the ends, the fragment was ligated into pcDNA3. Integrity of the mutation and the full-length cDNA was confirmed by sequencing.

**Expression of Wild-type and S440A Mutant 69-kDa ChAT in HEK293 Cells**—Monolayers of HEK293 cells were transfected with plasmid DNA containing inserts encoding wild-type and mutant 69-kDa human ChAT using the LipofectAMINE 2000 method (Life Technologies, Inc.). G418-resistant transformants were selected and tested for ChAT enzyme activity by radioenzymatic assay and ChAT protein by immunoblot. Cells were maintained in modified Eagle's medium containing 10% fetal calf serum, 50 units/ml penicillin/streptomycin, and 0.5 mg/ml G418 in humidified 5% CO2 at 37 °C.

**Immunoaffinity Columns and One-step Purification of 69-kDa ChAT**—Two different immunoaffinity columns were used for purification of purified native ChAT in an one-step purification protocol. The antibody used was a rabbit polyclonal antibody prepared to a peptide encoding the last 13 amino acids at the carboxyl terminus of human ChAT (called CTab) (7). The first column was prepared by attachment of Fab (antigen-binding fragments) of CTab to CNBr-Sepharose. Fab fragments were prepared by proteolytic cleavage of whole affinity-purified antibody with immobilized CNBr (50 mg/ml gel) was dissolved in 5 ml of antibody binding buffer (50 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 25 mM Na4EDTA, 10 mg/ml (biotinyl)-labeled phosphatidylserine) and added to 5 ml of Protein G-Sepharose. After washing with Tris-HCl, pH 8.2, and centrifuged at 35,000 rpm for 1 h with the supernatant yielding the flow-through from the Protein G-Sepharose column, were dialyzed subsequently applied to a Protein G-Sepharose column to separate the 3°C against three changes of 40 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 25 mM Na4EDTA, 10 mg/ml (biotinyl)-labeled phosphatidylserine) was added to the purified enzyme preparation (1–3 mg/ml) and incubated for varying times, then the phosphorylation reaction was stopped by addition of electrophoresis sample buffer. For the stoichiometry experiments, the concentration of purified ChAT was estimated from Coomassie Blue-stained gels using bovine serum albumin as a standard. One microgram (14.5 pmol) of purified ChAT was phosphorylated by PKC (0.4 milliunit) for varying times in the presence of 0.6 nmol of [γ-32P]ATP (15 μCi), as described above. Samples were run on one-dimensional SDS-PAGE gels, then proteins were transferred to nitrocellulose membrane; only residual radioactivity was retained in the gels after transfer as monitored by Cerenkov counting. Nitrocellulose membranes were exposed to film, and following brief (5 min) autoradiography, membranes were processed for immunoblotting. Specifically, areas corresponding to radioactive bands on autoradiography were cut from the membranes, and incorporated [32P]phosphate was quantified by Cerenkov counting.

**In Vivo Treatment of HEK293 Cells with PMA**—Cells were treated at ~50% confluence. 2 h before treatment, fresh medium was added to the cells, then the phorbol ester phorbolester 12-myristate 13-acetate (PMA) was diluted from a 1 mM stock in Me2SO to a concentration of 25 μM and added to the cells for varying times and at varying concentrations. Following treatment, cell lysates were prepared for measurement of total ChAT activity by sonication (3 × 15 s) in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM AEBSF, leupeptin/aprotinin/pepstatin at 10/25/10 μg/ml, 500 μM sodium orthovanadate, 10 mM sodium fluoride, and 250 μM eserin e sulf ate). For ChAT subcellular localization and activation studies, cells were treated for 2 h with 1 μM PMA.

**Subcellular Fractionation of HEK293 Cells**—Wild-type and mutant 69-kDa ChAT-expressing HEK293 cells were washed twice with ice-cold PBS then scraped into PBS and pelleted by centrifugation at 700 g for 5 min. Cells were gently resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 3 mM MgCl2, 10 mM NaCl, 1 mM AEBSF, leupeptin/aprotinin/pepstatin at 10/25/10 μg/ml, 500 μM sodium orthovanadate). The original post-nuclear supernatant was combined with the washes then centrifuged at 35,000 rpm for 1 h with the supernatant yielding the cytosolic fraction and the membrane pellets were washed three times in wash buffer containing 1% Nonidet P-40 by sonication (3 × 15 s). Purified nuclei were not used in the present study, because this subcellular fraction contains little 69-kDa ChAT (3).

**ChAT Activity Measurement**—ChAT activity was measured radioenzymatically by a modified method of Fonnum (8), as published previ

**One-dimensional SDS-PAGE**—One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5 or 10% gels according to the method of Laemmli (10). After electrophoresis, gels were stained with Coomassie Brilliant Blue or transferred to nitrocellulose for immunoblotting. For staining, gels were incubated in 0.05% Coomassie Blue-R250 in 10% acetic acid and 50% methanol for 20 min; in the case of samples to be prepared for mass spectrometry, gels were stained with acetic acid/methanol for an additional 3 h to ensure adequate removal of SDS.

**Western Blot Analysis**—For immunoblotting, proteins from SDS-PAGE gels were transferred onto nitrocellulose membranes in a semidry electrophoretic apparatus using transfer buffer (48 mM Tris, 39 mM glycine) containing 20% methanol. Nitrocellulose membranes were stained after protein transfer with 0.02% solution of Ponceau-S in 1%...
samples were solubilized in choline kinase reaction buffer (choline metabolites remaining in the aqueous phase. Choline esters were separated by C18 reverse phase HPLC, and fractions containing \[^{32}P\]phosphopeptides were identified by Cerenkov counting of all fractions. Masses of peptides present in these fractions were obtained by MALDI-MS (ToFSpec SE, Micromass, Inc., Beverly, MA), then compared with theoretical tryptic peptide masses for ChAT using the program GPMAW (Lighthouse Data, Denmark). The in-gel protein trypsinization, HPLC, and MALDI-MS analyses were performed at the Howard Hughes Medical Institute Biopolymer Facility/W. M. Keck Biotechnology Resource Laboratory at Yale University. Additional MALDI-TOF analysis was performed at the Molecular Medicine Research Centre Mass Spectrometry Laboratory at the University of Toronto. In this case, protein samples in SDS-polyacrylamide gel processed were identified by autoradiography. Following tryptic digestion of the isoforms by autoradiography. Following tryptic digestion of the resulting tryptic peptides were separated by two-dimensional SDS-PAGE using the method of Garrels (11) with isoelectric focusing gels containing 2% Ampholines, pH 3.5-10, and the second dimension run on 7.5 % separating gel (thickness 1 mm). Protein spots corresponding to \[^{3}P\]phosphorylated isoforms identified by autoradiography were cut from the Coomassie Blue-stained gel, rinsed, incubated with sequencing grade-modified (TPCK) trypsin (Promega), and reduced, and alkylated by carboxymethylation (12). The resulting tryptic peptide sequences for phosphorylation by PKC, including SYK (position 350), SYL (350–352), SYR (161–163), TNR (255–257), THR (283–285), PMA treatments, ChAT activity was measured in crude lysates of the cells. Data are expressed as the mean ± S.E. of five independent experiments with duplicate measurements. Curves were fit to data using GraphPad Prism. Statistical differences at the level of \(p < 0.05\) were determined by Student’s t test when compared with control values and denoted by asterisks.

**RESULTS**

**PKC-mediated Phosphorylation of 69-kDa ChAT**—In a previous study, we demonstrated that 69-kDa human ChAT is a substrate for a number of protein kinases, including PKC, with phosphorylation of purified recombinant ChAT in *vitro* by PKC leading to a 2-fold increase in catalytic activity of the enzyme (7). In the present study, we extended this observation to determine which isoforms of PKC phosphorylate ChAT. Using incorporation of \[^{32}P\]phosphate and autoradiography to monitor covalent modification by PKC isoforms, we observed that 69-kDa human ChAT was phosphorylated by PKC α, β-1, γ, δ, ε, and η but not by PKC β-2 (data not shown).

**Effect of Activation of PKC by PMA on 69-kDa ChAT in Situ**—To test the effect of activation of PKC on ChAT activity in *situ*, monolayers of HEK293 cells stably expressing 69-kDa human ChAT were treated with the phorbol ester PMA. As shown in Fig. 1, this resulted in a time- and dose-dependent activation of the recombinant enzyme. ChAT activity was significantly increased by 10 min with the effect becoming maximal at 157 ± 10% of control and reaching a plateau by 2 h (Fig. 1A). In terms of effective concentration, an EC\(_{50}\) value of about 0.3 μM was determined from the sigmoidal dose-response curve shown in Fig. 1B, with maximal increase in ChAT activity obtained at about 1 μM (158% ± 8%). The effect of PMA appeared to be biphasic with concentrations of PMA above 2 μM resulting in smaller increases in ChAT activity (data not shown).

**One-step Purification of 69-kDa Human ChAT**—Immunoaffinity columns prepared by covalent binding of Fab fragment of the anti-ChAT antibody CTab to CNBr-Sepharose or whole purified antibody to Protein G-Sepharose allowed isolation of highly purified enzyme in a single purification step. This preparation of purified native protein is comprised of the same enzyme isoforms as produced in cells, as demonstrated by immunoblots of two-dimensional SDS-PAGE gels (data not shown). The purity of ChAT obtained from the one-step immunoaffinity purification protocol using High-5 cell lysate is greater than 95% as demonstrated on Coomassie Blue-stained gels (Fig. 2), with a yield of at least 90% of total ChAT activity when compared with total activity in the crude extract.

**Identification of PKC Phosphorylation Site(s)**—The 69-kDa form of human ChAT contains 10 putative canonical consensus sequences for phosphorylation by PKC, including SYK (position 126–128), SYT (161–163), TNR (255–257), THR (283–285), SSR (346–348), SRK (347–349), SIR (440–442), SEK (476–478), SNR (532–534), and SSK (586–588). The strategy adopted for identification of functional PKC phosphorylation site(s) by mass spectrometry is outlined in Fig. 3. Purified recombinant ChAT was incubated under phosphorylating conditions with PKC and \([γ-^{32}P]ATP\) then separated by two-dimensional SDS-PAGE to allow identification of phosphorylated isoforms by autoradiography. Following tryptic digestion of the \[^{3}P\]-labeled ChAT isoforms, the resulting peptides were separate...
rated by HPLC, and two fractions were identified to contain $^{32}$P-labeled phosphopeptide. These were recovered and analyzed by MALDI-MS. One fraction contained a peptide with mass of 1390.43 (±0.2%), whereas the other contained a peptide with mass of 1237.30 (±0.2%). Comparison with the theoretical tryptic peptide masses for ChAT revealed two peptides with the calculated mass of 1391.764 having sequences LVPTYESASIRR (residues 432–443) and RLVPTYESASIR (residues 431–442). Another peptide with a calculated mass of 1235.663 had the sequence LVPTYESASIR (residues 432–442). These three peptides all contained the PKC consensus sequence SIR (residues 440–442) therefore identifying the candidate phosphorylation site serine 440. Further analysis using MALDI-TOF in linear and reflectron mode revealed an 80-Da shift in the mass of this peptide from predicted mass of 1391.764 to a measured mass of 1472.03, indicative of the presence of a phosphate group. Based on this confirmation of serine 440 as a putative phosphorylation site, we pursued functional analysis through mutagenesis of this residue.

**Phosphorylation of Wild-type and Mutant 69-kDa ChAT**—To investigate the biological role of phosphorylation of 69-kDa human ChAT at serine 440, we prepared a site-directed mutant in which serine 440 was changed to alanine (called S440A-ChAT). This mutant was expressed and purified from crude extract of High-5 cells on a CTab/Fab-CNBr-Sepharose column as described under “Experimental Procedures.” Crude extract (50 μg of protein) and purified enzyme (~2 μg of protein) were run on one-dimensional SDS-PAGE gels followed by Coomassie Blue staining. The purification yield was at least 90% of enzyme detected by measuring the enzymatic activity.

| Sample          | Total Activity | Protein | Specific Activity | Yield |
|-----------------|----------------|---------|------------------|-------|
| Crude Extract   | 94             | 58.2    | 1.6              | 100   |
| CTab Fab-CNBr   | 85             | 0.27    | 315              | 90    |

**FIG. 2.** Immunoaffinity purification of ChAT. ChAT was purified from crude extract of High-5 cells on a CTab/Fab-CNBr-Sepharose column as described under “Experimental Procedures.” Crude extract (50 μg of protein) and purified enzyme (~2 μg of protein) were run on one-dimensional SDS-PAGE gels followed by Coomassie Blue staining. The purification yield was at least 90% of enzyme detected by measuring the enzymatic activity.

**Subcellular Localization of ChAT—**Subcellular compartmentalization of wild-type and S440A-ChAT and the effect of activation of PKC in situ by PMA on this measure was determined in HEK293 cells stably expressing the two forms of the enzyme. Interestingly, subfractionation of cells into cytosolic and membrane components revealed striking differences in distribution of wild-type and S440A-mutant ChAT. The wild-type enzyme was present in both fractions, with 88% of total enzyme activity found in cytoplasm and the remaining 12% being membrane-associated; following washing with 350 mM NaCl, only residual enzyme activity (~0.5%) was recovered in the membrane fraction, suggesting that ChAT protein was ionically associated with the membranes. In contrast, all activity of the S440A-ChAT was recovered in the cytosolic fraction, with no measurable enzyme activity found in the membrane fraction. As shown in Fig. 5A, treatment of cells with 1 μM PMA for 2 h resulted in activation of wild-type ChAT in both membrane (165% of control) and cytosolic (148% of control) compartments. PMA treatment also led to activation of the S440A-ChAT in cytosol (125% of control) but did not result in appearance of detectable ChAT activity in the membrane fraction from these cells (Fig. 5A).

**FIG. 3.** Strategy for identification of PKC phosphorylation site(s) in ChAT using MALDI-MS. Masses obtained for $^{32}$P-labeled tryptic peptides derived from in-gel trypsin digestion of PKC-phosphorylated isoforms of ChAT were compared with theoretical masses for tryptic peptides of the enzyme in the database. A combination of this information with predicted consensus sequences for PKC phosphorylation sites allowed identification of one PKC phosphorylation site in 69-kDa human ChAT. Further studies used MALDI-TOF in linear and reflectron mode to analyze a mixture of tryptic peptides of ChAT; this revealed the presence of one phosphorylated peptide with a mass shift of about 80 Da (see “Results”).

Immunoblots for ChAT were performed on cytosolic and...
membrane samples from control and PMA-treated cells to determine whether the increase in ChAT activity was related to a change in the amount of enzyme protein in any of the fractions, or translocation of enzyme between cytoplasm and membrane. As indicated in Fig. 5B, treatment of HEK293 cells expressing either wild-type or S440A-ChAT with 1 μM PMA for 2 h did not result in changes in enzyme amount in cytosolic or membrane fractions, as analyzed by densitometry in seven independent experiments.

ACh Synthesis in PMA-treated HEK293 Cells—The capacity for cells expressing wild-type versus S440A-ChAT to synthesize [3H]ACh from [3H]choline in the presence of PMA stimulation was tested. ACh was not synthesized in wild-type HEK293 cells, with almost all [3H]choline taken up into the cells converted to phosphorylcholine or other choline metabolites not extracted from aqueous solution by sodium tetraphenylboron. In contrast, expression of wild-type or S440A-ChAT in HEK293 cells shifted choline metabolism so that only about 10–20% of [3H]choline transported into the cells was converted to these metabolites, with the remainder being metabolized to [3H]ACh or remaining as unmetabolized [3H]choline. Treatment of cells expressing wild-type and S440A-ChAT with 1 μM PMA for 2 h prior to incubation with [3H]choline resulted in

DISCUSSION

We found recently that purified recombinant 69-kDa human ChAT is a substrate for PKC, and that phosphorylation of the enzyme in vitro led to a 2-fold increase in activity (7). In the present study, we demonstrated for the first time that 1) 69-kDa human ChAT undergoes rapid regulation of its catalytic activity in response to activation of cellular PKC by phorbol ester PMA, 2) the enzyme is phosphorylated by PKC at residue serine 440 within a functional consensus sequence for PKC, and 3) mutation of serine 440 to alanine resulted in loss of binding of ChAT to membranes and attenuation of PMA-induced enhancement of ChAT enzymatic activity. Numerous reports in the literature indicate that ChAT is predominantly a cytosolic protein but that some fraction of the total neuronal enzyme associates both ionically and nonionically with plasma membrane (13). The proportion of enzyme that is membrane-bound appears to vary between species and at different stages of development (14, 15). The means by which ChAT binds nonionically to membranes has not been elucidated; it does not have obvious hydrophobic domains for association with the lipid bilayer, and the presence of covalent modifications such as a glycosylphosphatidylinositol linkage have been controversial (16–18). Eder-Colli and colleagues (19) reported recently that in Drosophila neurons amphiphilic ChAT had properties of a peripheral membrane protein and was removed from plasma membranes by alkaline carbonate
and urea, suggesting that it is anchored to membrane through association with other, unidentified membrane components. ChAT also binds ionically to cellular membranes, being liberated experimentally by alterations in salt concentration and pH. Changes in phosphorylation state could result in altered charge on the protein and changes in isoelectric point. We demonstrated previously that both 69- and 82-kDa purified recombinant ChAT is comprised of multiple isoforms with the more acidic isoforms being phosphorylated (7), but it has not been determined whether some isoforms bind to membrane more readily than others. Bruce and Hersh (4) reported that phosphorylation of human placental ChAT by multifunctional calcium-calmodulin (CaM) kinase altered its ionic association with synaptic membranes, with phosphorylated ChAT binding less well to membranes than the native protein. However, this finding requires further investigation, because it was only observed over a narrow NaCl concentration range from 5 to 20 mM, with equivalent amounts of unphosphorylated and phosphorylated ChAT bound to membrane fragments in the absence of NaCl or at 30 mM NaCl or above. In the present studies, we identified serine 440 as a PKC phosphorylation site that is involved in membrane association of ChAT. Under the subcellular fractionation conditions used in the present experiments, about 10–15% of total wild-type enzyme activity was membrane-bound. Mutation of this phosphorylation site eliminated membrane binding of ChAT, suggesting that addition of negative charge to the protein in this position facilitated interaction with charged groups on cellular membranes, or resulted in a protein conformation involved in interaction with other membrane proteins. It is likely that the site(s) on ChAT phosphorylated by CaM kinase is not serine 440 as it is not within a canonical CaM kinase consensus sequence, with phosphorylation at other sites potentially leading to differences in functional outcome for the phosphorylated enzyme. Testing this hypothesis awaits identification of CaM kinase phosphorylation site(s) in ChAT and investigation of their functional roles by mutagenesis studies.

Phosphorylation of ChAT by PKC either in vitro (7) or following PKC activation in situ resulted in enhanced catalytic activity. It is likely that the increase in activity of cytosolic and membrane-associated ChAT observed in PMA-treated cells is explained by this change in kinetics rather than by translocation of enzyme protein between subcellular compartments, particularly because we demonstrated that the amount of ChAT recovered in cytosolic and membrane fractions of HEK293 cells expressing either wild-type or S440A-ChAT was not altered by PKC activation. Phosphorylation of purified recombinant 69-kDa human ChAT by PKC in vitro led to a 2-fold increase in enzyme activity (7), compared with an increase in ChAT activity to ~160% of control in cells stably expressing the enzyme in which PKC was activated by PMA. This suggests that phosphorylation-related regulation of ChAT activity in the cellular context is more complex and may not be maximal when compared with that measured for the enzyme in vitro, with net phosphorylation in situ being a balance between the function of kinases and phosphatases. Moreover, it is not known to what level PKC was activated in cells by PMA, and how this compares to activity of PKC present under optimized phosphorylation reaction conditions in vitro. This finding with ChAT is similar to reports for another neurotransmitter-synthesizing enzyme tyrosine hydroxylase, where activation of the enzyme in situ in response to experimental manipulations that would activate protein kinases (20) is generally less than that observed following phosphorylation of the purified enzyme in vitro (21).

Mutation of serine 440 to alanine in 69-kDa ChAT did not completely eliminate phosphorylation of the enzyme by PKC. Incubation of S440A-ChAT with PKC under phosphorylating conditions resulted in one-half as much [32P]phosphate incorporation as that measured for wild-type ChAT. This suggests that there are two (or more) functional phosphorylation sites for PKC in 69-kDa human ChAT in vitro with serine 440 being one of these. Only one site was identified in these studies by MALDI-MS and MALDI-TOF suggesting that the tryptic peptide bearing the other phosphorylation site(s) was not recovered in the complex peptide mixture analyzed, or that the ChAT isoforms phosphorylated on this additional residue(s) were not analyzed. In the preparation of PKC-phosphorylated ChAT for mass spectrophotometric analysis, [32P]-labeled isoforms of the enzyme were recovered from a two-dimensional SDS-PAGE gel for tryptic digestion. It is possible that additional phosphorylated isoforms of the enzyme that fell below detection on autoradiography were omitted from analysis. It is also important to note that, in HEK293 cells expressing S440A-ChAT, PMA treatment resulted in increased activity of the mutant to about one-half the level of activation measured for the wild-type enzyme. This suggests that PKC-mediated phosphorylation at this additional site(s) is also involved in regulation of activity of ChAT. However, PMA treatment of cells expressing S440A-ChAT did not lead to mutant ChAT protein or activity becoming associated with the membrane fraction, as demonstrated in Fig. 5. This indicates that, although functional PKC phosphorylation site(s) in ChAT other than serine 440 may be involved in regulation of enzymatic activity, they are not involved in membrane association of the protein.

From the functional standpoint, it has been considered that membrane-bound ChAT may play a unique role in regulation of ACh biosynthesis being situated to acetylate choline efficiently upon uptake into the nerve terminal by sodium-coupled choline transporters (22). Studies employing a choline analogue that binds irreversibly to choline binding sites suggested that choline is transferred from the sodium-coupled choline transporter to membrane-bound ChAT, because this form of the enzyme is most inhibited in nerve terminals incubated with the compound (23). It has been difficult, however, to address the role of membrane versus cytosolic ChAT in ACh synthesis directly, and studies reported to date have attempted to modulate activity of selected subcellular pools of the enzyme and then monitor changes in ACh levels. For example, lowering nerve terminal chloride ion concentration dramatically reduced membrane-bound ChAT activity without changing activity of the cytosolic enzyme, but this did not alter resting ACh synthesis (24). It is important, however, to evaluate changes in neurotransmitter synthesis under conditions where demand for its production is increased; this is complicated experimentally as stimulation of ACh turnover by depolarization, for example, leads to simultaneous activation of high affinity choline uptake, ACh synthesis and release, and membrane-bound ChAT activity thereby making it difficult to dissect out relationships between enzyme activity and product formation.

Identification of serine 440 as a functional phosphorylation site in ChAT may be important in the context of human neurological disorders. A very recent report identified single nucleotide polymorphisms leading to point mutations in ChAT to be associated with myasthenic syndrome and apnea, with one of these involving the arginine residue at the +2 position to serine 440 being mutated to a histidine residue (25). This mutation leads to an enzyme with very high $K_m$ for both of its substrates. Because this arginine forms an important part of the canonical consensus sequence $^{440}$SIR$^{442}$ for PKC, it is unlikely that this mutant form of ChAT would be recognized and
phosphorylated by PKC at serine 440, leading potentially to altered regulation of the enzyme. Furthermore, it has been shown that there are reduced levels of PKC, or alterations in activity and distribution of its isozymes, that may play a role in selective degeneration of some neurons during aging and Alzheimer disease (26–28). Immunohistochemical studies have shown brain area-specific modifications in selected PKC isoforms in Alzheimer brain when compared with control subjects (29). After perturbation of cells by a range of stimuli, individual PKC isoforms translocate from cytoplasm to different subcellular sites, including nucleus, cytoskeleton, and plasma membrane, suggesting that they may mediate distinct cellular functions (30–32). The role of individual PKC isoforms in phosphorylation-mediated regulation of ChAT and cholinergic neuron function requires further investigation to establish basic mechanisms, and to identify modifications that may occur in disease.

Acknowledgments—We thank Dr. David Litchfield, Department of Biochemistry, University of Western Ontario for helpful discussion, and Dr. Y. Yang from the Molecular Medicine Research Centre Mass Spectrometry Laboratory, University of Toronto for mass spectrometric analysis.

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J. Biol. Chem. 2001, 276:22244-22250.
doi: 10.1074/jbc.M011702200 originally published online April 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011702200

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