Protein Kinase C Isoforms Differentially Phosphorylate Human Choline Acetyltransferase Regulating Its Catalytic Activity*

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Choline acetyltransferase (ChAT) synthesizes acetylcholine in cholinergic neurons; regulation of its activity or response to physiological stimuli is poorly understood. We show that ChAT is differentially phosphorylated by protein kinase C (PKC) isoforms on four serines (Ser-440, Ser-346, Ser-347, and Ser-476) and one threonine (Thr-255). This phosphorylation is hierarchical, with phosphorylation at Ser-476 required for phosphorylation at other serines. Phosphorylation at some, but not all, sites regulates basal catalysis and activation. Ser-476 with Ser-440 and Ser-346/347 maintains basal ChAT activity. Ser-440 is targeted by Arg-442 for phosphorylation by PKC. Arg-442 is mutated spontaneously (R442H) in congenital myasthenic syndrome, rendering ChAT inactive and causing neuromuscular failure. This mutation eliminates phosphorylation of Ser-440, and Arg-442, not phosphorylation of Ser-440, appears primarily responsible for ChAT activity, with Ser-440 phosphorylation modulating catalysis. Finally, basal ChAT phosphorylation in neurons is mediated predominantly by PKC at Ser-476, with PKC activation increasing phosphorylation at Ser-440 and enhancing ChAT activity.

Choline acetyltransferase (ChAT, EC 2.3.1.6)1 synthesizes the neurotransmitter acetylcholine (ACh) and serves as a phenotypic marker for cholinergic neurons. ChAT expression changes in normal aging and in neurological and psychiatric disorders such as Alzheimer disease and schizophrenia (1, 2). Several peptide and steroid hormones and growth/trophic factors regulate ChAT at the transcriptional level (3–6). For example, chronic administration of nerve growth factor causes hypertrophy of basal forebrain cholinergic neurons with increased ChAT mRNA and protein in aged rodents (5) and promotes recovery of cholinergic neurons following fimbria/fornix lesions (6).

Little is known, however, about mechanisms involved in short term regulation of ChAT function, with there being only a few reports of physiological perturbations that modulate its catalytic activity (7, 8). Protein kinase-mediated phosphorylation is a common mechanism that regulates enzymatic activity, subcellular compartmentalization, or interaction of a protein with other cellular proteins. ChAT is a substrate for multiple kinases, with phosphorylation by some kinases regulating its activity (9). Phosphorylation of purified 69-kDa human ChAT by protein kinase C (PKC) increases its activity 2-fold; this increase is attenuated in mutant ChAT in which serine-440 is changed to an alanine residue (10). Phosphorylation of ChAT at Ser-440 by PKC is also associated with altered membrane binding of the enzyme (10).

PKC comprises a family of serine/threonine kinases produced as isoenzymes that differ in mode of activation, substrate specificity (11, 12), cell/tissue-specific expression (13–15), and subcellular compartmentalization. PKC isoforms fall into three main groups, conventional (cPKC: α, βI, βII, and γ), novel (nPKC: δ, ε, η, and θ), and atypical (aPKC: ζ and η), defined by their structural homology and functional dependence on calcium, acidic phospholipids, and diacylglycerol (16–18). The biological actions and regulation of PKC isoenzymes in neurons are not fully understood, but it is known that different isoenzymes mediate diverse cellular responses. This appears to be defined by different resting and stimulus-induced subcellular localization and translocation patterns for the isoenzymes and different target substrates based on the recognition of different optimal phosphorylation consensus sequences.

Moreover, specific PKC isoenzyme expression and/or activity may be altered by pathology, including neurodegenerative diseases (19). Most isoforms of PKC are found in brain where they are involved in regulation of a wide range of neuronal processes, including ion channel gating, receptor desensitization, neurotransmitter release, synaptic efficacy, and some forms of learning/memory (20, 21). Importantly, various PKC isoform levels and activities are increased or decreased differentially during neurodegeneration. These changes have been linked to processes involved in cellular repair, but more often they relate to advancing pathology and neuronal death (19).

In the present study, we investigated phosphorylation of 69-kDa human ChAT by a panel of PKC isoforms and identified differences in the patterns of phosphorylation. We determined that some residues that are phosphorylated by PKC are required for regulation of catalytic activity of ChAT, with selective mutation of these residues resulting in attenuation or complete loss of enzymatic activity. Thus, differential modulation of activity of PKC isoforms in response to physiological

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§ This abbreviation is defined in Table I.

¶ This abbreviation is defined in Table II.

** This abbreviation is defined in Table III.
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One-dimensional SDS-PAGE and Immunoblotting—SDS-PAGE was performed on 7.5% separating gels according to the method of Laemmli (25). After electrophoresis, proteins were either stained with 0.05% Coomassie Brilliant Blue R-250 or transferred to nitrocellulose membranes for immunoblotting (transfer buffer: 48 mM Tris, 39 mM glycine containing 20% methanol). After transfer, membranes were saturated with 8% nonfat milk powder in phosphate-buffered saline, then probed with anti-ChAT CTab antibody (9) (1:200) for 1 h at room temperature. Membranes were then washed with phosphate-buffered saline containing 0.5% Triton X-100, with primary antibody detected by peroxidase-coupled secondary antibody and ECL chemiluminescence kit (Amersham Biosciences).

In-gel Tryptic Digestion and Sample Preparation—After separation by one-dimensional SDS-PAGE (gel thickness, 0.75 mm), proteins were stained briefly with Coomassie Blue and de-stained by washing over 3 h with at least five solvent changes (50% methanol, 10% acetic acid) to ensure removal of SDS. After the washes, with double-distilled water, bands corresponding to ChAT were excised from gels, reduced with dithiothreitol, and alkylated with iodoacetamide. In-gel tryptic digestion was then carried out over 20 h, as described previously (26, 27).

Two-dimensional Tryptic Phosphopeptide Mapping and Identification of Phosphorylated Residues—Two-dimensional phosphopeptide maps of ChAT were prepared as described previously (28, 29). Briefly, following the in-gel tryptic digestion, tryptic peptide maps were transferred to thin-layer cellulose (for thin-layer chromatography (TLC)) plates and electroblotted in the first dimension in water/acetate/formic acid (89.7:7.8:2.5, v/v, pHI 1.9) at 1000 V for 30 min. Plates were air-dried and developed in the second dimension in water/n-butanol/pyridine/acetate acid (30:37.5:25:7.5, v/v). Phosphopeptides were visualized by autoradiography. For further analysis, phosphopeptides were eluted from the electroblot using water/acetamide (4:1 v/v) and freeze-dried to 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 ZipTipC18TM according to the manufacturer’s instructions (Millipore) and eluted from the tip resin with 65% acetonitrile and 1% acetic acid.

One-dimensional phosphomonoamino acid analysis (electrophoresis at pH 1.9 water/acetate/formic acid, 89.7:7.8:2.5, v/v; 1500 V; 25 min) 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, boiled at 110 °C for 1 h, vacuum-dried at 45 °C, recovered in 2 μl of electrophoresis buffer, and spotted onto TLC plates.

MALDI-MS was performed on Micromass MALDI-R and LR mass spectrometers using Masslynx 3.5 or 4.0 and operating in positive ion mode. Calibration was performed with a standard mixture of peptides (angiotensin I, renin substrate, and adrenocorticotropic clip 18–39 (ACTH)). The sample in 0.1% trifluoroacetic acid was mixed 1:1 with a matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50:50 ACN/E-TOF mass spectrometry). For ESI-MS/MS analysis, phosphopeptides generated by phosphorylation of ChAT with PKC isoforms were recovered from cellulose plates by spraying into water/acetonitrile (4:1, v/v) solution and reduced to dryness in a vacuum centrifuge. Recovery of [32P]phosphate-labeled peptides was monitored by Cerenkov counting and was >90%. Dry samples were reconstituted prior to mass spectrometry analysis in a solution containing 10% acetonitrile or 0.1% formic acid or 0.1% acetic acid.

MALDI-MS was performed on Micromass Q-Tof or Micromass GLOBAL mass spectrometer using a standard analytical column (0.5 × 5 mm C18, LC Packings plus 75 μm × 15 cm column, LC Packings). Chromatographic separation was achieved using a ten-port switching valve that allowed sample loading onto a precolumn (0.5 × 5 mm, LC Packings) at high flow rates (30 μl/min) followed by changing valve position for elution of the analyte from the precolumn to an analytical column (300 μl/min) that eluted directly through a nanospray ion source. The mass spectrometry used was: A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile for sample elution; and C, 0.1% formic acid in high performance liquid chromatography grade water for sample loading. Peptides were eluted after a 3-min loading period using a linear gradient of 5–60% B over 32 min, 60–95% B over 5 min, 95% B for 10 min, 95–5% B over 5 min, and 5% B for 10 min. Instruments were calibrated using MS/MS spectra of Glu-fibrinopeptide-b. Experiments were carried out for the whole pro-
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RESULTS

Phosphorylation of ChAT by PKC Isoenzymes—Purified recombinant human 69-kDa ChAT is a substrate in vitro for each of seven PKC isoforms tested. However, phosphorylation of ChAT by different isoforms varies in both extent and pattern of phosphate addition with different amino acid residues utilized by some isoforms. As illustrated in Fig. 1a, phosphorylation of ChAT is greatest with cPKCa and nPKCBS, intermediate with cPKCs, and least with cPKCδ, βII, nPKCδ, and aPKCγ. In these experiments, [32P]phosphate incorporation into ChAT was quantification by Cerenkov counting of pieces of nitrocellulose membrane corresponding to ChAT protein after visualization by autoradiography (Fig. 1). Purified bacterially expressed ChAT (0.5 μg) was incubated for 60 min with conventional (α, β, βI, and γ), novel (δ and ε), or atypical (ζ) PKC isoforms in the presence of [γ-32P]ATP. At varying times, aliquots of reaction mixtures were taken for separation by SDS-PAGE to develop time-course profiles for ChAT phosphorylation. Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes for autoradiography followed by immunoblot with CTab antibody. Densitometry was performed on each immunoblot to quantify protein loading at varying time points, and ChAT bands were excised to measure [32P]phosphate. α, time course for [32P]phosphate incorporation into ChAT mediated by the seven PKC isoforms tested is expressed as counts per minute (cpm) at constant levels of ChAT protein. Data are the mean ± S.E. of three separate experiments. b, a representative autoradiograph (upper panel) and corresponding immunoblot (lower panel) are shown from one of these experiments; phosphorylation of ChAT varies with different PKC isoforms with the amount of ChAT protein constant.

tein digest or for synthetic peptides. Species corresponding to expected m/z values of phosphorylated species were subjected to MS/MS. Data were processed with MaxEnt3 to obtain singly charged de-isotoped spectra. All sequences were manually verified using the PepSeq module accompanying MassLynx 4.0.

Identification of Phosphorylated Residues—Based on analysis by mass spectrometry (MS) and mutagenesis, we identified four serine and one threonine residues that could be phosphorylated by PKC in neuroblastoma cells either in the presence or absence of the PKC activator PMA. Additional residues that could be phosphorylated by PKC would have to be revealed by conformational rearrangement of the protein in response to perturbation(s) not apparent from the current experimental approaches; using the minimal PKC recognition sequence ((R/K)X(S*/T*)nX(R/K)) (28), there are five

FIG. 1. ChAT is phosphorylated differentially by different PKC isoforms. Purified bacterially expressed ChAT (0.5 μg) was incubated for 60 min with conventional (α, β, βI, and γ), novel (δ and ε), or atypical (ζ) PKC isoforms in the presence of [γ-32P]ATP. At varying times, aliquots of reaction mixtures were taken for separation by SDS-PAGE to develop time-course profiles for ChAT phosphorylation. Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes for autoradiography followed by immunoblot with CTab antibody. Densitometry was performed on each immunoblot to quantify protein loading at varying time points, and ChAT bands were excised to measure [32P]phosphate. α, time course for [32P]phosphate incorporation into ChAT mediated by the seven PKC isoforms tested is expressed as counts per minute (cpm) at constant levels of ChAT protein. Data are the mean ± S.E. of three separate experiments. b, a representative autoradiograph (upper panel) and corresponding immunoblot (lower panel) are shown from one of these experiments; phosphorylation of ChAT varies with different PKC isoforms with the amount of ChAT protein constant.

FIG. 2. Two-dimensional phosphopeptide maps reveal different serine/threonine phosphorylation patterns of ChAT by PKC isoforms. Purified bacterially expressed ChAT (3 μg) was incubated with PKC isoforms under phosphorylating conditions, then separated by SDS-PAGE. ChAT protein was subjected to in-gel tryptic digestion with the resulting peptides analyzed by phosphopeptide and phosphoamino acid mapping. α, phosphopeptide maps of ChAT phosphorylated by cPKCa or nPKCBS, results obtained with cPKCa, βI, βII, and γ are identical to that shown for cPKCa, and those obtained with nPKCδ and aPKCζ are essentially identical to that shown for nPKCδ. b, phosphopeptides present in the numbered spots on autoradiographs in Panel a were recovered for phosphoamino acid analysis. Phosphoserine is found in all spots except number 12. Phosphothreonine is present in spots 6 and 12 as phosphopeptides associated with these spots co-migrate and are separated only upon site-directed mutagenesis. These data are based on mobility of ninhydrin-stained authentic phosphoamino acids that were added to hydrolysates prior to electrophoresis. Data are representative of at least four (α) and three (β) separate experiments with each of the cPKC and nPKC/aPKC isoforms tested.

Panel). To begin to investigate sites phosphorylated in ChAT by PKC, phosphorylation patterns were compared on two-dimensional phosphopeptide maps. Taken together, two separate patterns were observed, one with cPKCs α, βI, βII, and γ and the other with nPKCs β, ε, and aPKCζ. Representative phosphopeptide maps for cPKCs and nPKCBS are given in Fig. 2a. Phosphoamino acid analysis of phosphopeptides eluted from TLC plates showed that ChAT is phosphorylated on serine and threonine by cPKC, but only on serine by nPKC and aPKC (Fig. 2b).
more putative PKC phosphorylation sites in ChAT. All PKC phosphorylation sites identified in ChAT in this study correspond to (S*/T*)X(R/K); other optimal recognition motifs have been identified for different PKC isoforms in other substrate proteins (29, 30).

Phosphorylated residues were identified by MALDI-TOF and ESI-MS survey spectrum analyses; results from in-gel tryptic digests of purified ChAT phosphorylated by PKC isoforms are summarized in Table I. Four phosphopeptides were identified containing a total of four serine and one threonine residues as putative PKC phosphorylation sites. Liquid chromatography was required to separate isobaric peptides from phosphorylated ones to confidently identify phosphorylated sites. Phosphopeptides appeared to be low in abundance, making it difficult to obtain reliable sequence data. Moreover, sample handling had to be performed with extreme care; samples containing tryptic peptides were maintained in a dry state until analysis and could not be desalted using ZipTips due to peptide loss. To verify putative phosphorylation sites, peptides corresponding to the tryptic peptides identified were synthesized, incubated with PKC under phosphorylating conditions, and sequenced by tandem MS.

This step was particularly important with one of the peptides identified by MS that contains two serine residues (HMTQS346S347RKLIR), both of which lie within putative PKC consensus sequences. MS and MS/MS analyses of this peptide are shown in Fig. 3. Under the conditions used, this peptide was highly phosphorylated at a single site with a small amount of doubly phosphorylated species observed (Fig. 3, a and b). MS/MS analysis of m/z 479.57 that corresponds to the triply charged, singly phosphorylated peptide clearly shows a y ion series that overwhelmingly supports Ser-346 as the major phosphorylation site (Fig. 3, c and d).

<table>
<thead>
<tr>
<th>Observed m/z</th>
<th>Expected m/z</th>
<th>Tryptic peptide sequence</th>
<th>Putative phosphorylation site</th>
<th>Phospho-peptide spot number</th>
<th>PKC isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>658.39 (2+)</td>
<td>658.32</td>
<td>LVPTYESASIR442</td>
<td>Ser-440</td>
<td>1–5</td>
<td>All</td>
</tr>
<tr>
<td>718.88 (2+)</td>
<td>718.86</td>
<td>HMTQSSSRKLIR352</td>
<td>Ser-346, Ser-347</td>
<td>7–11</td>
<td>α, β, βII, γ</td>
</tr>
<tr>
<td>606.77 (2+)</td>
<td>606.80</td>
<td>TVLVKDSRN327</td>
<td>Thr-255</td>
<td>12</td>
<td>α, β, βII, γ</td>
</tr>
<tr>
<td>501.91 (3+)</td>
<td>501.91</td>
<td>AVTDHKAAPSE476</td>
<td>Ser-476</td>
<td>6</td>
<td>All</td>
</tr>
</tbody>
</table>

*Phosphopeptide(s) associated with spots 6 and 12 were co-localized using this two-dimensional electrophoresis-chromatography approach; similarly, phosphopeptides 2 and 11 were also co-localized.
sus sequences for PKC, arginine was found to be superior to lysine (31).

**Mutagenesis of PKC Isoenzyme-specific Phosphorylation Sites**—Serine and threonine residues identified in ChAT as phosphorylation sites for PKC isoforms were mutated to alanine residues either singly or in combination. This allowed us to test their role in regulation of ChAT activity, confirmed the MS data, and gave insight whether there are additional phosphorylation sites not detected by MS. Representative phosphopeptide maps from tryptic digests of ChAT phosphorylated by cPKCα (Fig. 4a, upper panel) or nPKCβ (Fig. 4a, lower panel) demonstrate loss of [32P]-labeled phosphopeptides associated with specific mutations. Table II summarizes the identity of phosphorylated residues associated with the individual phosphopeptide spots.

Thus, mutation of Ser-440 → Ala (mutant A) reduced [32P]phosphate labeling of purified ChAT by cPKC, nPKC, and aPKC; phosphopeptide spots lost correspond to numbers 1–5 for cPKC and numbers 1–3 for nPKC/aPKC seen for wild-type ChAT (Fig. 2). Mutation of Ser-346/347 → Ala in S440A-ChAT (mutant F) further reduced phosphorylation by cPKC, but not by nPKC/aPKC; phosphopeptide spots lost with this mutation include numbers 7–11 for cPKC in Fig. 2. The addition of Ser-476 → Ala to S440A/S346A/S347A-ChAT (mutant G) abolished phosphorylation by nPKC/aPKC; this reduced, but did not eliminate phosphorylation of ChAT by cPKC. The phosphopeptide spot lost with mutation of Ser-476 corresponds to number 6 in Fig. 2. Finally, mutation of Thr-255 → Ala combined with mutation of the four serine residues produced mutant ChAT (mutant H) that was not a substrate for cPKC under the in vitro conditions tested; the phosphopeptide spot lost with mutation of Thr-255 corresponds to number 12 in Fig. 2. Multiple phosphopeptide spots that are sometimes generated relating to a single phosphorylation site arise from one or more missed cleavages of the protein during trypsin digestion. This occurs most commonly when the phosphorylated residue is closely adjacent to an arginine or lysine that serves as the tryptic cleavage site (32).

To define effects of phosphorylation by PKC on ChAT function, we investigated the relationship between phosphorylation at certain residues or combinations of residues and enzymatic activity. Taken together, the [32P]phosphate incorporation data support results from the phosphopeptide analysis, and when viewed with activity data several interesting pieces of information emerge (results are shown in Fig. 4, b and c). First, incubation of wild-type ChAT with cPKCα leads to substantial [32P]phosphate incorporation and a 2-fold increase in catalytic activity. Second, of the single-site mutants studied (mutants A, B, C, and D), all have reduced [32P]phosphate incorporation, from a 20% decrease in T255A-ChAT (mutant D) to over 90% decrease in S476A-ChAT (mutant C). However, of these mutants only S440A-ChAT (mutant A) has reduced basal activity compared with wild-type enzyme. Moreover, cPKCα-mediated activation was retained in S346A/S347A-ChAT (mutant B) and T255A-ChAT (mutant D), but attenuated in S440A-ChAT and abolished in S476A-ChAT. Third, of the multiple-site mutants studied (mutants E, F, G, and H), those with S440A or S346/347A (mutants F, G, and H) have substantially less basal activity than wild-type ChAT. Incubation of mutants F, G, or H with cPKCα did not alter catalytic activity relative to the non-phosphorylated proteins. These mutations decreased [32P]phosphate incorporation by 60% compared with control for mutant F with Ser-476 and Thr-255 intact, to about 100% for mutant H with all sites mutated. Thus, some mutations, for example mutant F, may lower basal ChAT activity in a non-phosphorylation-dependent manner, perhaps by causing a conformational change in the protein that affects catalysis. Finally, mutation of Ser-476 (mutant C) almost completely abolished phosphorylation in vitro by cPKCα but, importantly, was not accompanied by a change in enzyme activity. Addition

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**Fig. 4.** Phosphorylation of ChAT on serine residue(s) by PKC is regulated hierarchically by phosphorylation at Ser-476. a, purified bacterially expressed ChAT proteins (3 µg) were incubated with PKC isoforms. Mutant ChAT proteins tested were: A, S440A; B, S346A; S347A; C, S476A; D, T255A; E, S476A; T255A; F, S446A; S347A, S440A; G, S440A, S346A, S347A, S476A; and H, S440A, S346A, S347A, S476A, T255A. Phosphorylated proteins separated by one-dimensional SDS-PAGE were digested by trypsin in-gel, with the resulting phosphopeptides separated in two-dimensions on TLC plates. Representative autoradiographs show phosphopeptide maps of ChAT mutants incubated with PKCα (upper panel) or PKCβ (lower panel); patterns essentially identical to cPKCα were obtained with other cPKC isoforms, and to nPKCβ with other nPKC/aPKC isoforms. Data are representative of at least three independent experiments for each ChAT mutant and each PKC isoform. b, ChAT mutants incorporated [32P]phosphate to variable extents compared with wild-type protein. Purified protein samples (1 µg) were incubated for 1 h with [γ-32P]ATP and cPKCα, then separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting to verify equivalent ChAT content. ChAT bands were excised and counted for [32P]phosphate. Data are expressed as mean ± S.E. of five experiments, with statistically significant differences at p ≤ 0.05 compared with wild-type ChAT (denoted by asterisks) determined by one-way ANOVA with Dunnett’s post-hoc test. c, purified wild-type and mutant ChAT proteins (1 µg) were incubated under phosphorylating conditions in the absence or presence of cPKCα. ChAT activity was measured and expressed relative to unphosphorylated wild-type enzyme. Data are mean ± S.E. of four experiments. Statistically significant differences were determined at the level of p ≤ 0.05 by one-way ANOVA with Tukey’s post-hoc test for multiple comparison. *, differences relative to corresponding unphosphorylated proteins; #, differences between activity of unphosphorylated wild-type enzyme and mutants A, F, G, and H.
of T255A to S476A-ChAT (mutant E) abolished ChAT phosphorylation, indicating that residual phosphorylation of S476A-ChAT is on Thr-255; this is supported by observations with mutants G and H in which the four serine residues were mutated in the absence or presence of the T255A mutation, respectively. Interestingly, mutant E had catalytic activity not different from wild-type ChAT, even though phosphorylation by cPKC\( \alpha \) was abolished and activity of mutant E was not enhanced by cPKC\( \alpha \).

Importantly, phosphorylation of ChAT by PKC was regulated hierarchically through Ser-476; mutation of Ser-476 to Ala (mutant C) blocked phosphorylation by cPKCa (Fig. 5a) and S476A-ChAT do not yield detectable phosphorylation at any sites identified in wild-type ChAT. These data are representative of two independent experiments with similar results. b, ChAT specific activity was measured in lysates of IMR32 cells transiently expressing the proteins. Results are expressed as mean ± S.E. of four experiments. Statistically significant differences at \( p \leq 0.05 \), denoted by asterisks, were determined by one-way ANOVA with Dunnett’s post-hoc test compared with wild-type ChAT.

**TABLE II**

Identity of amino acid residues associated with phosphopeptide spots from tryptic digests of wild-type and mutant ChAT proteins

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Residues mutated</th>
<th>Phosphopeptide spots deleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>S440A</td>
<td>1–5</td>
</tr>
<tr>
<td>B</td>
<td>S346A, S347A</td>
<td>7–11</td>
</tr>
<tr>
<td>C</td>
<td>S476A</td>
<td>1–11 (cPKC); all (nPKC/aPKC)</td>
</tr>
<tr>
<td>D</td>
<td>T255A</td>
<td>12*</td>
</tr>
<tr>
<td>E</td>
<td>S476A, T255A</td>
<td>All spots deleted</td>
</tr>
<tr>
<td>F</td>
<td>S346A, S347A, S440A</td>
<td>1–5, 7–11</td>
</tr>
<tr>
<td>G</td>
<td>S440A, S346A, S347A, S476A</td>
<td>All, except 12*</td>
</tr>
<tr>
<td>H</td>
<td>S440A, S346A, S347A, S476A, S476A, T255A</td>
<td>All spots deleted</td>
</tr>
</tbody>
</table>

*Thr-255 was phosphorylated by cPKCs, but not by nPKCs or aPKCs.

**FIG. 5.** Phosphorylation, but not activity, of ChAT is partially recovered by substitution of Ser-476 by glutamate. a, purified bacterially expressed wild-type and mutant ChAT proteins (3 \( \mu \)g) were incubated under phosphorylating conditions with PKC, then digested with trypsin for two-dimensional phosphopeptide analysis. Representative phosphopeptide maps are shown for wild-type, S476A- and S476E-ChAT following phosphorylation by cPKCa (upper panel) and nPKC\( \delta \) (lower panel). Phosphorylation of Ser-440 is partially reconstituted in S476E-ChAT seen as phosphopeptide spots that co-migrate with spots 1, 3, and 5 of wild-type ChAT. A small amount of phosphate incorporation corresponding to Thr-255 is seen in cPKCa phosphorylated S476A-ChAT (spot 12). nPKC\( \delta \)-treated S476A- and S476E-ChAT do not yield detectable phosphorylation at any sites identified in wild-type ChAT. These data are representative of two independent experiments with similar results. b, ChAT specific activity was measured in lysates of IMR32 cells transiently expressing the proteins. Results are expressed as mean ± S.E. of four experiments. Statistically significant differences at \( p \leq 0.05 \), denoted by asterisks, were determined by one-way ANOVA with Dunnett’s post-hoc test compared with wild-type ChAT.
residues by PKC by mutating Ser-476 to Glu. As illustrated in Fig. 5a, comparison of phosphopeptide maps of purified wild-type, S476A- and S476E-ChAT phosphorylated by cPKCs revealed that phosphorylation of serine residues in S476E-ChAT is partially recovered when phosphoserine 476 is replaced by glutamic acid. Specifically, spots 1, 3, and 5 that correspond to phosphorylated Ser-440 and spot 12 corresponding to phosphorylated Thr-255 in wild-type ChAT were phosphorylated by cPKCs in S476E-ChAT but not in S476A-ChAT. Interestingly, phosphorylation of S346/S347 by cPKC-α did not occur in S476E-ChAT. Although a quantitative comparison was not carried out, recovery of phosphorylation with S476E-ChAT constituted about 5% of that obtained with wild-type ChAT. Of note, there was no recovery of phosphorylation when S476E-ChAT was incubated with nPKCs.

Partial recovery of phosphorylation of Ser-440 in S476E-ChAT did not translate to altered specific activity of this mutant protein. ChAT activity in lysates of IMR32 cells transiently expressing either S476A- or S476E-ChAT was significantly reduced compared with wild-type ChAT (Fig. 5b). This suggests that S476E-ChAT is not an effective substrate for PKC isoenzymes leading to reconstitution of the phosphorylation pattern of wild-type ChAT. Moreover, partial recovery of phosphorylation of ChAT expressed in cells was insufficient to restore control basal catalytic activity.

Several single nucleotide polymorphisms have been identified in ChAT with some causing loss-of-function mutations and failure of cholinergic transmission (34–36). Of relevance to the present study, Arg-442 is mutated to histidine in cases of episodic apnea resulting in inactive ChAT (34). As the minimal consensus sequence recognized by PKC is (R/K)X(R/K) (28), we determined if Arg-442 is required for phosphorylation of ChAT at Ser-440 by PKC. To accomplish this, two ChAT mutants, Arg-442 → His (R442H-ChAT) and Arg-442 → Ala (R442A-ChAT) were made. Phosphopeptide maps in Fig. 6a show that

![Image](http://www.jbc.org/)

**Fig. 6.** Arg-442 is critical for phosphorylation of ChAT at Ser-440 and for catalytic activity of the enzyme. a, representative phosphopeptide patterns obtained from tryptic digests of wild-type and R442H-ChAT after phosphorylation by cPKCs. Substitution of other cPKC isoenzymes for cPKCs yielded similar results, and essentially identical data were obtained for R442A-ChAT. Phosphopeptide maps for R442H-ChAT do not have spots 1–5 containing phosphorylated Ser-440. b, lysates of IMR32 cells expressing S440A-, R442A-, or R442H-ChAT have significantly reduced ChAT activity compared with wild-type, S467A- (mutant C), S346/347/440/476A/T255A-ChAT (mutant H) and wild-type ChAT. Changing the single mutation S440A to S440E restored enzyme activity to that of wild-type ChAT, but when this change was made in combination with R442H no recovery of enzymatic activity was obtained. Data are expressed as mean ± S.E. of five experiments. Equivalent expression of ChAT proteins in transiently transfected IMR32 cells was verified by immunoblot (data not shown).

**Fig. 7.** Basal and stimulated phosphorylation of ChAT in IMR32 cells is attributed in part to actions of PKC. IMR32 cells expressing wild-type and mutant ChAT were lysed for analysis. Mutant ChAT proteins tested were: A, S440A; B, S346A and S347A; C, S476A; D, T255A; E, S476A and T255A; G, S440A, S346A, S347A and S476A; and H, S440A, S346A, S347A, S476A and T255A. a, ChAT specific activity was measured in lysates, with protein expression assessed by immunoblot and densitometry (data not shown). Results are the mean ± S.E. of five separate experiments with duplicate determinations; asterisks denote statistically significant differences compared with wild-type-ChAT at p = 0.05 by one-way ANOVA with Tukey’s post-hoc multiple comparison test. b, an autoradiograph (upper panel) and immunoblot (lower panel) shows differential basal phosphorylation of immunoprecipitated wild-type and mutant ChAT from IMR32 cells grown in the presence of [32P]phosphate for 3 h (representative of three independent experiments). c, ChAT immunoprecipitated from cell lysates was separated by SDS-PAGE, then phospho-ChAT bands were excised from gels and digested by trypsin. Phosphopeptide maps for wild-type, S467A- (mutant C), S346/347/440/476A/T255A-ChAT (mutant H) are shown. Also, to determine the extent to which ChAT is phosphorylated in unstimulated cells by PKC, cells expressing wild-type ChAT were treated with PKC inhibitor H7 (10 μM). These data are representative of three separate experiments. d, specific activity of wild-type ChAT was measured in lysates of IMR32 cells grown for 6 h in the absence (control) or presence of PKC inhibitors H7 (10 μM) or epidermal growth factor receptor fragment 651–658 peptide (10 μM). Data are expressed as mean ± S.E. for five experiments. Asterisks denote statistically significant differences compared with untreated cells at p = 0.05 by one-way ANOVA with Dunnett’s post-hoc test.
phosphorylation of ChAT at Ser-440 was abolished in R442H-ChAT (phosphopeptide spots 1–5); identical results were obtained for R442A-ChAT (data not shown). This is accompanied by loss of catalytic activity for R442H- and R442A-ChAT expressed in IMR32 cells, compared with wild-type enzyme (Fig. 6b). Importantly, decreased activity of S440A-ChAT was restored to that of wild-type ChAT, when acetylation associated with phosphorylation of Ser-440 was simulated by mutation of Ser-440 → Glu (S440E-ChAT). To assess the relative roles of Ser-440 compared with Arg-442 in catalytic activity of ChAT, we constructed the double mutant S440E/R442H-ChAT. Although changing Ser-440 to glutamate restored basal catalytic function to S440A-ChAT, this did not occur when the acidic residue was substituted in conjunction with the R442H mutation (Fig. 6b). Thus, Arg-442, not phosphorylation of Ser-440, appears primarily responsible for basal ChAT activity, with the phosphorylation state of Ser-440 modulating catalysis.

Phosphorylation and Regulation of ChAT Activity in Situ by PKC—Wild-type and mutant ChAT proteins were expressed in IMR32 cells and isolated for analysis to study effects of PKC-mediated phosphorylation of the enzyme in situ. Specific activity of ChAT mutants with PKC phosphorylation sites removed singly or in combination was measured in IMR32 cell lysates relative to wild-type ChAT (Fig. 7a). It is important to evaluate these data in context with activity data of purified bacterially expressed proteins in Fig. 4c, because expression of ChAT proteins in mammalian cells is likely accompanied by phosphorylation of the protein, whereas proteins expressed in bacteria are not phosphorylated. First, effects of some mutations (A, B, D, G, and H) on activity of ChAT proteins expressed in human neural cells parallel results obtained for proteins purified from bacteria. Second, notable differences were found for mutants C and E, with specific activity of these mutants being significantly reduced compared with wild-type ChAT when expressed in IMR32 cells, but not when expressed in bacteria.

We also determined the extent to which basal phosphorylation of ChAT could be attributed to PKC. To begin, we screened incorporation of [32P]phosphate into wild-type and mutant ChAT immunoprecipitated from unstimulated IMR32 cell lysates. Wild-type ChAT undergoes substantial [32P]phosphorylation compared with mutants C and H in which either Ser-440 or -H (S440A, S346A, S347A, S476A, and T255A) ChAT were transiently transfected into IMR32 cells that stably express the human sodium-coupled choline transporter, CHT1. This model was chosen because it allows reconstitution of wild-type and mutant forms of the ACh-synthesizing enzyme with CHT1, which provides the substrate choline. Previously, we showed that PMA treatment increased conversion of [3H]choline to [3H]ACh in HEK 293 cells expressing wild-type ChAT, but not S440A-ChAT (10). In the present study, we compared synthesis of [3H]ACh from [3H]choline in cells expressing wild-type or mutant ChAT. As predicted from data shown in Fig. 7a, synthesis of [3H]ACh was decreased by each of the ChAT mutants tested when compared with wild-type enzyme (mutant A, 18.8 ± 7.9%; mutant C, 31.8 ± 10.9%; and mutant H, 10.4 ± 8.8% of that measured in wild-type ChAT-expressing cells; mean ± S.E. from three or four separate experiments). Interestingly, however, the magnitude of this decrease for mutants A and C was greater than would be expected based on the in vitro ChAT activity data (Fig. 4c) or ChAT-specific activity data (Fig. 7a).

**DISCUSSION**

Phosphorylation of neurotransmitter-synthesizing enzymes serves as a regulatory mechanism underlying rapid changes in neuronal communication. For example, tyrosine hydroxylase catalyzes the rate-limiting step in catecholamine synthesis and is phosphorylated by at least seven serine/threonine kinases, including PKC (37). Activation of tyrosine hydroxylase occurs in situ, and phosphorylation at several residues, with a complex relationship between phosphorylation sites, stoichiometry of phosphorylation, and biological outcome (38). By comparison, little is known about phosphorylation of ChAT and modulation of its activity under normal or pathological conditions. Based on published reports (9, 10, 26, 39–41), ChAT is a substrate for multiple kinases in vitro, and in situ ChAT is phosphorylated constitutively and in response to cell perturbations. In hippocampal nerve terminals, ChAT phosphorylation is partially Ca2+- and cAMP-dependent (39, 40), and lowering cytosolic Ca2+ decreases incorporation of [32P]phosphate (39); the one or more protein kinases that phosphorylate ChAT in situ were not identified.

The present study provides a new framework for regulation of ChAT by phosphorylation and the role of PKC in cholinergic neurotransmission. It was established that: 1) PKC contributes substantially to basal phosphorylation of ChAT expressed in unstimulated neural cells; this likely represents direct phosphorylation of ChAT by PKC; or could involve protein kinases such as mitogen-activated protein kinase that are modulated by PKC; 2) phosphorylation of ChAT by PKC is organized hierarchically, with phosphorylation of Ser-476 required for
phosphorylation by PKC at other serine residues to proceed; 3) PKC isoforms modify ChAT differentially with cPKCs phosphorylating Ser-346/347, Ser-440, Ser-476, and Thr-255 and nPKC/aPKC phosphorylating only Ser-440 and Ser-476; 4) Ser-440 and/or Ser-346/347 play important roles in maintenance of basal catalytic activity and in PKC-mediated stimulation of ChAT activity, whereas Ser-476 and Thr-255 appear not to be required (directly) for this function; 5) Ser-346/347 are involved in modulating the extent of phosphorylation of ChAT on other residues; [32P]phosphate addition to Ser-346/347 comprises only a small proportion of total phosphate observed on autoradiographs, but deletion of these residues reduces phosphate incorporation by over 60% (mutant B); and 6) the relative abundance of phosphorylation at specific sites differs under in vitro versus in situ conditions, and for basal versus stimulated conditions in cells; Ser-440 is phosphorylated predominantly when purified ChAT is incubated with PKC, Ser-476 is phosphorylated primarily in unstimulated cells, and increased phosphorylation of ChAT in phorbol 12-myristate 13-acetate-treated cells is largely on Ser-440.

Proteins can undergo hierarchical phosphorylation by kinases to regulate their physiological functions. For example, tau is a substrate for multiple kinases with in vitro phosphorylation proceeding sequentially, first by protein kinase A and subsequently by paired-helical filament-associated protein kinase; tau is not a substrate for paired-helical filament-associated protein kinase without prior phosphorylation by protein kinase A or after phosphorylation by other kinases, including PKC, protein kinase CKII, mitogen-activated protein kinase, glycogen synthase kinase 3, p34cdc2, and cdk5 (42). Functional studies related to phosphorylation of translational inhibitor 4E-BP1 in vivo show a strict order for phosphate addition, but kinases involved in this hierarchical phosphorylation were not determined (43). Moreover, the pattern of phosphate addition to some proteins, for example viral NS1 protein, can vary depending on which PKC group or isoform is mediating the phosphorylation (44).

The current studies reveal Ser-476 as a key phosphorylation site for PKC in ChAT-regulating phosphorylation of other serine residues by PKC in a hierarchical manner. In this context, Ser-476 has two important functions. First, when not phosphorylated, Ser-476 in association with Ser-440 and Ser-346/347 is involved in maintaining basal ChAT activity; this is likely related in some manner to phosphorylation-dependent regulation of active-site conformation. In support of this, unphosphorylated bacterially expressed ChAT with the four serine PKC phosphorylation sites identified, Ser-367, Ser-347, and Thr-255, are also surface-accessible, thereby facilitating phosphorylation without requiring substantial conformational rearrangement of the enzyme. In summary, transition of a protein between phosphorylation states provides a range of opportunities for functional regulation, including through modification of catalytic activity, interaction with other proteins and cellular structures, and subcellular trafficking and compartmentalization. This is particularly important in the context of neurons to facilitate rapid replenishment of transmitters for chemical communication. Neurochemistry of cholinergic neurons is specialized in that Ach is not transported back into the nerve terminal following release and must be synthesized de novo after each action potential. Phosphorylation of ChAT by PKC appears to be an important mechanism that could provide a level of control required for maintenance of cholinergic neurotransmission.

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Protein Kinase C Isoforms Differentially Phosphorylate Human Choline Acetyltransferase Regulating Its Catalytic Activity
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