The Neuropeptide Processing Enzyme EC 3.4.24.15 Is Modulated by Protein Kinase A Phosphorylation*

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The metalloendopeptidase EC 3.4.24.15 (EP24.15) is a neuropeptide-metabolizing enzyme expressed predominantly in brain, pituitary, and testis, and is implicated in several physiological processes and diseases. Multiple putative phosphorylation sites in the primary sequence led us to investigate whether phosphorylation affects the specificity and/or the kinetics of substrate cleavage. Only protein kinase A (PKA) treatment resulted in serine phosphorylation with a stoichiometry of 1.11 ± 0.12 mol of phosphate/mol of recombinant rat EP24.15. Mutation analysis of each putative PKA site, in vitro phosphorylation, and phosphopeptide mapping indicated serine 644 as the phosphorylation site. Phosphorylation effects on catalytic activity were assessed using physiological (GnRH, GnRH1–9, bradykinin, and neurotensin) and fluorimetric (MCA-PLGPDL-Dnp and or- thoamino benzoate; CKII, casein kinase II; Dnp, dinitrophenyl; GnRH, gona- dthic, I-arginyl-[1-((2,4-dinitrophenyl)-ethylenediamine]-5-isoquinolinesulfonamide-HCl; Lys-C, endoproteinase Lys-C; MADLI/TOF, matrix-assisted laser desorption-ionization-time of flight; MCA, 7-methoxycoumarin-4-acetyl; NT, neurotensin; PAGE, polyacryl- amide gel electrophoresis; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; QFS, 7-methoxycoumarin-4-acetyl-[2-((2,4-dinitrophenyl)ethylenediamine]; QFS, 7-methoxy- cFP-AAF-pAB, N-[2-((2,4-dinitrophenyl)ethyl]-5-isoquinolinesulfonamide-HCl; Lys-C, endoproteinase Lys-C; MADLI/TOF, matrix-assisted laser desorption-ionization-time of flight; MCA, 7-methoxycoumarin-4-acetyl; NT, neurotensin; PAGE, polyacryl- amide gel electrophoresis; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; QFS, 7-methoxycoumarin-4-acetyl-[2-((2,4-dinitrophenyl)ethylenediamine]; QFS, 7-methoxy-
phosphorylation, or by another kinase activated through PKA (18, 19). Indeed, the amino acid sequence of EP24.15 contains PKA, CKII, and PKC consensus phosphorylation motifs (20), suggesting that the enzyme may be a kinase substrate in mammalian cells. To build on the indirect observation of possible kinase influences on EP24.15 activity in rat PC12 cells (17), we sought to determine the role of phosphorylation upon EP24.15, specifically examining its effect on neuropeptide hydrolysis. Similarly, it would be important to determine if phosphorylation is a conserved event in other neuroendocrine/peptide hydrolysis model systems, such as in AtT-20 mouse pituitary cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were purchased from Sigma unless otherwise noted.

**Protein Expression and Mutagenesis**

For PKC—PKC was purified from Rat 6 fibroblast cell lines overexpressing PKC (epsilon calcium independent) or PKC (beta calcium dependent) (gift of Dr. Robert Krauss, Mount Sinai School of Medicine) and assays were performed as described elsewhere (24, 25), using 3 μg of EP24.15, each condition was assayed in triplicate. EGF receptor peptide (RRKTRLRR) served as positive control.

For PKC—PKC was purified from Rat 6 fibroblast cell lines overexpressing PKC (epsilon calcium independent) or PKC (beta calcium dependent) (gift of Dr. Robert Krauss, Mount Sinai School of Medicine) and assays were performed as described elsewhere (24, 25), using 3 μg of EP24.15, each condition was assayed in triplicate. EGF receptor peptide (RRKTRLRR) served as positive control.

**Phosphorylation Assays**

For PKA—5–30 units of PKA/μg of EP24.15 (1 unit = amount of enzyme required to transfer 1 pmol of phosphate to Kemp tide substrate LRRASLG in 1 min at 30 °C, PKA catalytic subunit, New England Biolabs, Beverly, MA) were incubated in PKA reaction buffer (50 mM Tris, 10 mM MgCl₂, 0.3 mM dithiothreitol, pH 7.5). Kemp tide (LRRASLG) served as positive control.

For CKI—50–300 units of CKII/μg of EP24.15 (1 unit = amount of enzyme required to transfer 1 pmol of phosphate to RRREEEEEE peptide in 1 min at 30 °C, New England Biolabs) were incubated in CKI reaction buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM MgCl₂, 0.3 mM dithiothreitol, pH 7.5). The peptide RRREEEEEE served as positive control.

**SDS- and Native-PAGE**

Samples for SDS-PAGE were heated in 2 x sample buffer at 65 °C for 5 min. The proteins were separated on an 8% SDS-polyacrylamide gel as described previously (22) with modifications. 5–40 ng of EP24.15 (either phosphorylated or non-phosphorylated) was incubated at 37 °C with varying amounts of QFS and QF7 (4.4–17.6 μM) in a final volume of 635 μL. Kinetic parameters (Kₐ, Vₘₐₓ, kₐ, and k₋₋/Kₐ) were evaluated using the double-reciprocal plot method of Lineweaver and Burk (29).

**Quantitation of Incorporated Phosphate**

Prior to inactivation of the kinase assays (described above), a 2-μl aliquot was spotted onto P81 cellulose phosphate paper (Life Technologies, Grand Island, NY) (representing total counts). The sample was allowed to dry, washed with 75 mM phosphoric acid (specific incorporation) (4 x 25 μl, where no more label was eluted), counted, and moles of phosphate incorporated per mole of EP24.15 ± S.E. calculated. To ensure phosphorylation saturation, controls included: decreasing substrate concentration, increasing kinase concentrations, increasing ATP concentration, and examining time course reactions by PhosphorImager analyses for time-dependent saturation of signal. There was no incorporation in the absence of kinase. Additionally, after saturation of phosphate incorporation (90 min, 10 units of PKA), an additional 10 units of PKA was added, demonstrating that saturation was not due to kinase depletion.

**Kinetic Determinations Using the Fluorimetric Substrates QFS and QF7**

EP24.15 enzymatic activity was determined under discontinuous assay conditions with the quenched fluorescent substrate QFS (27) and QF7 (28), as described previously with modifications. The non-phosphorylated EP24.15 enzyme (control) used for all kinetic determinations underwent identical kinase reaction conditions (described above), except that ATP was excluded. All determinations were done using two independent protein preparations and two independent phosphorylation reactions. Total substrate hydrolysis was less than 10%. 6.8 ng of EP24.15 (either phosphorylated or non-phosphorylated) was incubated at 37 °C with varying amounts of QFS or QF7 (4.4–17.6 μM) in a final volume of 635 μL. Kinetic parameters (Kₐ, Vₘₐₓ, kₐ, and k₋₋/Kₐ) were evaluated using the double-reciprocal plot method of Lineweaver and Burk (29).

**Kinetic Determinations Using Physiological Peptides GnRH, GnRH₁₋₉, Bradykinin, and Neurotensin**

EP24.15 activity was determined under discontinuous assay conditions by quantification of substrate product peaks via high-performance liquid chromatography as described previously (22) with modifications. 5–40 ng of EP24.15 (phosphorylated and non-phosphorylated) was incubated at 37 °C with varying concentrations of peptide substrate (11.2 μM to 1 μM for GnRH, 10–100 μM for GnRH₁₋₉, and NT, and 2–100 μM for bradykinin and bradykinin-amide). For NT assays, both cleavage products, NT₁₋₉ and NT₂₋₁₃, were used as standards. Total substrate hydrolysis was less than 10%. Kinetic parameters were evaluated using Lineweaver and Burk plots (29).

**Determination of Enzyme Inhibitor Constants for cFP-AAF-pAAB**

The inhibition constant of EP24.15 for the specific active site-directed inhibitor, cFP-AAF-pAAB, was determined with 25 ng of EP24.15 (either phosphorylated or non-phosphorylated) incubated at 37 °C with QFS (4.4 μM final concentration) and varying concentrations of cFP-AAF-pAAB (0–100 nM) in reaction buffer (125 mM NaCl, 0.3 mM dithiothreitol, 25 mM Tris-HCl, pH 7.5) in a final volume of 635 μL. Reactions were terminated after 30 min by the addition of 115 μl of 0.5 M sodium formate, pH 3.5. EP24.15 activities were determined as described above and the inhibition constant or Kᵢ for phosphorylated and non-phosphorylated enzyme was evaluated using the method of Dixon (30).

**In Vivo Labeling and Immunoprecipitation**

Rat pheochromocytoma PC12 cells (grown as described previously, Ref. 17) and mouse pituitary AtT-20 cells (grown as described previously, Ref. 31) were cultured in 6-well plates (Nunc, Naperville, IL) until 70% confluent, serum-deprived for 10 h in phosphate-free media (Mediatech, Herndon, VA), and incubated in pregassed phosphate-free Dulbecco’s modified Eagle’s medium containing 1 mCi/ml of [³²P]orthophosphate (PerkinElmer Life Sciences) for 6 h. For kinase activation/inhibition experiments, 100 μM forskolin (β₃-acetoxy-1α,6,9-trihydroxy-8,13-epoxy-labd-14-en-11-one) was added 30 min prior to harvesting, and 20 μM H89 (Calbiochem, La Jolla, CA), a PKA-selective inhibitor, was preincubated on cells for 4 h prior to forskolin activation, respectively. EP24.15 was then immunoprecipitated with antibodies as described previously (21) with the modification of RIPA buffer containing phosphatase inhibitors (10 mM sodium orthovanadate, 100 mM fenvalerate, 1 mM Microcystin-LR) (Calbiochem, La Jolla, CA) in the presence of 25 μl of the affinity purified anti-EP24.15 antibody (21). Immunoprecipitation reactions were electrophoresed on an 8% SDS-polyacrylamide gel (23). Gels were dried under vacuum and exposed to film or phosphoscreen for further quantitation.
Phosphoamino Acid Analysis

Immunoprecipitated, \(^{32}\)P-labeled EP24.15 from either PC12 or ArT-20 cells, and in vitro PKA phosphorylated EP24.15 was extracted from SDS-PAGE gels (26). 200 \(\mu\)l of 6 M HCl was added, tubes purged with nitrogen gas, capped, and then incubated at 110 °C for 1 h (32). The hydrolyzed samples were dried by vacuum centrifugation. Hydrolyzed amino acids were mixed with phosphoamino acid standards (Ser(P), Thr(P), and Tyr(P)) (ICN Biomedicals, Aurora, OH). Samples were spotted on 20 \(\times\) 20-cm\(^2\) cellulose TLC plates (EM Science, Gibbstown, NJ), dried, and run at least 15 cm in a proprionic acid, 1 M NH\(_4\)OH, isopropyl alcohol (45:17.5:17.5) solvent system (33). Amino acid standards were visualized using a ninhydrin spray (0.25% in acetone), and experimental phosphoamino acids visualized by autoradiography.

CNBr Cleavage of Phosphorylated EP24.15

In vitro phosphorylated EP24.15 was separated from free \(^{32}\)P\textsubscript{ATP} by Sephadex G75 gel filtration (Amersham Pharmacia Biotech, Piscataway, NJ). Crystalline cyanogen bromide was prepared in 70% trifluoroacetic acid, and cleavage performed (34). Cleavage fragments were chromatographed on an analytical (0.6 \(\times\) 50 cm) Bio-Gel P10 Fine (Bio-Rad) column eluted isocratically in 0.1 M ammonium bicarbonate (pH 8.0) under denaturing conditions at 0.5 ml/min. 0.5-ml fractions were collected and radioactivity quantitated in a scintillation counter. Mass estimates of cleavage fragments were deduced by a plot of the relative elution constant (\(k_{el}/k_0\) versus log(MW)) of calibrated molecular weight standards prior to, and after the sample was chromatographed.

Proteolytic Cleavage of Phosphorylated EP24.15 and MALDI-TOF Mass Spectrometry

Trypsin and endoproteinase Lys-C (Roche Molecular Biochemicals, Indianapolis, IN) were reconstituted and incubated (as per the manufacturer’s instructions) with in vitro phosphorylated EP24.15. The digested (phospho)peptides were desalted using a ZipTip (Millipore, Bedford, MA), divided into aliquots, and resuspended in 5 \(\mu\)l of 10 mg/ml \(\alpha\)-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, with angiotensin as an internal standard. To further enhance detection of phosphorylated peptides, a portion of the peptide mixture was dissolved in a 1:1 1 mM ammonium citrate/matrix solution (35).

Identification and Sequencing of Mouse EP24.15 cDNA

A \(\lambda\) ZapII mouse pituitary cDNA library was purchased (Stratagene, La Jolla, CA) and insert size determined by screening using 15.5 \(\times\) 10\(^5\) recombinants and was screened as described previously with modifications (38). After plaque formation, plates were overlaid first with one nitrocellulose filter (BA82 Schleicher and Schuell, Keene, NH), for 1 min, and then a second filter was placed for 2 min. These duplicate filters were probed with the entire coding sequence for the rat EP24.15 cDNA (36), labeled by random priming (Rediprime II, Amersham Pharmacia Biotech). Filters were washed stringently to 0.1 \(\times\) SSC, 0.1% SDS, 65 °C. From the first screen (-2.8 \(\times\) 10\(^6\) plaques), 27 positive plaques were identified. Subsequent rescoring produced 14 positive plaques which were purified, and placed through a tertiary screen. Bluescript plasmid containing the cloned insert were excised by superinfection with R408 helper phage (36). cDNA inserts ranged in size from 1.1 to 2.3 kilobases. Restriction analysis and probing with 5’ and 3’ random primed labeled cDNA fragments indicated that one of the clones contained a full-length insert. This clone was sequenced in both directions using the ABI Bigdyte terminator sequencing kit (PerkinElmer Life Sciences, South Plainfield, NJ). Data were analyzed using ABI Sequencing Analysis 3.3 (PerkinElmer Life Sciences), and Sequencer 3.1.1 (Gene Codes, Ann Arbor, MI) computer programs. Sequence alignments were made with the CLUSTAL program (39).

RESULTS

EP24.15 Is a Substrate for PKA Phosphorylation—Examination of the EP24.15 primary sequence revealed putative consensus phosphorylation sites (20), including those for PKA, PKC, and CKII (refer to Fig. 5). These motifs suggested that EP24.15 could be a potential substrate for phosphorylation. To test this hypothesis, rat recombinant EP24.15 and control substrates were incubated with various protein kinases. Whereas the incubation of EP24.15 with PKC (both \(\beta\) and \(\delta\) isotypes) and CKII did not cause incorporation of phosphate into the protein (versus positive control substrates) (Fig. 1A), incubation with PKA yielded a rapid and saturable incorporation of phosphate at the correct mass of 77 kDa (Fig. 1B). The phosphorylated residue was confirmed to be serine by phosphoamino acid analysis (Fig. 1C). This incorporation yielded an overall stoichiometry of 1.11 \(\pm\) 0.12 mol of phosphate/mol of EP24.15, consistent with one primary site of phosphate incorporation. A time course of phosphorylation (performed see “Experimental Procedures”) from 15 to 240 min indicated that saturation of incorporated label occurred within 90 min (data not shown) under the assay conditions. To ensure saturable labeling conditions, components of the kinase reaction were varied in an attempt to increase the molar ratio (Table I). Doubling the ATP concentration, PKA concentration, and priming the reaction with additional PKA at 90 min did not effect the stoichiometry of the reaction. These results suggested that the system was at saturation for subsequent kinetic analyses.

Phosphorylation of EP24.15 Introduces Alterations in Enzyme Kinetic Parameters—The possibility that PKA phosphorylation of EP24.15 may effect kinetic parameters toward various substrates was examined. A significant 46% decrease in the specificity constant was observed for the QF7 fluorimetric substrate (18 \(\times\) 10\(^5\) M \(\text{cat/s}\) versus 10 \(\times\) 10\(^5\) M \(\text{cat/s}\), \(n = 4, p < 0.05\)) upon PKA phosphorylation. Another fluorimetric substrate was examined (QFS, see Table II), and following phosphorylation, a 44% decrease in the specificity constant (\(k_{cat}/K_m\))
was observed, nearly identical to the result seen with QF7. Similarly, a 38% decrease in the specificity constant was observed for GnRH$_{1-9}$ ($p < 0.09$). The specificity constant of neurotensin also decreased, and indicated the same trend, but was not significant.

Strikingly, phosphorylation caused a 7-fold increase in the $K_m$ and $k_{cat}$ (with the corresponding increase in $V_{max}$) parameters measured for GnRH (Table II). The possibility that the 7-fold increases observed with the kinetic values for GnRH (not seen with GnRH$_{1-9}$) were a function of a newly formed charge interaction between the COOH-terminal residue on the peptide and the phosphate group on EP24.15 was investigated. Another EP24.15 substrate, bradykinin (RPPGFSPFR), and a synthetic bradykinin analog containing an amide-blocked COOH terminus (RPPGFSPPR-NH$_2$) were used for similar kinetic analyses. The carboxyl-terminal charge of these substrates were analogous to GnRH$_{1-9}$ and GnRH, respectively. Following phosphorylation, the $K_m$ of EP24.15 for bradykinin increased from 3.8 to 13 $\mu$M upon phosphorylation, while for bradykinin-amide it decreased from 82 to 50 $\mu$M, and thus did not replicate the findings for GnRH.

To examine whether the alterations in the kinetic parameters of EP24.15 for GnRH were caused by a change in active site accessibility due to phosphorylation, the $K_m$ was determined with an EP24.15-specific active site-directed inhibitor, cFP-AAF-pAB, the design of which is based on the hydrophobic core (40). In vitro phosphorylation conditions were altered in an attempt to further saturate EP24.15 phosphorylation site(s) (see "Experimental Procedures"). To build on earlier findings (17) and confirm the phosphorylation site on EP24.15 using [$\gamma^32P$]ATP and then cleaved by cyanogen bromide. This treatment yielded a phosphorylated 2-kDa fragment which was detectable by scintillation counting of fractions eluted from a size exclusion chromatography column calibrated before and after the CNBr-cleaved fragments were separated (Fig. 2B).

Furthermore, phosphorylated and non-phosphorylated enzyme was prepared (see "Experimental Procedures"), and subjected to specific (trypsin and Lys-C) proteolytic cleavage and mass analysis by MALDI-TOF mass spectrometry. Lys-C digestion yielded a peptide whose mass (2484 daltons) corresponded to the fragment 637–659 with the addition of a 80-dalton phosphate moiety (Fig. 2C). Trypsin digestion of PKA-phosphorylated EP24.15 yielded a peptide whose mass (1846 daltons) corresponded closely to fragment 643–659 with the addition of a 80-dalton phosphate moiety (an actual addition of 84 daltons) (Fig. 2C). This fragment also included the serine at residue 644, a consensus PKA site. Other miscut fragments from the analysis of trypsin digestions also indicated serine 644-containing fragments with the addition of an 80-dalton moiety. These miscut fragments included fragment 657–664, the mass of which measured 9162 (the theoretical mass of 9085 + 77 daltons), as well as fragment 643-675, the mass of which measured 3687 (the theoretical mass of 3606 + 81 daltons) (Fig. 2C). No +80-dalton adducts to corresponding fragments were noted elsewhere in the spectra of either the trypsin or Lys-C proteolytic digestions.

**TABLE I**

| Substrate | Sequence | $K_m$ (M) | $V_{max}$ pmol/min/mg | $k_{cat}$ s$^{-1}$ | $k_{cat}/k_m$ $10^{-3}$M$^{-1}$s$^{-1}$ |
|-----------|----------|----------|-----------------------|------------------|----------------------|
| QFS       | Mca-PL:GPDL-Dop | NP$^a$  | 8.0 $\pm$ 3.0 | 1.0 $\pm$ 0.15 | 1.3 $\pm$ 0.20 | 1.5 |
| GnRH$_{1-9}$ | pGlu-HWSY:GLRP | P$^b$ | 12 $\pm$ 3.0 | 0.74 $\pm$ 0.07 | 1.0 $\pm$ 0.11 | 0.82 |
| GnRH | pGlu-HWSY:GLRGP-NH$_2$ | NP | 57.3 $\pm$ 0.7 | 5.6 $\pm$ 0.68 | 7.2 $\pm$ 0.60 | 13 |
| NT | pGlu-LYENKPR:RPYIL | P | 7.3 $\pm$ 1.0 | 4.5 $\pm$ 0.42 | 5.7 $\pm$ 0.50 | 7.8$^c$ |

$^a$ NP, not phosphorylated.

$^b$ P, phosphorylated.

$^c$ $p < 0.05$.

$^d$ $p < 0.09$.

$^e$ $p < 0.01$.  

**TABLE II**

| Substrate | Sequence | EP24.15 $V_{max}$ pmol/min/mg | $k_{cat}$ s$^{-1}$ | $K_m$ M | $k_{cat}/K_m$ $10^{-3}$M$^{-1}$s$^{-1}$ |
|-----------|----------|-----------------------|------------------|--------|----------------------|
| QFS       | Mca-PL:GPDL-Dop | NP$^a$  | 8.0 $\pm$ 3.0 | 1.0 $\pm$ 0.15 | 1.3 $\pm$ 0.20 | 1.5 |
| GnRH$_{1-9}$ | pGlu-HWSY:GLRP | P$^b$ | 12 $\pm$ 3.0 | 0.74 $\pm$ 0.07 | 1.0 $\pm$ 0.11 | 0.82 |
| GnRH | pGlu-HWSY:GLRGP-NH$_2$ | NP | 57.3 $\pm$ 0.7 | 5.6 $\pm$ 0.68 | 7.2 $\pm$ 0.60 | 13 |
| NT | pGlu-LYENKPR:RPYIL | P | 7.3 $\pm$ 1.0 | 4.5 $\pm$ 0.42 | 5.7 $\pm$ 0.50 | 7.8$^c$ |

$^a$ NP, not phosphorylated.

$^b$ P, phosphorylated.

$^c$ $p < 0.05$.

$^d$ $p < 0.09$.

$^e$ $p < 0.01$.  

**EP24.15 Is Phosphorylated by PKA in Vivo in Rat PC12 and Mouse AT-20 Cells**—To build on earlier findings (17) and con-
firm PKA action on rat EP24.15 in vivo, rat PC12 cells were incubated with $^{32}$Porthophosphate and EP24.15 was immunoprecipitated. Analysis of the immunoprecipitate by polyacrylamide gel electrophoresis and autoradiography revealed a labeled protein band of 77-kDa present in cell extracts (Fig. 3A). The labeled band was extracted from the gel and subjected
to phosphoamino acid analysis which revealed only serine phosphorylation (Fig. 3B), consistent with serine/threonine kinase action. To test if EP24.15 is PKA phosphorylated in vivo, PC12 cells were subjected to a kinase activation and inhibition paradigm (Fig. 3C). Forskolin stimulation resulted in a 38% increase in 32P labeling. Preincubation with the PKA-selective inhibitor H89 dropped 32P labeling to 58% of basal (vehicle) levels, despite the presence of forskolin. Western blot autoradiograms showed no change in the protein expression levels secondary to the treatments (data not shown).

Because murine AtT-20 cells are an important cell biological model for the study of EP24.15 regulation (27, 31), we sought to determine whether PKA phosphorylation of EP24.15 was conserved across species, and specifically in this mouse model. Mouse pituitary AtT-20 cells were incubated with [32P]orthophosphate and EP24.15 was immunoprecipitated (Fig. 3D). These cells also revealed only serine phosphorylation on EP24.15 protein (Fig. 3E). Again, a kinase activation and inhibition paradigm was employed (Fig. 3F) to determine if PKA is a kinase acting on EP24.15. Upon stimulation of AtT-20 cells with forskolin, a 35% increase in 32P labeling was observed. When the cells were preincubated with the PKA-selective inhibitor H89, the 32P labeling dropped to 17% of basal (vehicle). In parallel, it was confirmed by scanning densitometry of the Western blot autoradiograms that there was no change in the protein expression due to the treatments (data not shown).

Molecular Cloning of Murine EP24.15 Confirms Conservation of Serine 644 PKA Phosphorylation Site—To determine whether the serine 644 phosphorylation site is conserved in mouse EP24.15, and to validate the experiments in the AtT-20 model neuropeptide cell line, a mouse pituitary cDNA library was screened. A full-length mouse EP24.15 cDNA clone was isolated and sequenced (Fig. 4). On the nucleic acid level, the mouse and rat coding sequences were 92.9% identical. The mouse and rat amino acid sequences shared 96.7% identity and 97.1% similarity (Fig. 5) and both species encoded a protein of 687 amino acids. The structural features are as reported for the rat form of the enzyme (38), and serine 644 was conserved (Fig. 5).

**DISCUSSION**

Whereas there are many possible mechanisms by which peptidase activity may be regulated, the presence of numerous putative phosphorylation sites on EP24.15 led us to examine whether phosphorylation plays a role in its modulation. Of the kinases studied, only PKA elicited significant phosphorylation (Fig. 1B). The effects of the in vitro PKA phosphorylation of EP24.15 on enzyme kinetics with different substrates was assessed. The 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decline in the 46, 44, and 40% decrease in activity has been...
EP24.15 Is Phosphorylated by PKA

PKA sites indicated: PKA sites are Rat EP24.15 (accession number P24155) (Ref. 35) and mouse EP24.15.

PKA phosphorylation site, serine 644, is indicated by curved number underlines. Casein kinase II sites have been postulated to approach −10 mM in synaptosomal vesicles. Therefore, it is quite plausible that peptide concentrations in the synapse at the point of vesicular release can approach the in vivo $K_{m}$ range of −10 μM described here and by others (45), as well as 1 mM upon phosphorylation. Overall, these findings suggest that phosphorylation can have a significant impact on EP24.15 activity by preventing its saturation at the time of pulsatile release, and hence, GnRH hydrolysis. If EP24.15 exists in the hypophysial portal blood both in a phosphorylated and non-phosphorylated state, the effective substrate concentration range of this enzyme would be substantially broadened.

We next sought to understand the nature of the biophysical changes conferred by phosphorylation of EP24.15. None of the kinetic changes outlined (Table II) would appear to be the result of limited substrate accessibility to the active site, given the nearly identical $K_{d}$ determinations with phosphorylated and non-phosphorylated EP24.15 for the active site-directed inhibitor cFP-AAF-pAB. We further hypothesized that the major alterations in kinetic parameters observed with GnRH as compared with GnRH$_{1–9}$, were perhaps due to a direct interaction of the phosphate group of the enzyme with the carboxyl-terminal amide of the substrate peptide (this glycine-amide being absent in GnRH$_{1–9}$), where there might be repulsion and conformational alterations with respect to the carboxyl moiety in GnRH$_{1–9}$. In this context, we examined the kinetics of both phosphorylated and non-phosphorylated EP24.15 with bradykinin-amide and bradykinin in a manner identical to that for GnRH and GnRH$_{1–9}$, respectively. The $K_{m}$ of EP24.15 for bradykinin increased upon phosphorylation, while for bradykinin-amide it decreased, not in agreement with the relative changes seen with GnRH and GnRH$_{1–9}$. This would suggest that the major changes observed in the kinetic parameters for GnRH upon EP24.15 phosphorylation are not likely a charge-induced phenomenon. There may exist other, as yet unknown, binding mechanisms which differentiate between the two substrates (GnRH and GnRH$_{1–9}$). More importantly, it indicates that the phosphorylation of EP24.15 alters its neuropeptide kinetic profile and substrate specificity.

Systematic site-directed mutagenesis of all putative serine PKA phosphorylation sites to alanine, indicated serine 644, is indicated by number sign, r, rat; m, mouse; :, residue conserved.

reported for caspase-9 (15), which has significant downstream consequences in the apoptotic cascade.

There are compelling physiological data (2–4) establishing that the half-life of GnRH increased 8-fold (2). Additionally, peripheral infusion of the EP24.15 inhibitor augmented GnRH-dependent luteinizing hormone surge in rats (4). GnRH secretion from the hypothalamus in a pulsatile fashion is critical for the proper release of luteinizing hormone and follicle stimulating hormone from the pituitary, and the resultant control of mammalian reproduction (42). As such, understanding the kinetic aspects of GnRH degradation at the release site by EP24.15 is fundamentally important in understanding GnRH pulse waveform regulation. In this study, there was a 7-fold increase in $V_{max}$ and $k_{cat}$ (and correspondingly, $V_{max}$) observed with GnRH upon EP24.15 phosphorylation. These changes imply that the affinity of GnRH binding was reduced upon phosphorylation, but once bound, the substrate appears to be turned over more rapidly. It is possible that the phosphorylated enzyme has the versatility to expeditiously handle large increases in GnRH concentration (during the large increase in amplitude, concomitant with GnRH pulsatile release), without becoming rapidly saturated at these substrate concentrations. The insulin peptide concentration in secretory vesicles has been measured to be approximately 40 mM just prior to release (43). In another study (44), the concentration of neuropeptide achieved at the site of release at the synapse has been postulated to approach −10 mM in synaptic vesicles.
EP24.15 Is Phosphorylated by PKA

fragment contained an internal arginine (647), situated amino
to a proline residue, a combination of residues which is known
to cleave very inefficiently with trypsin. Furthermore, limited
proteolysis with endoprotease Lys-C yielded spectra with a
full cut fragment (637–659), consistent with serine 644 phos-
phorylation (Fig. 2C). Serine 644 was conserved between rat
and mouse, a finding paralleled by the in vivo data demonstrat-
ing that PKA is contributing to EP24.15 phosphorylation in
both rat and mouse cell lines. In the human EP24.15 sequence,
6 of 7 putative PKA sites are conserved, but not the serine 644
consensus site. Nonetheless, when human M17 neuroblastoma
cells were subjected a similar PKA kinase activation/inhibition
scheme, EP24.15 likewise was phosphorylated by PKA (data
not shown). Importantly, in a fashion similar to rat and mouse
(Fig. 3), the PKA phosphorylation of EP24.15 still occurs in vivo
in human cells.

Homology modeling studies of EP24.15 based on the bacte-
rial enzymes, thermolysin, and neutral protease (previously
solved to atomic resolution by x-ray diffraction), indicate the
presence of a 4-helix bundle structural motif in the carboxy-
terminal 80–95 residues.2 This motif has been previously mod-
eled by homology to the related metalloenzymes enkephaline-
as and angiotensin converting enzyme (46). In this model, serine
644 would reside near the carboxyl end of the second helix in a
4-helix bundle, a structural motif present in many proteins
(reviewed in Ref. 47). The closest distance approximation of
serine 644 to the active site zinc is approximately 17 Å, seem-
ingly too far for a direct steric effect in the active site. This
interpretation is consistent with the unchanged EP24.15 inhib-
itor (cFP-AAF-pAB) $K_i$ data upon phosphorylation, although it
is possible a longer range conformational change in the protein
is modulated through this structural motif.

As our initial studies characterizing the phosphorylation and
inhibition of EP24.15 activity had been performed in vitro
with recombinant enzyme, therefore it was important to determine
if phosphorylation of EP24.15 occurs in mammalian cells. In-
terestingly, an earlier study in rat pheochromocytoma (PC12)
cells treated with cAMP analogues showed a decrease in the
soluble specific activity of EP24.15 without a decrease in the
amount of EP24.15 protein (17). We extend this observation by
specifically demonstrating decreases in EP24.15 enzyme activ-
ity by PKA phosphorylation in vitro, and by demonstration of
the PKA phosphorylation of EP24.15 in PC12 cells. We further
explored whether the PKA phosphorylation of EP24.15 is con-
served in a commonly used mouse neuroendocrine cell model.
Utilizing the AtT-20 mouse pituitary cell line, our studies indi-
cated that EP24.15 can be phosphorylated by PKA. As was
the case in the PC12 cells, EP24.15 phosphorylation was en-
hanced by forskolin treatment, and inhibited by the PKA-
selective inhibitor H89 (48) concomitant with forskolin treat-
ment. The complete cDNA cloning of mouse EP24.15 (Fig. 4)
and alignment with the rat sequence (Fig. 5) indicated the
perfect conservation of the PKA phosphorylation site, serine
644.

It is also possible that phosphorylation may regulate EP24.15
function by subcellular targeting and/or expression at the
plasma membrane (27), the nucleus (4, 49), or other cellular
locations, either directly or via protein-protein interactions.
For example, enkephalinase (EC 3.4.24.11), a related enzyme,
can be phosphorylated by casein kinase II, and subsequently
co-associates with a tyrosine-phosphoprotein complex in Nalm
6 (lymphoblastic leukemia) cells, suggesting a role for this
peptidase in signal transduction pathways (50). Previously, a

\[^{2}\text{M. J. Glucksman, M. Ciscio, and J. L. Roberts, manuscript in preparation.}\]
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EP24.15 Is Phosphorylated by PKA