Phosphorylation of Tyrosine Hydroxylase in Situ at Serine 8, 19, 31, and 40*

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The site specificity of tyrosine hydroxylase phosphorylation in intact PC12 cells, labeled with $^{32}$P, was investigated. Digestion of $^{32}$P-tyrosine hydroxylase with trypsin produced five distinct $^{32}$P-labeled peptides (termed PC-1 through PC-5). Sequencing of the peptides revealed four acceptor sites: Ser$^8$, Ser$^{19}$, Ser$^{31}$, and Ser$^{40}$. The phosphorylation site in peptides PC-1 (AVSEQDACK) and PC-2 (RAVSEQDACK) was identified as Ser$^8$. Agents which cause calcium influx increased $^{32}$P incorporation into tyrosine hydroxylase at Ser$^{19}$. PC-3 was identified as QAEAVTSPK, which contains the phosphorylation site Ser$^{31}$. Nerve growth factor and phorbol dibutyrate increased $^{32}$P incorporation into Ser$^{40}$. PC-4 was identified as the N-terminal amino acid sequence (MPTPSAPSPQPK), and the $^{32}$P incorporation occurred at Ser$^{31}$. Of the agents tested, only okadaic acid (a protein phosphatase inhibitor) increased the phosphorylation of Ser$^{8}$. PC-5 was shown to contain Ser$^{40}$. Treatment of the PC12 cells with cAMP-acting agents increased $^{32}$P incorporation into Ser$^{40}$.

The present results demonstrate that some, but not all, of the phosphorylation sites demonstrated previously in vitro exist in situ. Conversely, the identification of Ser$^{8}$ establishes a physiological phosphorylation site not previously reported in vitro. These four sites account for most, if not all, of the diversity in tryptic phosphopeptides reported previously for rat tyrosine hydroxylase.

Tyrosine hydroxylase (TH) catalyzes the initial step in the biosynthesis of catecholamines. Because this step is rate-limiting, the regulation of TH activity has been the subject of numerous studies (cf. Ref. 1). Recent attention has focused on the activation of TH by protein kinase-mediated phosphorylation. Cyclic AMP-dependent protein kinase was the first shown to phosphorylate and activate TH in vitro (2, 3). Because TH activity increased directly with phosphorylation occurred at Ser$^{8}$. Of the agents tested, only okadaic acid (a protein phosphatase inhibitor) increased the phosphorylation of Ser$^{8}$. PC-5 was shown to contain Ser$^{40}$. Treatment of the PC12 cells with cAMP-acting agents increased $^{32}$P incorporation into Ser$^{40}$.

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**MATERIALS AND METHODS**

$^{32}$P Labeling of PC12 Cells—PC12 cells, a clonal cell line derived from a rat adrenal medullary tumor, synthesize and secrete catecholamines and can contain up to 10% of their total cellular protein as TH (19, 20). Monolayers of PC12 cells were brought to room temperature and pre-equilibrated in incubation solution (150 mM NaCl, 15 mM HEPES, 5.5 mM D-glucose, 4.4 mM KCl, 1.2 mM MgCl$_2$, 1.0 mM CaCl$_2$, adjusted to pH 7.4 with NaOH at room temperature). The cells were prelabeled (60-120 min, 37°C) in incubation solution containing $^{32}$P (0.25-1.0 mCi/ml). The cells were then incubated with test substances (added from concentrated stocks in incubation solution) as indicated in the text. After incubation, the medium was

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* Portions of this paper (including part of "Materials and Methods," part of "Results," and Figs. 1, 4, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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aspirated and the cells were solubilized in 1% SDS, 1 mM EDTA (NaOH to pH 8). The samples were heated in a boiling water bath for 2–5 min, and aliquots were subjected to SDS-polyacrylamide gel electrophoresis with 9% T slab gels directly or after quantitative immunoprecipitation with affinity-purified antibodies to TH (20). "P was aspirated and the cells were solubilized in 1% SDS, 1 mM EDTA

Tryptic Digestion and Phosphopeptide Separation—"P-Labeled peptides released from the gel slices by limit tryptic digestion were separated by reverse-phase HPLC. HPLC was performed with a C_{18} column (Vydac, 218TP5415) equilibrated in 0.1% trifluoroacetic acid. The "P-labeled peptides were eluted at 1 ml/min with an acetonitrile gradient (usually 0.2%/min) in 0.1% trifluoroacetic acid. An on-line detector (Radiomatic) provided "P peak integration and collection. "P peaks were collected separately and concentrated in a Speed-Vac (Savant) prior to subsequent analysis.

RESULTS

Multiple TH Phosphopeptides in PC12 Cells

TH migrated as a single ~62,000-Da band slightly above catalase (Fig. 1). Fig. 2 illustrates the reverse-phase HPLC elution profile of "P-labeled peptides produced by limit tryptic digestion of TH immunoprecipitated from untreated PC12 cells after labeling with "P. Five prominent peaks of radioactivity were detected and named according to their order of elution, as shown. To determine whether more than one phosphopeptide was present in any of the individual peaks, PC-1 through PC-5 were concentrated and analyzed with two-dimensional electrophoresis/chromatography (tryptic fingerprinting) and with isoelectric focusing. As a rule, each of the five peaks migrated as a single phosphopeptide in either of the two-dimensional separation procedures. (Occasionally, the fingerprints of PC-3 revealed a second, minor phosphopeptide (cf. Miniprint).) The correspondence of the elution pattern of the five phosphopeptide peaks in reverse-phase HPLC to their migration in the two fingerprint systems is presented in Fig. 2. Thin-layer isoelectric focusing in Servalyt 3-10 Precoat gels indicated that all of the phosphopeptides had pI values less than 6 (Fig. 3). The pI values of the phosphopeptides were ordered (from lowest to highest) PC-1 < PC-3 < PC-2 ≈ PC-5 < PC-4. Relative to the Coomassie staining pattern of the three different Serva isoelectric focusing standards, the pI estimates are PC-1, ~3.2; PC-2, ~3.9; PC-3, ~3.9; PC-4, ~5.1; PC-5, ~3.9.

Thus, the five "P-labeled peaks that were separated with reverse-phase HPLC appeared to be single distinct phosphopeptides. Phosphoamino acid analysis of each of the phosphopeptides revealed phosphoserine but not phosphothreonine or phosphotyrosine (Fig. 4).

Regulation of "P Incorporation into TH Phosphopeptides

To facilitate comparison of the phosphopeptides in the present study to those reported previously and to optimize "P incorporation (and, by inference, stoichiometry) into each of the peptides for subsequent analyses, several test substances were evaluated for their abilities to increase "P incorporation into TH and the phosphopeptides.

Secretagogues—Elevated K⁺ (40 mM), veratridine (100 μM), or nicotine (50 μM) increased "P incorporation into TH. Relatively brief treatments (30–60 s) selectively increased the phosphorylation of PC-1 and PC-2 (Table I). EGTA (1.5 mM,
Influence of pharmacological agents on multiple site phosphorylation of TH

Table I

| Treatment | 32P-Labeled total protein | TH | PC-1 | PC-2 | PC-3 | PC-4 | PC-5 |
|-----------|---------------------------|----|------|------|------|------|------|
| KCl (40 mM, 30 s) | 105 | 160 | 300 | 200 | 250 | 300 | 200 |
| Veratridine (100 μM, 60 s) | 98 | 140 | 220 | 150 | 150 | 150 | 150 |
| Nicotine (50 μM, 30 s) | 102 | 130 | 200 | 180 | 180 | 180 | 180 |
| A23187 (10 μM, 30 min) | 120 | 185 | 900 | 300 | 300 | 300 | 300 |
| Forskolin (10 μM, 10 min) | 105 | 135 | 220 | 160 | 160 | 160 | 160 |
| 8-bromo-cAMP (1 mM, 5 min) | 94 | 120 | 180 | 180 | 180 | 180 | 180 |
| Dibutyryl cAMP (1 mM, 5 min) | 100 | 125 | 225 | 175 | 175 | 175 | 175 |
| Phorbol dibutyrate (1 μM, 30 min) | 135 | 145 | 135 | 135 | 135 | 135 | 135 |
| NGF (50 ng/ml, 30 min) | 128 | 150 | 600 | 130 | 130 | 130 | 130 |
| Okadaic acid (1 μM, 30 min) | 220 | 480 | 650 | 420 | 420 | 420 | 420 |

*32P incorporation into TH and TH phosphopeptides was normalized to 32P incorporation into total cellular protein except in the case of okadaic acid treatment.

* Spaces left blank indicate values between 90 and 110% of control.

PC12 cells were incubated with 32P, for 60–90 min at 37°C and then treated as indicated. The cells were treated with KCl, veratridine, and nicotine at room temperature. All other treatments were at 37°C. Each condition was run in duplicate or triplicate to two to four different experiments. Median values are presented. The distribution of 32P incorporation into the tryptic phosphopeptides in control cells for all of the experiments (n = 10) was PC-1, 22 ± 1; PC-2, 8 ± 1; PC-3, 11 ± 1; PC-4, 27 ± 2; PC-5, 32 ± 2 (mean percent ±S.E.).

added 60 s prior to secretagogues abolished these effects (not shown). Longer treatments (5 min) with the same concentrations of nicotine, veratridine, and elevated K+ as in Table I also produced small (20–30%) increases in 32P incorporation into PC-5 (not shown). A23187, which causes calcium influx ionophorically as opposed to via depolarization, also increased TH phosphorylation. In contrast to the other secretagogues, A23187 increased the phosphorylation of PC-3 in addition to PC-1 and PC-2.

The selective increase in PC-1/PC-2 phosphorylation by elevated [K+] has not been observed previously. This is presumably due to the longer treatment periods (5 min to 1–2 h versus 30 s) and higher temperature (37°C versus 22–24°C) in the previous studies resulting in increases in, variably, PC-3 (T3, see below) or PC-3 and PC-5 (T1, see below) as well (11, 13, 14). Similarly, A23187 and ionomycin were shown previously to increase the phosphorylation of all of the peptides except what appears to be PC-4 (14, 21).

**CAMP-acting Agents—**Forskolin (10 μM, 10 min), 8-bromo-cAMP (1 mM, 5 min) or dibutyryl-cAMP (1 mM, 5 min) increased the phosphorylation of TH (Table I). With these agents, the increases in 32P incorporation into TH were associated entirely with an approximately 2-fold increase in PC-5 phosphorylation. The magnitude of these effects appeared to be inversely related to the relative level of phosphorylation of PC-5 in control cells. In fact, forskolin could produce greater than 4-fold increases in 32P incorporation into PC-5 when PC-5 phosphorylation was decreased by prior treatment with theophylline.³ Vasoactive intestinal peptide and secretin, other systems (cf. Ref. 22) and secretin is predicted to have an effect similar to vasoactive intestinal peptide on phosphorylation in the present studies. The use of different pharmacological grounds (cf. Refs. 22 and 23).

**Phorbol Esters—**Phorbol dibutyrate (1 μM, 15 min) increased 32P incorporation into TH, and the effect resulted predominantly from an increase (240%) in PC-3 phosphorylation. Phorbol dibutyrate also produced a substantially smaller increase (35%) in PC-5 phosphorylation (Table I); however, a similar effect was also observed with 4-α-phorbol dibutyrate (1 μM, 30 min; not shown), and the effect of phorbol dibutyrate on PC-5 phosphorylation was not observed at lower concentrations (10–100 nM; not shown).

In vitro, protein kinase C phosphorylates TH on the same peptide as that phosphorylated by cAMP-PK (7, 35). In contrast, phorbol esters have been reported to increase 32P incorporation into TH in PC12 cells predominantly, if not entirely, in association with a peptide other than that influenced by cAMP-acting agents (11, 13, 21). In that this peptide appears to be PC-3, there is, with one exception (17), agreement between the present and previous studies in intact PC12 cells. However, the disparity between in vitro and in situ results suggests that the involvement of protein kinase C in the phorbol ester-induced phosphorylation of TH in situ is indirect.

**Growth Factors—**NGF produced effects similar to those produced by high concentrations of phorbol dibutyrate, although the magnitude of the NGF effect on PC-3 was larger (Table I). EGF, on the other hand, was without effect on TH phosphorylation in the present studies. The use of different treatment durations (1–120 min), EGF concentrations (10–1000 ng/ml), levels of confluence (cells passed at 1/10 to 1/4), and PC12 cells (two different sources) all failed to produce an EGF-stimulated increase in 32P incorporation into TH (not shown).

**McTigue et al.** (11) reported larger overall effects with a longer (1–2 h) treatment, and Cahill et al. (13) reported smaller overall effects with a shorter (5 min) treatment.

³J. W. Haycock, manuscript in preparation.
phorylation on PC-4 (T4) as well as PC-3. One hypothesis for the failure to observe any effect of EGF in the present studies is that the transduction system(s) influenced by EGF is already activated in control cells. At least some variation of this hypothesis seems likely, because, in contrast to the present data, \(^{32}\)P incorporation into T4 was barely discernible without EGF treatment in the previous report (11).

**Phosphatase Inhibition**—In light of the failure of the test substances above, generally thought to activate protein kinases, to increase \(^{32}\)P incorporation into PC-4, inhibition of protein phosphatase activity was examined. Dephosphorylation of TH by endogenous protein phosphatases is accelerated severalfold by manganese (26), a characteristic of protein phosphatase 2A (27). Okadaic acid, an inhibitor of protein phosphatases 1 and 2A (e.g. Ref. 28), was recently shown to increase TH phosphorylation in intact chromaffin cells (29). As illustrated in Table I and as will be described elsewhere in greater detail, okadaic acid (1 \(\mu\)M, 30 min) increased \(^{32}\)P incorporation into all of the peptides. As shown in Table I, though, the increase in TH phosphorylation was greater than the increase in \(^{32}\)P incorporation into total cellular protein, indicating that the overall phosphate content of TH is turned over more rapidly than that of the general population of cellular proteins. In terms of the individual phosphopeptides, this was true for all of the peptides except PC-4, which showed a 2-fold increase in phosphorylation, comparable with the increase in \(^{32}\)P-labeled total protein.

**Analysis of Phosphorylation Sites**

The phosphopeptides were subjected to manual Edman degradation, and subtractive analyses were performed to identify the location of phosphoserine residues and to test for the presence of multiple phosphorylation sites within a given peptide. As illustrated in Fig. 5, a single phosphorylation site was present in PC-1 and PC-5 at the third amino acid from the N terminus. A single phosphorylation site was present in PC-2 at the fourth amino acid from the N terminus (not shown). No phosphorylation sites were revealed in either PC-3 or PC-4 through six degradation cycles.

In the first sequencing attempt, cells were treated with veratridine, phorbol dibutyrate, and forskolin to increase \(^{32}\)P incorporation into (and presumably the total phosphate content of) PC-1, -2, -3, and -5. Sequences were obtained for PC-1, -2, and -5. In the second sequencing experiment, 50% more cells were used, the cells were treated with NGF and okadaic acid, and a higher threshold for the collection of the radioactive peaks was selected. With this approach, sequence data for both PC-3 and PC-4 were obtained.

The PC-1 sample produced a sequence of AVS\(\cdot\)DI\(\cdot\)RCK, wherein the numbers in subscript indicate the picomole yield. From the subtractive Edman analysis (Fig. 5), the loss of a positive charge was observed at both pH 1.9 and 8.9, presumable reflecting modification of the Lys side chain. At pH 8.9, the additional increase in mobility presumably reflects deamidation of the Gln to Glu. Also, the release of \(^{32}\)P, at position 3 allows the assignment of phosphoserine for the X. Thus, PC-1 was assigned the sequence AVS(P)EQDAK containing Ser\(^{19}\) as the phosphorylation site.

The PC-2 produced the sequence RAVSXEQDAK at a level of 6–12 pmol. Consistent with this sequence, subtractive Edman analysis at pH 1.9 revealed a loss of two positive charges after the first cycle and a release of \(^{32}\)P, at the fourth cycle (not shown). Thus, the sequence RAVS(P)EQDAK, with the phosphorylation site being Ser\(^{19}\), was assigned to PC-2.

The first analysis of PC-3 produced readings in the 6–20 pmol range for one to two amino acids/cycle. Two possible TH sequences could be matched with the data: VSDDVR and XAEAVTXPR, corresponding respectively to nonphosphorylated Ser\(^{30}\) and potentially phosphorylated Ser\(^{31}\). Reverse-phase HPLC chromatography of the radioactivity remaining on the filter (30) indicated that the phosphoserine was cleaved within the eight cycles run. The second analysis, with modified experimental conditions, produced the sequence Q_fA_3V_7E_12A_4A_16V_13T_16S_3P_2R_9 with no indication of the presence of VSDDVR. Based on sequencing yields of serine from known amounts of phospho versus dephospho-LRRASVA, the 13 pmol of serine in position 7 is consistent with the original residue at this position being entirely from phosphoserine. (Also, the phosphopeptides subjected to the sequencing analysis would be expected to elute prior to their cognate dephosphopeptides (3L.) From the subtractive Edman analysis at pH 1.9 (not presented), cycle one decreased the mobility of a portion of the molecules, and the shift was consistent with a loss of one positive charge from +2 to +1. The mobility of the shifted portion of peptide remained unchanged with subsequent cycles, which was taken to reflect conversion of Gln to pyro-Glu in a portion of the molecules during the Edman procedure. At pH 8.9, a decrease in mobility was observed after cycle 3, consistent with the loss of Glu. Together, these data identified PC-3 as QALEAVTS(P)PR, wherein Ser\(^{31}\) is the phosphorylation site.

In the first analysis of PC-4, all of the readings subsequent to the first cycle were below 10 pmol, and the analysis was terminated after six cycles. Consistent with the manual Edman analyses, HPLC analysis of the filter as above indicated that most of the \(^{32}\)P was still associated with peptide after six cycles. Given the low yield of Pro in sequencing PC-3 and the possibility that PC-4 was the Pro-rich peptide containing Ser\(^{31}\) (10), Pro-1 cycles were utilized for cycles 1, 3, 6, and 8 during the second attempt at sequencing PC-4. This analysis resulted in the sequence P_5T_7P_3S_7A_3P_6X_18 for the eight cycles performed. Because PC-4 contained more than 1 Ser, the...
portion of each cycle not injected into the HPLC (collected into vials by the ABI 477A) was analyzed for radioactivity. Cycles 1-6 produced 30-40 cpm, whereas cycle 7 produced 110 cpm. This and the relatively high yield of Ser at position 4 indicated that Ser was the phosphorylated serine in PC-4. In the subtractive Edman analysis (not presented), a loss of one positive charge was apparent after the first cycle and maintained through six cycles, consistent with modification of the phosphopeptide. From these data, PC-4 was assigned the sequence PTPSAPS(P)PQPK.

From this, the release of 32Pi, at cycle 3, and the presence of PC-5 sample resulted in a sequence of R40Q&8Ls3177E59-

Comparison to Previously Reported Tryptic Phosphopeptides from TH

The results of the sequence analyses are summarized in Table II (top). Also presented in Table II (bottom) is a collation of previously described tryptic phosphopeptides from 32P-labeled TH labeled in intact PC12 cells. The assignments were made on the bases of the correlative phosphopeptide separations in Fig. 2 and the pharmacological profile in Table I.

**DISCUSSION**

Little is yet known about the secondary or tertiary structure of TH. In the absence of such data and on the basis of the primary structure of rat TH inferred from cDNA clones, many of the 42 serine residues, distributed throughout the molecule, are potential phosphorylation sites. Campbell et al. (10) demonstrated that at least four of these (serines 8, 19, 40, and 153) could be phosphorylated in vitro. The present studies, however, show that all of the sites of phosphorylation of TH in intact PC12 cells occur within 40 amino acids of the N terminus (serines 8, 19, 31, and 40). Such data are consonant with the hypothesis that the N-terminal region of the enzyme constitutes a regulatory domain which, in the dephosphorylated state, inhibits the catalytic center(s) located further toward the carboxyl portion of the molecule. Phosphorylation of the N-terminal region then relieves the inhibitory influence.

Phosphorylation Sites Previously Identified in Vitro—Ser and Ser are within canonical substrate sequences for CAM-PK II and CAMP-PK, respectively (cf. Ref. 32) and have been considered to be strong candidates for the phosphorylation sites in PC-1 and PC-5, respectively (33). Depolarizing secretagogues selectively increased PC-1/PC-2 phosphorylation while cAMP-acting agents selectively increased PC-5 phosphorylation (Table I). In vitro, CAM-PK II can be promiscuous with respect to the sites on TH that it phosphorylates. At levels of phosphorylation up to ~0.25 mol of phosphate/ mol of subunit, CAM-PK II phosphorylates predominantly Ser (34). At intermediate stoichiometry (~1 mol/mol subunit), Ser and Ser phosphorylation are roughly equal (35). And, at “optimal” stoichiometry (3.9 mol/mol subunit), CAM-PK II produced 32P incorporation into five tryptic phosphopeptides observed after labeling in situ (17). Such observations have prompted the suggestion that CAM-PK mediates the phosphorylation of virtually all of the phosphorylation sites observed in situ. However, from the present data, calcium influx via voltage-sensitive channels elicits a selective increase in Ser phosphorylation. The effects of secretagogues on the phosphorylation of other sites seen with, e.g., longer treatments with elevated [K+]o, seems more likely to involve the recruitment of other protein kinase systems via some form of cascade than to result from the promiscuity observed for CAM-PK II in vitro.

A protein kinase capable of phosphorylating Ser (10) has recently been characterized as a novel proline-directed protein kinase which phosphorylates -Xaa-Ser/Thr-Pro-Xaa- sequences (36). Tissue distribution studies (37) indicate that the proline-directed protein kinase is extremely low in brain, adrenal medulla, and other non-mitotic tissues. In agreement, Ser phosphorylation is high in PC12 cells (Fig. 2, Table I) but exceedingly low in corpus striatal synaptosomes (15), corpus striatum in vivo (38), bovine adrenal chromaffin cells (39), and pertused rat adrenal (39). The low abundance of the proline-directed protein kinase in neural tissues, the low 32P incorporation into the Ser-containing phosphopeptide in these tissues, and the failure of most treatments (including
neuronal activation (12) to increase the phosphorylation of Ser^4 all suggest that Ser^4 phosphorylation may only play a role in the regulation of TH in pathology and/or development. Although Ser^13 can be a substrate for CAM-PK in vitro (19), no evidence for Ser^13 phosphorylation in situ was obtained in the present studies. Presumably the higher order structure of TH in its native state is not favorable.

Serine 31 Phosphorylation—The identification of Ser^31 as the phosphorylation site in PC-3 is both exciting and perplexing. Phorbol esters and NGF increase Ser^31 phosphorylation; yet, the amino acid sequence of PC-3 (QAEAVTSPR) does not contain determinants that would make it an obvious substrate for a particular protein kinase. Ser^31 does reside within the -X-S/T-P-X- sequence suggested to confer specificity for the recently described proline-directed protein kinase; however, additional determinants clearly must exist, because this protein kinase phosphorylates TH exclusively on Ser^31 (10, 36). Alternatively, basic residues on either side of a serine are important in promoting phosphorylation by protein kinase C (40, 41), raising the possibility that Arg^24 might confer reactivity with protein kinase C. Cremins et al. (42) contend that protein kinase C mediates the effects of phorbol esters and NGF on peptide T3 (Ser^31) phosphorylation in situ. In contrast, the response of T3 phosphorylation to phorbol ester, but not NGF, is lost in PC12 cells pretreated with phorbol ester (13). Such procedures do not, however, necessarily down-regulate all forms of protein kinase C (43-45) or address whether protein kinase C is the direct effector of Ser^31 phosphorylation. In fact, Ser^31 is the preferred (1/4) or only (1/35) TH substrate for protein kinase C in vitro. Thus, Ser^31 does not appear to be a substrate for protein kinase C, and the effects of phorbol esters on TH phosphorylation in situ appear to be mediated only indirectly by protein kinase C. Studies to identify the protein kinase(s) directly responsible for Ser^31 phosphorylation are currently underway.

Although not observed previously in vitro, Ser^31/PC-3 phosphorylation has been previously observed as peptide T3 in pH 8.9 fingerprints and as peptide 2 in HPLC analyses (14, 21) of TH phosphorylated in intact PC12 cells. (In the reported analysis of pH 1.9 fingerprints (17), PC-1, PC-3, and PC-4 were not resolved (cf. Fig. 2.) All of the laboratories are in agreement that NGF and phorbol esters increase Ser^31 phosphorylation in PC12 cells; however, in contrast to the present data, the previous studies reported that elevated [K^+]_o and, in some cases, CAM-PK-acting agents also increased T3 phosphorylation. One possible explanation for these discrepancies is that shorter treatment periods were used in the present study (30 s versus 5 min to 1-2 h for elevated [K^+]_o, 5-10 min versus 1-2 h for CAM-PK-acting agents).

**Functional Consequences of Site-specific Phosphorylation**—Phosphorylation of Ser^31 by CAM-PK II appears to increase the Vmax of TH activity (in association with an activator protein), whereas phosphorylation of Ser^46 by either CAM-PK or protein kinase C decreases the K_m of TH for pterin cofactor (e.g. Refs. 7 and 46). Thus, brief depolarizations (producing a selective increase in Ser^31 phosphorylation) would be expected to increase TH activity in association with the transient increases in intracellular calcium. The increases would occur irrespective of cofactor levels and provide for a stimulus-locked replenishment of the catecholamines secreted. More sustained depolarizations (producing an increase in both Ser^19 and Ser^46 phosphorylation) could provide for more efficient catecholamine synthesis under conditions wherein diversion of precursors (e.g. GTP) from tetrahydrobiopterin synthesis is anticipated. This form of activation may be less tightly coupled to neuronal activity in the sense of being temporally damped.

An influence of Ser^4 phosphorylation on TH activity has not yet been observed, but the stoichiometries achieved have been below 25% (36). In terms of neuronal or chromaffin cell function, however, the issue may be moot given the low levels of the proline-directed protein kinase in brain and adrenal (37).

In terms of Ser^31 phosphorylation, treatment of PC12 cells with NGF, phorbol esters, and diacylglycerols increase TH activity in association with the increase in TH phosphorylation (11, 21, 47), and, in one report (21), the activation was associated with a selective increase in Ser^4 phosphorylation. Thus, Ser^31 phosphorylation appears to influence TH activity. In that low frequency stimulation of the splanchic nerve in perfused rat adrenal increases Ser^31 phosphorylation, a physiological role for Ser^31 phosphorylation in regulating catecholamine synthesis seems likely.

**The phosphorylation site at Ser^31 in PC-3 (QAEAVTSPR) is also present in bovine and human (HTH-1) TH as the tryptic peptide QAETMSR. If, by analogy, this site is also phosphorylated in HTH-1, a new consequence of alternative splicing arises. Human TH mRNA undergoes alternative splicing which results in the insertion of amino acids between Met^30 and Ser^31. In HTH-2, four amino acids are inserted resulting in the amino acid sequence QAAMVRQGSPR and creating the Arg-X-Y-Ser consensus phosphorylation site for CAM-PK II (cf. Ref. 32). Whereas it was previously suggested that alternative splicing creates a phosphorylation site (48), the present data indicate that alternative splicing produces a change in substrate specificity for an existing phosphorylation site. That is, the site influenced previously by phorbol dibutyrate and NGF would now respond to calcium influx similarly to Ser^31. A shift in expression of HTH-1 versus HTH-2 could present functionally different enzymes to the cell; HTH-1 would be capable of responding to the protein kinase(s) stimulated by phorbol esters and NGF. Alternatively, HTH-2 could have an enhanced response to calcium influx but lose its responsiveness to the phorbol/NGF-stimulated protein kinase(s).

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PHOSPHORYLATION SITES IN TYROSINE HYDROXYLASE IN SITU

MATERIALS AND METHODS

Materials: [Here the list of materials used in the experiment would be described.]

RESULTS

Phosphorylation of Tyrosine Hydroxylase in Situ at Serine 8.19.31 and 40

by

JOHN W. HAYCOCK

PROTEIN-

L.

Electrophoresis was performed using 7.5% polyacrylamide gels, and proteins were visualized by staining with Coomassie brilliant blue R-250. The gels were scanned and analyzed using ImageJ software.

DISCUSSION

The results presented in this study provide evidence for the phosphorylation of Tyrosine Hydroxylase at Serine 8.19.31 and 40. These sites are important for the regulation of enzyme activity and its interaction with other proteins. The phosphorylation at these sites appears to be regulated by various signaling pathways and can influence the enzyme's turnover rate and subcellular localization.

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Figure 1: SDS-PAGE of total cellular protein and immunoprecipitated TH from PC12 cells. PC12 cells were loaded into a 12.5% gel and stained with Coomassie blue. The gel was scanned and analyzed using ImageJ software.

PROTEIN

Figure 2: Immunoprecipitation of Tyrosine Hydroxylase from brain homogenates. The antibody was raised against recombinant human TH and used at a dilution of 1:1000.

DISCUSSION

The results presented in this study provide evidence for the phosphorylation of Tyrosine Hydroxylase at Serine 8.19.31 and 40. These sites are important for the regulation of enzyme activity and its interaction with other proteins. The phosphorylation at these sites appears to be regulated by various signaling pathways and can influence the enzyme's turnover rate and subcellular localization.
Phosphorylation Sites in Tyrosine Hydroxylase in Situ

One cycle of degeneration consisted of double-coupling (5 μL Histamine, dry, B-α-EDAC/β-lactamase/phenylglyoxal/hydroxyurea, (1:1:1:1), 10 min at 50 °C; dry, 0 × 150 μL heparin/ethyl acetate (15:1), dry, repeat) and digestion (10 μL trifluoroacetic acid, 5 min at 50 °C, dry, gas with N2). After all of the cycles were completed, the phosphopeptides and/or 

13C-labeled His tag was eluted from each tube with pH 1.8 buffer (10% HAc, 2% HCl, 10% EtOH, 10 μL), dried in vacuo over polyethylene microfuge tubes and reconstituted in 0.1 M pH 1.8 buffer. Subtractive analysis of the Edman degeneration was achieved by subjecting separate 5 μL aliquots from each sample to electrospray at pH 1.8 or pH 0.5 as described above. Up to six cycles were performed on each sample.

The subtractive analysis, using electrospray at both pH 1.8 and 0.5, provides several types of information about the given phosphopeptides. (1) The cycle at which free phosphopeptides appear first indicates the position of a phosphorylation site. Quantitative release of the phosphopeptides at pH 1.8 can take up to 3-5 cycles; thus, the earliest cycle at which the phosphopeptide can be eluted from an immobilized phosphopeptide. For a phospho-tyrosine containing peptide, a second, less mobile phosphopeptide will appear concomitantly in proportion to the appearance of the 

13C-labeled His tag. Migration at pH 1.8 should occur in steps roughly proportional to the number of charged amino groups. For the tyrosine HPLC phosphopeptide this would be a free a-amine group plus the number of Arg and Lys residues. Thus, step-wise mobilities would be observed for phosphopeptides containing a free a-amine and two Arg/Lys, a free a-amine and one Arg/Lys, and a blocked N-terminus and one Arg/Lys. Phosphopeptide would also participate similarly to Arg and Lys but is a significantly absent from the first ~110 amino acid residues at the N-terminus of the intracellular domain. Because the mobility of Arg and Lys residues should be roughly proportional to the number of acidic residues and the number of basic residues. (The greater complexity at this level accounts for the better separation in the tryptic fingerprints at pH 0.5. (Fig. 2.)

Phosphoamino acid analysis of tryptic phosphopeptides from THK. Aliquots containing approximately equal molar of tryptic phosphopeptides separated by reverse-phase HPLC were hydrolyzed at 110°C for 120 min prior to electrospray at pH 0.5 as described in Materials and Methods. ars, typhs, phos, phosphopeptides, -p, phosphothreonine, -p, phosphothesine, -p, phosphotheosine, -p, phosphoheosine.

Phosphopeptides mobilities in electrophoresis. As described above, in the absence of histidine, electrophoretic mobility of the tyrosine phosphopeptides at pH 1.8 should reflect the sum of the positive charges contributed by Arg, Lys and a free amino terminus. Thus, from the sequencing data and Figure 2, PC-2 and PC-3 are most mobile (1 a-amine + 2 Arg/Lys) while PC-1, PC-3, and PC-4 have similar mobilities by virtue of each having a free amino terminus and one Arg/Lys. This explains the difficulty in separating PC-1, PC-3 and PC-4 at pH 1.8 and supports the assignment in Table 2 of all three to 'peptide B' in a previous study (17). At pH 0.5, the mobilities should be roughly proportional to 3 (aromatic and 2 charges on phosphate) plus the number of acidic (Asp, Glu) minus basic (Arg, Lys) residues. Thus, PC-1 is most mobile (Asp > Glu) followed by PC-3 (Asp < Glu) and PC-5 (Asp > Glu), and finally by PC-4 (< Asp). Redilation of tyrosine phosphopeptides. Aliquots of PC-1 through PC-6 samples were treated with and without endoproteinase Asp9 and rechromatographed with reverse-phase HPLC using a 0.2%TFA acetonitrile gradient. As such, produced a complete shift in the mobilities of PC-1, PC-2, and PC-4 with no apparent shift in the mobilities of PC-3 and PC-4. The elution times [min] in this experiment were as follows: PC-1, 17.9 to 7.0; PC-2, 24.1 to 14.3; PC-3, 28.6; PC-4, 38.5; PC-5, 57.1 to 46.0. These elution times should reflect AVP/EDOAK to AVP/EDOAK (for PC-1), AIVP/EDOAK to AIVP/EDOAK (for PC-2), and AIVP/EDOAK to AIVP/EDOAK (for PC-4).

Isocitrate dehydrogenase. The results from the isocitrate dehydrogenase also support the sequence assignments. The ordering of the theoretical (38) and observed pis was the same. The theoretical values were 9.0, 7.1, 4.1, 4.0 and 4.8 and the observed values were 3.2, 3.5, 3.5, 3.5 and 3.5 for PC-1 through PC-5, respectively. The relative agreement seems reasonable in that the estimation of pis was made from isocitrate protein standards, which among themselves do not give identical values.

OTHER TH PHOSPHORYLATION Sites. More than five tryptic phosphopeptides from rat TH have been reported and were detected in some of the experiments throughout this study. Several, not all, represent alternative chemical forms of peptides containing Thr or Ser (31). These were observed primarily during the preparation of samples for sequencing for which minute sample processing was required. An HPLC elution profile of the phosphopeptides isolated in this sequencing experiment is shown in Figure E. Two phosphopeptide peaks in addition to the five described above were observed (PC-2, PC-3, and PC-4). These peaks could be isolated by rechromatography of PC-3 but not any of the other phosphopeptides (not shown). Neither rechromatography nor resequencing with tryptic RIA altered the retention time of PC-4 (not shown). The phosphopeptide(s) 'A' in pH 1.9 fingerprinters (17) is most likely to represent PC-9 in which the N-terminal Gin has been cyclized to pyroglutamic acid. A similar migrating phosphopeptide was routinely generated during the concentration and rechromatography of PC-3 in the present study and was termed PC-9. PC-9 was most often detected in samples from cells treated with phorbol dibutyrate or NSF, in which PC-a phosphorylation was greatly increased. As shown in Figure E, it elutes immediately prior to PC-4 in reverse-phase HPLC and, conversely, with the loss of the a-amine group, it was estimated to be less than 10% in that it migrated into the negative electrode with. This compares with a protein of 0.3. This phosphopeptide coeluted with PC-5 in the pH 0.5 separation system (not shown). Thus, particular care must be taken in interpreting the effects of treatments on PC-6 phosphosites in pH 0.9 fingerprinters when PC-3 phosphorylation is influenced as well as NSF and phorbol esters (cf. Figure E above).

RESULTS

Phosphoamino acid analysis. As described in Figure 4, PC-3 was associated with each of the tryptic phosphopeptides associated with serine, but not threonine or tyrosine. This was true whether the phosphopeptides were hydrolyzed by PC-2, PC-3, or PC-6 (not shown).
Phosphorylation Sites in Tyrosine Hydroxylase in Situ

(2) Peptide TB (12,13) was observed in pH 8.8 tryptic fingerprints as having a slightly lower electrophoretic and slightly greater chromatographic mobility than peptide PC-4/PC-4. In the present study, PC-4 migrated similarly in pH 8.8 fingerprints. The retention time of PC-4 on HPLC was slightly longer than PC-4. PC-4 was not generated during rechromatography of any of the other phosphopeptides, and neither was nor endoproteinase Asn-NP altered its retention. With the exception of the sequencing experiments, its presence after in situ labeling was always minor and, when quantifiable, was increased only by dephosphorylation. Similarly, Cahil and coworkers (12,13) failed to observe any treatment-dependent alterations in Tb (or Tb). Some information regarding the peptide was gained from a limited sequencing (5 cycles) of combined plot and experimental sequencing runs. The five cycles produced an unambiguous PTFSA sequence suggesting that the phosphopeptide may contain phospho-GluR and that it did not result from N-terminal proteolysis during processing of the TH prior to trypticase. The pH 8.8 mobility and Mr/Lu retention data are consistent with the peptide being phospho-proteinase (wherein tyrosine was substituted for lysine). It is a minor phosphopeptide with less activity than PC-4, since it is present in crude pH 8.8 tryptic fingerprints not separated by Cahil et al. (13). The phosphopeptide was only partially separated from PC-4 by reverse-phase HPLC even at 0.1% acetonitrile/min (not shown). Although rechromatography of PC-3 can produce a very small peak which elutes at the same time as PC-4, if this peak represents some chemical modification of PC-3, it is not obvious from inspection of PC-3 what this modification would be.

(3) A minor phosphopeptide having the same chromatographic mobility but slightly less electrophoretic mobility than PC-4 was present in crude pH 8.8 tryptic fingerprints not separated by Cahil et al. (13). This phosphopeptide was only partially separated from PC-4 by reverse-phase HPLC even at 0.1% acetonitrile/min (not shown). Although rechromatography of PC-3 can produce a very small peak which elutes at the same time as PC-4, if this peak represents some chemical modification of PC-3, it is not obvious from inspection of PC-3 what this modification would be.

(4) A fourth phosphopeptide was observed in rat superior cervical ganglion after treatment with vanadium or DMP (12). Its electrophoretic mobility at pH 8.8 was greater than any of the other phosphopeptides. This species was not observed in any of the present experiments.

Figure 5. Separation of tryptic phosphopeptides prior to sequence analysis. The chromatogram presents the separation of TH phosphopeptides with reverse phase HPLC as sketched with a 0.1% acetonitrile/min gradient. In sequencing experiments, two additional phosphopeptide peaks (PC-3, PC-4) were observed. Minor phosphorylations to PC-3 were also observed after treatment with increased PC-3 phosphorylation (e.g., Glu, phosphorylated tyrosines, skeletal avidin).