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 (AV-SEQDAK) and PC-2 (RAVSEQDAK) was identified as Ser$^8$. Agents which cause calcium influx increased $^{32}$P incorporation into tyrosine hydroxylase at Ser$^8$. PC-3 was identified as QAEEV1SPK, which contains the phosphorylation site Ser$^{31}$. Nerve growth factor and phorbol dihydroxy increased $^{32}$P incorporation into Ser$^{31}$. PC-4 was identified as the N-terminal amino acid sequence (MP)TPSAPSPDPK, and the $^{32}$P incorporation occurred at Ser$^8$. Of the agents tested, only okadaic acid (a protein phosphatase inhibitor) increased the phosphorylation of Ser$^{31}$. Treatment of the PC12 cells with CAMP-acting agents increased $^{32}$P incorporation into Ser$^{31}$.

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$^{31}$ 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 tyrosine hydroxylase (2, 9). Because TH activity increased directly with phosphate incorporation up to $\sim$0.7 mol of phosphate/mol of TH subunit (4), a "one-site one-kinase" model was suggested for the regulation of TH activity. In situ, however, multiple-site phosphorylation of TH was demonstrated (5) and, subsequently, several other protein kinase systems were shown to phosphorylate and activate TH in vitro (6–9).

Campbell and co-workers (10) identified four phosphorylation sites in rat pheochromocytoma TH in vitro. Ser$^{19}$ was phosphorylated by CAM-PK II; Ser$^{31}$ was phosphorylated by CAMP-PK, protein kinase C, and, to a small extent, CAM-PK II. Minor phosphorylation of two other sites was also demonstrated. A protein kinase activity associated with the partially purified TH phosphorylated Ser$^{40}$, and CAM-PK phosphorylated Ser$^{130}$ in a proteolytically degraded TH preparation.

Tryptic digestion of TH after phosphorylation in situ in rat pheochromocytoma cells (PC12) also produced four phosphopeptides (11). However, a different separation technique was used, thus comparison of these peptides to the sites identified in vitro (10) was not possible. Subsequent studies of the multiple-site phosphorylation of TH in situ have reported anywhere from three to seven tryptic phosphopeptides (12–17). Thus, neither the number of phosphopeptides nor a strong inference regarding which phosphorylation sites might be represented by each of the phosphopeptides has been possible.

In the present report, multiple-site phosphorylation of TH in intact PC12 cells has been analyzed and the properties of the peptides bearing the phosphorylation sites have been characterized in a number of separation systems, allowing comparison among the previous studies. Furthermore, the high level of TH expression in the PC12 cells provided sufficient material for determining the amino acid sequences of the phosphopeptides, allowing assignment of phosphorylation sites to the different phosphopeptides (18).

**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...
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). \(^{32}\)P incorporation into TH was quantified by Cerenkov or liquid scintillation spectroscopy of gel slices containing the TH band, localized by autoradiography.

**Tryptic Digestion and Phosphopeptide Separation.** \(^{32}\)P-Labeled peptides released from the gel slices by limit tryptic digestion were separated by reverse-phase HPLC. HPLC was performed with a C18 column (Vydac, 218TP5415) equilibrated in 0.1% trifluoroacetic acid. The \(^{32}\)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 \(^{32}\)P peak integration and collection. \(^{32}\)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 \(^{32}\)P-labeled peptides produced by limit tryptic digestion of TH immunoprecipitated from untreated PC12 cells after labeling with \(^{32}\)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 pIs 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, ~3.5; PC-4, ~5.1; PC-5, ~3.9.

Thus, the five \(^{32}\)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 \(^{32}\)P Incorporation into TH Phosphopeptides**

To facilitate comparison of the phosphopeptides in the present study to those reported previously and to optimize \(^{32}\)P incorporation (and, by inference, stoichiometry) into each of the peptides for subsequent analyses, several test substances were evaluated for their abilities to increase \(^{32}\)P incorporation into TH and the phosphopeptides.

**Secretagogues—**Elevated K" (40 mM), veratridine (100 \(\mu\)M), or nicotine (50 \(\mu\)M) increased \(^{32}\)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, 3-10 Precoat gels indicated that all of the phosphopeptides had pIs 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, ~3.5; PC-4, ~5.1; PC-5, ~3.9.

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Influence of pharmacological agents on multiple site phosphorylation of TH

Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P-Labeled total protein</th>
<th>% of control</th>
<th>TH</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (40 mM, 30 s)</td>
<td>105</td>
<td>160</td>
<td>300</td>
<td>320</td>
</tr>
<tr>
<td>Veratridine (100 mM, 60 s)</td>
<td>88</td>
<td>140</td>
<td>220</td>
<td>240</td>
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<tr>
<td>Nicotine (50 μM, 30 s)</td>
<td>102</td>
<td>130</td>
<td>200</td>
<td>185</td>
</tr>
<tr>
<td>A23187 (10 μM, 30 min)</td>
<td>190</td>
<td>185</td>
<td>900</td>
<td>210</td>
</tr>
<tr>
<td>Forskolin (10 μM, 10 min)</td>
<td>105</td>
<td>135</td>
<td>160</td>
<td>220</td>
</tr>
<tr>
<td>8-bromo-cAMP (1 mM, 5 min)</td>
<td>94</td>
<td>120</td>
<td>180</td>
<td>130</td>
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<tr>
<td>Dibutyryl cAMP (1 mM, 5 min)</td>
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<td></td>
</tr>
<tr>
<td>Phorbol dibutyrate (1 μM, 30 min)</td>
<td>135</td>
<td>145</td>
<td>340</td>
<td>135</td>
</tr>
<tr>
<td>NGF (50 ng/ml, 30 min)</td>
<td>128</td>
<td>180</td>
<td>600</td>
<td>130</td>
</tr>
<tr>
<td>Okadaic acid (1 μM, 30 min)</td>
<td>220</td>
<td>480</td>
<td>650</td>
<td>420</td>
</tr>
</tbody>
</table>

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

$^b$ Spaces left blank indicate values between 90 and 110% of control.

PC12 cells were incubated with $^32$P, 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 in two to four different experiments. Median values are presented. The distribution of $^32$P 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.).

Phosphorylation Sites in Tyrosine Hydroxylase in situ

The increase in TH phosphorylation by forskolin and cAMP-acting agents (7, 11, 13, 23-25) occurred predominantly from an increase (240%) in PC-3 phosphorylation. Phorbol dibutyrate, 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) 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 previously shown to increase the phosphorylation of all of the peptides except what appears to be PC-4 (14, 21).

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 $^32$P incorporation into TH (1-2 h) treatment, and Cahill et al. (13) reported 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 $^32$P 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 ½ or ¾), and PC12 cells (two different sources) all failed to produce an EGF-stimulated increase in $^32$P incorporation into TH (not shown).

Several laboratories have shown that NGF increases TH phosphorylation in PC12 cells (11, 13, 23-25). In the two studies which analyzed tryptic phosphopeptides, both also reported increases in PC-3 (T3) and PC-5 (T1) with the effects on PC-3 being similarly larger than those on PC-5. 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.

McTigue et al. (11) reported that EGF increased TH phos-
Phosphorylation 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 μM, 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 in 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 subtractive 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 subtractive 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 $A_9V_7E_6Q_12D_1A_3K_6$, wherein the numbers in subscript indicate the pi-
comole yield. From the subtractive Edman analysis (Fig. 5), the loss of a positive charge was observed at both pH 1.9 and 8.9, presumably 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 RAVXEQDAK 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 XAEEAVTXPR, corresponding respectively to nonphosphorylated Ser$^{29}$ 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 first cycles run. The second analysis, with modified experimental conditions, produced the sequence $Q_9A_1E_2D_3A_4V_5T_6S_7P_8R_9$, with no indication of the presence of VSDDVR. Based on the 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 sequencing analysis would be expected to elute prior to their cognate phosphopeptides (31).) 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 QAEAVTS(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$^{3}$ (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 $P_9T_7P_9S_8D_1A_3P_8X_9P_8$, for the eight cycles performed. Because PC-4 contained more than 1 Ser, the

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**Fig. 5. Subtractive analysis of the tryptic phosphopeptides after manual Edman degradation.** The phosphopeptides isolated by rechromatography on reverse-phase HPLC at 0.1% acetonitrile/min were subjected to manual Edman degradation as described under "Materials and Methods." $^{32}$P standards and aliquots of the samples were spotted on thin-layer cellulose plates and subjected to electrophoresis at pH 1.9 and 8.9. Representative autoradiograms of PC-1 and PC-5 are presented. ori, origin. The origin for the pH 8.9 bottom compartment was cropped from the photographs.
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 phosphorlated 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 side chain of Lys presumed to be at the C-terminal of the phosphopeptide. From these data, PC-4 was assigned the sequence PTPSAPSPQPKGFRRAVESQDAGKEAVTSPRFIGRQGSLIDARK.

The PC-5 sample resulted in a sequence of R40QELs3177E59-DaAeRo. From the subtractive Edman analysis (Fig. 5), the loss of a positive charge at cycle 1 was indicated by a decrease in mobility at pH 1.9 and an increase in mobility at pH 8.9. From this, the release of 32P, at cycle 3, and the presence of a single serine having a low yield characteristic of phosphoserine, PC-5 was assigned the sequence RQS(P)LIEDAR containing Ser* as the phosphorylation site. From this, the release of 32Pi, at cycle 3, and the presence of one positive charge was indicated by a decrease in mobility at pH 1.9 and an increase in mobility at pH 8.9. From this, the release of 32P, at cycle 3, and the presence of a single serine having a low yield characteristic of phosphoserine, PC-5 was assigned the sequence RQS(P)LIEDAR containing Ser* as the phosphorylation site. From this, the release of 32Pi, at cycle 3, and the presence of one positive charge was indicated by a decrease in mobility at pH 1.9 and an increase in mobility at pH 8.9. From this, the release of 32P, at cycle 3, and the presence of a single serine having a low yield characteristic of phosphoserine, PC-5 was assigned the sequence RQS(P)LIEDAR containing Ser* as the phosphorylation site.

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 the enzyme inferred from cDNA clones, the secondary or tertiary structure of the enzyme inferred from cDNA clones, the secondary or tertiary structure of the enzyme inferred from cDNA clones, the secondary or tertiary structure of the enzyme inferred from cDNA clones, the secondary or tertiary structure of the enzyme inferred from cDNA clones, the secondary or tertiary structure of the enzyme inferred from cDNA clones. 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+], 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 (33), and perfused rat adrenal (39). The low abundance of the proline-directed protein kinase in neural tissues, the low 32P incorporation into the Ser*-containing phosphopeptides in these tissues, and the failure of most treatments (including...
neuronal activation (12) to increase the phosphorylation of Ser⁴ all suggest that Ser⁴ phosphorylation may only play a role in the regulation of TH in pathology and/or development. Although Ser³¹ can be a substrate for CAM-PK in vitro (19), no evidence for Ser³¹ 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 Ser³¹ as the phosphorylation site in PC-3 is both exciting and perplexing. Phorbol esters and NGF increase Ser⁴ 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. Ser³¹ 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 Ser⁴ (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 Arg⁶⁵ 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 (Ser³¹) phosphorylation in situ. In contrast, the response of T⁴ 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 Ser³¹ phosphorylation. In fact, Ser⁴ is the preferred (17) or only (7, 35) TH substrate for protein kinase C in vitro. Thus, Ser³¹ 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 not indirectly by protein kinase C. Studies to identify the protein kinase(s) directly responsible for Ser⁴ phosphorylation are currently underway.

Although not observed previously in vitro, Ser³¹/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 in agreement that NGF and phorbol esters increase Ser⁴ phosphorylation in PC12 cells; however, in contrast to the present data, the previous studies reported that elevated [K⁺], and, in some cases, CAMPK-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⁺], 5-10 min versus 1-2 h for CAM-PK-acting agents).

Functional Consequences of Site-specific Phosphorylation—Phosphorylation of Ser³¹ by CAM-PK II appears to increase the Vₘ₉₉ of TH activity (in association with an activator protein), whereas phosphorylation of Ser⁴ by either CAM-PK or protein kinase C decreases the Kₘ of TH for pterin cofactor (e.g. Refs. 7 and 46). Thus, brief depolarizations (producing a selective increase in Ser⁴ 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 Ser⁴ and Ser³¹ 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 dampened.

An influence of Ser⁴ 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 Ser³¹ 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 Ser⁴ phosphorylation. Thus, Ser⁴ phosphorylation appears to influence TH activity. In that low frequency stimulation of the splanchnic nerve in perfused rat adrenal increases Ser³¹ phosphorylation, a physiological role for Ser³¹ phosphorylation in regulating catecholamine synthesis seems likely.

The phosphorylation site at Ser³¹ in PC-3 (QAEAVTSPR) is also present in bovine and human (HTH 1) TH as the tryptophic peptide QAEMVMR. 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 Met⁹⁰ and Ser³¹. In HTH-2, four amino acids are inserted resulting in the amino acid sequence QAEMVRGQPSR 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 Ser³¹. 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 it responsiveness to the phorbol/NGF-stimulated protein kinase(s).

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11. The proline-directed protein kinase does, however, phosphorylate Ser³¹ when the sequence is presented as a synthetic peptide (P. R. Vulliet, personal communication).
Phosphorylation Sites in Tyrosine Hydroxylase in Situ


Phosphorylation Sites in Tyrosine Hydroxylase in Situ

JOHN W. HAYCOCK

MATERIALS AND METHODS

Materials. [32P]PO4 was from New England Nuclear (NEC 3A, 100 mCi/mM). Herpes pdm 1p was from Gailard Belanger. Trypsin (TPCK-treated) for routine digestion was from Cooper Biochemical. For other applications, proteases were purchased from Sigma, St. Louis, Mo. Interleukin-1 (IL) was from R & D Systems, Minneapolis, Minn. [3H]adrenaline, [3H]noradrenaline, [3H]serotonin, [3H]tyramine, [3H, 32P]adenosine 16-mer, and [3H, 32P]adenosine 23-mer were obtained from Amersham Nuclear. [3H]adenosine 16-mer was from Amersham Nuclear. [3H]adenosine 23-mer was from Amersham Nuclear. [3H]adenosine 23-mer was from Amersham Nuclear. [3H]adenosine 23-mer was from Amersham Nuclear.

Methods. For the [32P]PO4 incubation, cells were cultured on gelatinized coverslips for 24 h in DMEM supplemented with 10% fetal calf serum. At the end of the incubation period, the media were removed, and the cells were washed twice with PBS. The coverslips were then placed in 0.5 M HCl for 20 min at 4°C. The cells were then washed three times with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. The coverslips were then incubated in 50% methanol containing 0.1% acetic acid for 10 min at 4°C. The samples were then dehydrated through a series of ethanol concentrations (50%, 70%, 95%, and 100%) and embedded in paraffin. The paraffin blocks were then cut into 5-μm-thick sections and mounted on slides.

Analysis of [32P]PO4. Aliquots of the SDS-solvilized samples were taken for determination of protein and [32P]PO4 incorporation into total cellular protein. As noted, [32P]PO4 incorporation into TH and its tyrosine-peptide was normalized to total of these measurements. The latter was measured by spiking samples within a gradient on filter paper, using the filter paper with 10% [3H]adrenaline, 20% ethanol, and ethidium, and then measuring the filter-bound radioactivity by liquid scintillation spectroscopy. Respectively, the two measurements were used to correct for well-to-well differences in growth rate and for potential treatment-dependent changes in e.g., the specific activity of endogenously generated [32P]PO4-ATP, using some of the larger tyrosine-TH samples. Although such differences were also evident from protein bands and autoradiograms of gels of total cellular protein, they are not easily quantified by densitometry. Furthermore, they will go unnoticed in samples processed directly to immunoprecipitation. [Assay of ATP content] or [32P]PO4 incorporated into cellular protein was made in a few of the older studies (13, 31), but these studies have not been apparently taken into consideration in more recent studies. When a given treatment condition was determined not to influence [32P]PO4 incorporation into total cellular protein, this measurement was used in subsequent experiments to normalize [32P]PO4 incorporation into TH- and the tyrosine-peptide.

Immunoprecipitation. Aliquots of protein A-bearing immunoprecipitations (Immunoprecipitin, Invitrogen, San Diego, CA) were prepared by sequential incubations (15 min, room temperature) in PBS (15 mM NaCl, 1 mM KH2PO4, 4 mM EMPI, 0.5% [32P]PO4) 0.05% [32P]PO4 (30, 0.3% trypsin), containing 5% normal horse serum and 0.08% BSA, PBS containing 0.1% acrylamide/bis [Sigma, RIA grade], and PBS alone. After centrifugation, pellets were suspended in PBS containing 50 μg affinity-purified antibody/μl, incubated for 30 min, and washed twice with PBS. The immunoprecipitate was stored at -20°C for up to 1 week or 0°C prior to use.

Methods. Conditions for quantitative (100%) immunoprecipitation and recovery of TH were established using Western blots and varied only to some extent with immunoprecipitation assay (20). Solubilized cell samples were brought to 435-1000 μl, the final composition of which was Herpes pdm 1p (61, H-1; 33, 0.05% [32P]PO4), 0.05% [32P]PO4 (30, 0.3% trypsin), containing 5% normal horse serum and 0.08% BSA, PBS containing 0.1% acrylamide/bis [Sigma, RIA grade], and PBS alone. After centrifugation, pellets were suspended in PBS containing 50 μg affinity-purified antibody/μl, incubated for 30 min, and washed twice with PBS. The immunoprecipitate was stored at -20°C for up to 1 week or 0°C prior to use. After centrifugation, the pellets were resuspended in SDS-PAGE sample buffer and heated for 3 min in a boiling water bath.

In the solubilized cell samples subjected to SDS-PAGE, high background and the presence of other [32P]PO4 bands close to TH usually resulted in variable and erroneously high "blank" values and visually prevented accurate determination of [32P]PO4 incorporation into TH in these samples. Thus, [32P]PO4 was usually quantified after immunoprecipitation. Figure 1 illustrates the protein staining pattern and [32P]PO4 incorporation into total cellular protein, before and after immunoprecipitation, and into TH, immunoprecipitated from the sample. Whereas a protein-staining band3 was detected in the 63 kDa region after immunoprecipitation, the [32P]PO4 (TH) was quantitatively recovered.

Background did not, however, interfere with analysis of the [32P]PO4 peaks in the TH band area. Background [32P]PO4, taken from the 60-61,000 Da region of gel lanes from total cellular protein samples after TH had been immunoprecipitated (Fig. 1, lanes 3 and 3), was analyzed primarily in the first 24 min (20%) and during the acetylation ramp at the end of each reverse-phase HPLC gradient elution (30%). The remaining radioactivity eluted efficiently in between.
Phosphorylation Sites in Tyrosine Hydroxylase in Situ

One cycle of degradation consisted of double-coupling (5 μM HEPES, pH 7.4, 8 μM DTT, 1% Triton X-100, 10 min at 50 °C, 10 × 150 μg heparin/ethyl acetate (15/1), dry, and deglycosylate and lyophilize trifluoroacetic acid, 5 min at 50 °C, dry, gas with N2) after all of the cycles were completed, 32P-peptide and/or 32P, was eluted from each tube with pH 1.9 buffer (5% Hac, 2% HCl, 100 μL), dried to phosphorylcontaining ligase tubes and resuspended in 10 μl of pH 1.9 buffer. Substrate analysis of the Edman degradation was achieved by selecting separate 5 μl aliquots from each sample to electrophoresis at pH 1.9 or pH 3.9 as described above. Up to six cycles were used in most cases.

The subtractive analysis, using electrophoresis at both pH 1.9 and 3.9, provides several types of information about a given phosphopeptide. (1) The cycle at which free 32P, first appears indicates the position of a phosphorylation site. Quantitative release of the 32P can be up to 100% of the cycle; thus, the earliest cycle at which 32P release occurs is taken as the site. (2) At pH 3.9, a diglycosylated peptide can be distinguished from a monophosphorylated peptide. For diglycosylated peptide, a second, less mobile peptide will appear concomitantly inordinant proportion to the appearance of the 32P from the monoflaminopyridylated ester. (3) Migration at pH 1.9 should occur in steps roughly proportional to the number of charged amino groups. For the tyrosyl TH phosphopeptides this would be a free α-amino group plus the number of Arg and Lys residues. Thus, step-wise lower mobilities would be observed for peptides containing a free α-amino and two Arg/Lys, a free α-amino and one Arg/Lys, and a blocked N-terminus and one Arg/Lys. The reverse would also participate similarly to Arg and Lys but is considered absent from the free 

Phosphorosine motifs in electrophoresis. As described above, in the absence of heparin, electrophoretic mobility of the tyrosyl phosphopeptides at pH 1.9 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-1 and PC-3 are most mobile (1 α-amino + 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 3.9 and suggests the assignment in Table 2 of all three to "peptide B" in a previous study (17). At pH 3.9, the mobilities should be roughly proportional to 3 (carboxyl 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 + Lys) followed by PC-3 (Asp + Glu + Lys) and PC-5 (Asp + Glu), and finally PC-4 (Asp).

Reduction of tyrosyl phosphotyrosines. Aliquots of PC-1 through PC-5 samples were treated with 10% pyridine (pH 5.5) and stored at 55 °C for 1 h in 1 μL of sample buffer in a sealed vial. The sample was centrifuged at 10,000 × g for 30 min at 4 °C and the supernatant was removed and applied to a 20 μL aliquot of a 30 μL aliquot of a 30 μL aliquot of a 30 μL aliquot of a 30 μL aliquot of a 30 μL aliquot of a 30 μL aliquot of a 30 μL aliquot of a 30 μL aliquot

RESULTS

Sequence Analysis. Additional evidence that the amino acid sequences represent the phosphopeptides is as follows.

Phosphopeptide acid analysis. As shown in Figure 4, 32P associated with each of the tyrosyl phosphopeptides was isolated, but not tyrosine or tyrosine. This true whether the phosphopeptides were hydrazoxyen for 60, 120, 180 or 240 min (not shown).
Phosphorylation Sites in Tyrosine Hydroxylase in Situ

(2) Peptide TB (12,13) was observed in pH 8.9 tryptic fingerprints as having a slightly lower electrophoretic and slightly greater chromatographic mobility than peptide TC/PC-4. In this present study, PC-4 migrated similarly in pH 8.9 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 trypsin nor endoprotease Asp-N 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 edeine acid. Similarly, Cahil and coworkers (12,13) failed to observe any treatment-dependent alterations in TB (or TE.) Some information regarding the peptide was gained from a limited sequencing (5 cycles) of combined in vitro and experimental sequencing runs. The five cycles produced an unambiguous PTH-ethyl sequence suggesting that the phosphopeptide may contain phospho-Glu8 and that it did not result from N-terminal proteolysis during processing of the TH prior to trypticolysis. The pH 8.9 mobility and retention data are consistent with the peptide being dimerko-leucine (wherein tryptophan is replaced by dihydroxy methionine) (12). However, HPLC of the TH shows PC-4 to be more acidic than PC-4. (3) A minor phosphopeptide having the same chromatographic mobility but slightly lower electrophoretic mobility than PC-4 was present in some pH 8.9 electrophoretic fingerprints presented by Cahill et al. (13). This phosphopeptide was only partially separated from PC-4 by reverse-phase HPLC even at 0.1% acetic acid/min (not shown). Although derivatives of PC-4 can produce a very small peak which elutes at the same time as PC-4, if this peptide represents some chemical modification of PC-4, it is not obvious from an inspection of PC-4 what the modification would be.

(4) Lastly, a seventh TH phosphopeptide was observed in rat superior cervical ganglion after treatment with veratridine or DBH (12). Its electrophoretic mobility at pH 8.9 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.](http://www.jbc.org/)

The chromatogram presents the separation of TH phosphopeptides with reverse phase HPLC as skilled with a 0.1% acetic acid/min gradient. In sequencing experiments, two additional phosphopeptide peaks (PC-2, PC-4) were observed. Minor phosphorylation at PC-3 was also observed after treatments which increased PC-3 phosphorylation (i.e., NOS, phosphodiesterases, skeletal wild).
Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40.
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