Tyrosine Hydroxylase in Rat Brain Dopaminergic Nerve Terminals

MULTIPLE-SITE PHOSPHORYLATION IN VIVO AND IN SYNAPTOSOMES*

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Tyrosine hydroxylase, which catalyzes the initial step in catecholamine biosynthesis, is phosphorylated at serines 8, 19, 31, and 40 in intact pheochromocytoma (PC12) cells (Haycock, J. W. (1990) J. Biol. Chem. 265, 11682–11691). After 32P labeling of rat corpus striata in vivo or rat corpus striatal synaptosomes, 32P incorporation into tyrosine hydroxylase occurred predominantly at serines 19, 31, and 40. Electrical stimulation (30 Hz, 20 min) of the medial forebrain bundle (containing the afferent dopaminergic fibers) increased 32P incorporation into each of the three sites. Brief depolarization of the synaptosomes with elevated [K+], (20–60 mM, 5–30 s) or veratidine (50 μM, 2 min) produced a selective increase in 32P incorporation into Ser23. Phorbol 12,13-dibutyrate (1 μM, 5 min) increased 32P incorporation into Ser31, and AMP-acting agents such as forskolin (10 μM, 5 min) increased 32P incorporation into Ser40. In contrast, 32P incorporation into Ser5, which was usually detectable but very low, was not regulated either in vivo or in situ by any of the activators of signal transduction pathways. In synaptosomes, the only treatment found to increase Ser5 phosphorylation was okadaic acid (a protein phosphatase inhibitor), which increased 32P incorporation into all four phosphorylation sites. Thus, three different signal transduction systems appear to mediate the physiological regulation of tyrosine hydroxylase phosphorylation at three different sites.

Activation of catecholamine-containing neurons triggers both the secretion of catecholamines and an acceleration of catecholamine biosynthesis to replenish endogenous stores that have been lost via secretion (e.g. Ref. 1). This increase in catecholamine biosynthesis may result from the phosphorylation and activation of tyrosine hydroxylase (cf. Ref. 2). Tyrosine hydroxylase (TH; EC 1.14.16.2; L-tyrosine, tetrahydropterine:oxygen oxidoreductase (3-hydroxylating)) is the initial enzyme in the biosynthesis of catecholamines (3), and its activity appears to be rate-limiting therein. Recently, TH has been shown to be a substrate in vitro for a number of protein kinases, and the phosphorylation of TH by each of these protein kinases can lead to an increase in the catalytic activity of TH (cf. Ref. 4). TH is also phosphorylated in situ, and secretagogues increase both the phosphorylation and activity of TH (2, 5, 6).

Multiple-site phosphorylation of TH has been demonstrated in situ for bovine adrenal medullary chromaffin cells, rat PC12 cells, and rat superior cervical ganglia (7–9). Serine residues comprise the phosphate acceptor sites in each of the various tryptic phosphopeptides that have been isolated from rat TH, and these residues have recently been identified as serines 8, 19, 31, and 40 (10). However, in each of these catecholaminergic tissues, the proportion of TH in compartments relevant to the regulation of catecholamine biosynthesis is either small (superior cervical ganglion) or unknown (chromaffin cells and PC12 cells). Thus, it has been difficult to determine whether the observed phosphorylation and activation of TH represent biochemical events related to catecholamine biosynthesis in subcellular compartments relevant to secretion.

The analysis of TH phosphorylation and activity in neurons or isolated brain synaptosomes should essentially eliminate the contribution of TH from somal compartments. Several laboratories have described the regulation of catecholamine biosynthesis in synaptosomes from catecholamine-rich brain areas such as the corpus striatum (e.g. Refs. 11–13). Depolarization of striatal nerve terminals increases dopamine biosynthesis and activates TH (e.g. Refs. 14–16). And, this activation is similar to that produced by conditions which promote calcium-dependent protein phosphorylation (17). Until very recently (18–20), however, nothing was known regarding the phosphorylation of TH in striatal terminals. The present studies characterize the multiple-site phosphorylation of TH in dopaminergic nerve terminals, both in vivo and in synaptosomes, and establish the site-specificity of stimulation-dependent increases.

MATERIALS AND METHODS*

Most of the methods and materials used in the present study have been described previously in detail (10). Salient methods are described in brief immediately below, and details of additional or modified methods are presented under "Miniprint Supplement."

32P Labeling of Rat Corpus Striatum in Vivo—Carrier-free 32P (0.5–

The abbreviations used are: TH, tyrosine hydroxylase; CAM-protein kinase II, calcium/calmodulin-dependent protein kinase II; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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2 mCi) in 150 mM NaCl (5 μl) was delivered simultaneously into each caudate-putamen of anesthetized rats over 30 min via stereotaxically placed cannulae. The cannulae were withdrawn and a bipolar electrode was placed unilaterally into the medial forebrain bundle. Sixty min after the infusion of 32P, electrical stimulation (30 Hz, 200 μA, 3 ms, biphasic square wave) was applied for 20 min. At the end of the stimulation period, and with stimulation still on, rapid cryofixation of the corpus striata in situ was achieved by pressure injection of liquid nitrogen into the cranium. Heads were removed and immersed in liquid nitrogen, after which the forebrains were removed, warmed to −14 to −16 °C, and sectioned (1 or 2 mm thickness). Tissue punches from the vicinity of the cannulae tips were solubilized in hot 1% SDS and heated for 5 min in a boiling water bath.

32P Labeling of Corpus Striatal Synaptosomes—Pellets of crude or purified synaptosomal fractions were resuspended in incubation solution (150 mM NaCl, 15 mM Hepes, 5.5 mM d-glucose, 4.4 mM KCl, 1.2 mM MgCl2, 1.0 mM CaCl2, pH 7.4, with NaOH at room temperature and gassed with hydrated 100% O2) at 1 or 2 mg protein/ml and incubated for 15 min at 37 °C. Carrier-free 32P was added to a final concentration of 2 or 4 mCi/ml and the incubation was continued for 45 min. Aliquots (100 μl) of the tissue were added to 100 μl of prewarmed incubation solution that contained test substance at twice the desired final concentration. Treatment was terminated by adding 20 μl of 10% SDS, 10 mM Tris-EDTA, pH 8, and samples were heated in a boiling water bath for 2–5 min.

Analysis of 32P Incorporation—Aliquots of the solubilized samples were taken for determinations of total protein, 32P incorporation into total protein and TH protein levels. 32P-labeled TH was isolated by SDS-PAGE after immunoprecipitation from the remainder of the sample and, in some cases, transferred electrophoretically to nitrocellulose. 32P incorporation into TH was quantified by liquid scintillation counting of gel slices or Cerenkov counting of nitrocellulose pieces that were excised after autoradiographic localization of the 32P-labeled TH bands.

Tryptic Digestion and Phosphopeptide Separation—32P-Labeled peptides released from the gel slices or nitrocellulose pieces by limit tryptic digestion were separated by reverse-phase HPLC on a C18 column equilibrated in 0.1% trifluoroacetic acid. The 32P-labeled peptides were eluted at 1 ml/min with an acetonitrile gradient (0.1 or 0.2%/min) in 0.1% trifluoroacetic acid. An on-line detector (Radiomatic) provided 32P peak integration and collection. 32P peaks were collected separately and concentrated in a Speed-Vac (Savant) prior to subsequent analysis.

RESULTS

Identity between TH Phosphorylation Sites in Rat Brain and PC12 Cells

When striata were prelabeled in vivo with 0.5–2 mCi 32P, a single M, ~62,000 32P-labeled band was immunoprecipitated from solubilized striata by affinity-purified rabbit anti-TH (Fig. 1, left). Similar results have been presented for 32P-labeled TH from striatal synaptosomes (see Fig. 1 in Ref. 18). In both cases, 32P incorporation into TH could not be discerned with one-dimensional SDS-PAGE separations without immunoprecipitation. Immunoblots developed with affinity-purified sheep anti-TH also reacted with a single M, ~62,000 band in striatal tissue processed according to either the in vivo protocol (Fig. 1, right) or the synaptosomal protocol (not presented).

When sufficient 32P incorporation was present in tryptic digests of immunoprecipitated TH after 32P-labeling in vivo, the resulting 32P-labeled peptides were separated either by reverse-phase HPLC or two-dimensional electrophoresis/chromatography. With reverse-phase HPLC of the tryptic digest, five peaks of radioactivity were detected and named according to their order of elution, as shown in Fig. 2A (left). As shown in Fig. 2B (left), the elution profile of 32P-labeled tryptic TH phosphopeptides after labeling striatal synaptosomes with 32P was identical to that seen after labeling in vivo. Furthermore, these profiles were identical to that of TH phosphopeptides labeled in intact PC12 cells (cf. Fig. 6 in Ref. 10). Based on these separation patterns and additional data below, the phosphopeptides from TH labeled in rat corpus striatum in vivo and in synaptosomes appeared to be identical to those in TH after labeling intact PC12 cells with 32P. Thus, by virtue of the sequence data from the PC12 TH phosphopeptides (10), the following phosphorylation sites were inferred: CS-1 and CS-2, Ser31; CS-3 and CS-5, Ser21; CS-4, Ser6; and CS-5, Ser6 (Fig. 2C).

Hydrolysis (110 °C, 2 h, 6 n HCl) of each of the five peaks separated by reverse-phase HPLC revealed phosphoserine but not phosphothreonine or phosphotyrosine (not presented). In two-dimensional tryptic fingerprints (Figs. 2, A and B, right), five spots having the same pattern as those from PC12 TH were separated.

The facility with which larger amounts of 32P incorporation into TH could be achieved with the synaptosomal preparations allowed additional characterization of the peptides to support their identities with those from TH labeled in intact PC12 cells. For example, mixing experiments with the eluted peaks prior to two-dimensional electrophoresis/chromatography allowed identification of the five phosphopeptide peaks in reverse-phase HPLC in terms of their migration in the two-dimensional fingerprint system (Fig. 2B, right).

Thin-layer isoelectric focusing of the striatal TH phosphopeptides in Servalyt 3–10 Precoat gels allowed the following pl values to be assigned—CS-1, ~3.1; CS-2, ~3.8; CS-3, 3.5; CS-4, 5.0–5.5; CS-5, 4.0–4.2. This compares with the following values obtained from PC12 cells: PC-1, ~3.2; PC-2, ~3.9; PC-3, ~3.8; PC-4, ~5.1; PC-5, ~3.9 (10).

The phosphopeptides were subjected to six cycles of manual Edman degradation, and subtractive analyses were performed to identify the location of phosphorylserine residues relative to the NH2-terminal of the peptide and to test for the presence of multiple phosphorylation sites within a given peptide (10). After each cycle of degradation, separate aliquots of the reaction products were subjected to electrophoresis at pH 8.9 and 1.9. A single phosphorylation site was indicated for CS-1, CS-2, and CS-5 at positions 3, 4, and 3, respectively. No phosphorylation sites were revealed in either CS-3 or CS-4 for the six cycles. The phosphorylation sites in the PC peptides are at 3, 4, 7, 7, and 3 for PC-1 through PC-5, respectively (10). In addition, the changes in mobilities of the different CS peptides that occurred in a cycle-dependent fashion (not presented) were identical to those occurring with the PC peptides (10).

A final similarity between the CS and PC peptides was demonstrated by subjecting the tryptic peptides to additional proteolysis with other endoproteases and then rechromatography by reverse phase HPLC. These data are presented in Table II.

32P Incorporation into TH in Vivo

The effects of electrical stimulation of the medial forebrain bundle, using stimulation parameters previously shown to activate TH and increase catecholamine biosynthesis rates (14, 15), on overall 32P incorporation into TH are shown in Table III. Electrical stimulation increased the relative 32P incorporation into TH in 16 of 17 rats. The average increase was 91% (S.E. = 18%).

In unstimulated striata, the distribution of 32P incorporation into the four sites was as follows: Ser31, 26 ± 2; Ser31, 57 ± 2; Ser6, 4 ± 1; and Ser6, 14 ± 1 (mean percent ± S.E., n =
Electrical stimulation of the medial forebrain bundle increased $^{32}$P incorporation into three of the four sites: Ser$^{19}$, 236 ± 50; Ser$^{20}$, 176 ± 36; and Ser$^{21}$, 194 ± 42 (mean percent of control ± S.E., n = 9). $^{32}$P incorporation into Ser$^{21}$ from both the stimulated and unstimulated samples was quantifiable in only five of the nine rats. In these five pairs of samples, $^{32}$P incorporation into Ser$^{21}$ in the stimulated samples was 109 ± 222% of control.

During the several years over which the procedures for direct side-to-side comparisons of $^{32}$P incorporation into each of the TH phosphopeptides were being developed, the effects of electrical stimulation were evaluated by expressing $^{32}$P incorporation into each of the phosphopeptides (that could be quantified) relative to CS-3, the largest peak in unstimulated samples. It was noted and reported previously (21) that electrical stimulation decreased the ratio of $^{32}$P incorporation into CS-1 relative to that in CS-3. In the data from the previous paragraph, however, electrical stimulation produced a larger increase in Ser$^{19}$ phosphorylation than in Ser$^{21}$ phosphorylation. Reanalysis of all of the data in which Ser$^{19}$ (CS-1 plus CS-2) and Ser$^{21}$ (CS-3) phosphorylation could be quantified is shown in Table IV. All of the earlier animals, in which electrical stimulation decreased $^{32}$P-Ser$^{19/20}$/P-Ser$^{21}$, were labeled and stimulated in one of the author’s laboratories, whereas all of the later animals, in which electrical stimulation increased the ratio, were labeled and stimulated in the other author’s laboratory. Although the stimulation parameters were operationally matched in the two laboratories, it seems likely that the effective stimulation strength may have been different between the experimental setups (see below).
TABLE II
Redigestion of tryptic phosphopeptides

| Peptide | Protease | Predicted sequence | Retention |
|---------|----------|--------------------|-----------|
| PC/CS-1 | Control  | AVS(P)EQDAK        | 18        |
|         | AspN     | AVS(P)E            | 7         |
|         | GluC     | AVS(P)E            | 8         |
| PC/CS-2 | Control  | RAVS(P)EQDAK       | 24        |
|         | AspN     | RAVS(P)EQ          | 14        |
|         | GluC     | RAVS(P)E           | 15        |
| PC/CS-3 | Control  | QAEEAVTS(P)PR      | 29        |
|         | AspN     | QAEEAVTS(P)PR      | 29        |
|         | GluC     | AVTS(P)PR          | 29        |
| PC/CS-4 | Control  | PTPSAPS(P)QPK      | 37        |
|         | AspN     | PTPSAPS(P)QPK      | 37        |
|         | GluC     | PTPSAPS(P)QPK      | 37        |
| PC/CS-5 | Control  | RQS(P)LIEDAR       | 57        |
|         | AspN     | RQS(P)LIE          | 46        |
|         | GluC     | RQS(P)LIE          | 46        |

Phosphorylation of TH in Situ in Rat Striatal Synaptosomes

In a previous study (18), incubation of crude striatal synaptosomes with \(^{32}P\), resulted in a time-dependent incorporation of \(^{32}P\) into TH for at least 45 min, and multiple phosphopeptide peaks were separated by reverse-phase HPLC. Synaptosomes, while not an entirely physiological model system for investigating nerve terminal function, were used in the present study in an attempt to distinguish the participation of different signal transduction pathways in the multiple-site phosphorylation observed in vivo.

Effects of Depolarization—One obvious correlate of activation of the medial forebrain bundle would be the depolarization of striatal terminals. Thus, the effects of elevated [K\(^+\)], and veratridine were evaluated. In a previous study, elevated [K\(^+\)], increased \(^{32}P\) incorporation into TH and, for the conditions used (40 mM, 30 s), the increase was restricted to CS-1 and CS-2 (18). In the present studies, a number of conditions were varied in an attempt to increase the relative magnitude of the K\(^+\)-stimulated increase in TH phosphorylation and, thereby, the possible involvement of phosphorylation sites in addition to Ser\(^{19}\). At relatively short treatment durations, the effects of elevated [K\(^+\)], and veratridine were both restricted to Ser\(^{19}\) phosphorylation (Table V). The effects of elevated [K\(^+\)], on Ser\(^{31}\) phosphorylation were rapid (<5 s) and concentration-dependent. At longer treatment durations (up to 4 min) an increase in Ser\(^{19}\) phosphorylation was also observed (Table V). With treatment durations approaching that used for electrical stimulation in vivo (20 min), the magnitude of the K\(^+\)-dependent increases in Ser\(^{19}\) phosphorylation became smaller, resulting in greater relative increases in Ser\(^{19}\) versus Ser\(^{31}\) phosphorylation (not presented). While a 10–20-min exposure of synaptosomes to elevated [K\(^+\)], undoubtedly produces a number of effects in addition to depolarization, these data suggest a possible explanation of the laboratory-to-laboratory differences in the relative effects of electrical stimulation on Ser\(^{19}\) versus Ser\(^{31}\) phosphorylation presented in Table IV. For example, a weaker effective stimulation strength would bias toward observing a relatively greater effect upon Ser\(^{19}\) phosphorylation. Such a biphasic increase in Ser\(^{19}\) but not Ser\(^{31}\) phosphorylation has been observed in chromaffin cells during prolonged exposures to acetylcholine.

TABLE III
Effects of electrical stimulation of \(^{32}P\) incorporation into tyrosine hydroxylase in vivo

Corpus striata were labeled with \(^{32}P\), (0.5–2 mCi/side) as described under “Materials and Methods.” Electrical stimulation was applied to an electrode situated in the medial forebrain bundle on one side of the brain prior to freezing of the striata in situ (see “Miniprint Supplement”). Protein levels, \(^{32}P\) incorporation into total protein, TH levels, and \(^{32}P\) incorporation into TH were quantified, and normalized values for \(^{32}P\) incorporation into TH were calculated as illustrated in Table VI.

| Experiment | Rat no. | \(^{32}P\) incorporation into TH | Control | Stimulated | % control |
|------------|---------|-------------------------------|---------|------------|-----------|
| IV-6       | 1       | 15\(^{9}\)                      | 16\(^{9}\) | 150        |
|            | 2       | 31\(^{9}\)                      | 260     |
| IV-7       | 1       | 8\(^{9}\)                       | 190     |
|            | 2       | 8\(^{9}\)                       | 293     |
| IV-8       | 1       | 31\(^{9}\)                      | 151     |
| IV-15      | 1       | 37\(^{9}\)                      | 157     |
|            | 2       | 22\(^{9}\)                      | 123     |
| IV-17      | 1       | 33\(^{9}\)                      | 270     |
|            | 2       | 46\(^{9}\)                      | 109     |
| IV-18      | 1       | 66\(^{9}\)                      | 82      |
|            | 2       | 80\(^{9}\)                      | 315     |
|            | 3       | 56\(^{9}\)                      | 196     |
|            | 4       | 39\(^{9}\)                      | 318     |
| IV-19      | 1       | 42\(^{9}\)                      | 121     |
|            | 2       | 66\(^{9}\)                      | 226     |
|            | 3       | 47\(^{9}\)                      | 155     |
|            | 4       | 81\(^{9}\)                      | 142     |

* These values do not include a correction for TH levels.

\(^{32}P\) incorporation into Ser\(^{19}\) versus Ser\(^{31}\)

Corpus striata were labeled with \(^{32}P\), (0.5–2 mCi/side) as described under “Materials and Methods” prior to unilateral electrical stimulation of the medial forebrain bundle. Limit tryptic peptides from \(^{32}P\)-labeled TH were separated by reverse-phase HPLC, and \(^{32}P\) incorporation was quantified with an on-line radiochemical detector. Values are ratio of \(^{32}P\) incorporation into Ser\(^{19}\) divided by Ser\(^{31}\).

| Experiment | Laboratory | \(^{32}P\) incorporation into TH | Control | Stimulated |
|------------|------------|-------------------------------|---------|------------|
| IV-1       | A          | 0.55                          | 0.45    |
| IV-6       | A          | 0.55                          | 0.42    |
| IV-7       | A          | 0.67                          | 0.44    |
| IV-15      | A          | 0.44                          | 0.42    |
| IV-17      | A          | 0.45                          | 0.33    |
| IV-18      | B          | 0.62                          | 0.83    |
|            | B          | 0.33                          | 0.62    |
|            | B          | 0.56                          | 0.67    |
|            | B          | 0.55                          | 0.77    |
| IV-19      | B          | 0.21                          | 0.48    |
|            | B          | 0.37                          | 0.50    |

Corpus striata were labeled with \(^{32}P\), (0.5–2 mCi/side) as described under “Materials and Methods” prior to unilateral electrical stimulation of the medial forebrain bundle. Limit tryptic peptides from \(^{32}P\)-labeled TH were separated by reverse-phase HPLC, and \(^{32}P\) incorporation was quantified with an on-line radiochemical detector. Values are ratio of \(^{32}P\) incorporation into Ser\(^{19}\) divided by Ser\(^{31}\).
was without effect upon either basal or K-stimulated ³²P incorporation into TH (not presented).

Vasoactive intestinal polypeptide and related peptides also appear to act via cAMP in a number of catecholaminergic tissues (cf. Ref. 27). In PC12 cells, superior cervical ganglia, and perfused rat adrenal glands, vasoactive intestinal polypeptide causes a selective increase in Ser⁴⁰ phosphorylation (9, 10, 28). In the present studies, however, vasoactive intestinal polypeptide (1 μM, 15 min) was without effect on ³²P incorporation into TH at any of the sites (not presented).

In contrast, the adenylate cyclase/cAMP-dependent protein kinase system per se appeared to be both intact and capable of activation in that treatment of the synaptosomes with either forskolin, 8-bromo-cAMP, or dibutyryl cAMP produced a selective increase in Ser⁴⁰ phosphorylation (Table V).

Effects of Other Agents on TH Phosphorylation—The gradual increase in Ser⁴⁰ phosphorylation in response to elevated [K⁺], suggested that a signal transduction pathway more temporally dampened than the calcium influx/CAM-protein kinase II activation was being recruited in parallel. In PC12 cells, the phosphorylation of Ser³¹ was increased by treatment with nerve growth factor or phorbol esters (10). As shown in Table V, phorbol 12,13-dibutyrate selectively increased Ser³¹ phosphorylation in the striatal synaptosomes. Nerve growth factor (50 ng/ml, 5–15 min) failed to produce an effect (not presented).

Finally, that the phosphorylation of all four phosphorylation sites could be simultaneously increased was demonstrated by treatment of the synaptosomes with okadaic acid, an inhibitor of phosphatases 1 and 2A (Table V).

**Discussion**

TH is phosphorylated in *vivo* at different sites by a number of different protein kinases (cf. Ref. 25). To the extent that the phosphorylation is associated with an increase in TH activity, each of these protein kinases has, in turn, been suggested to mediate the physiological regulation of TH (cf. Ref. 25). However, an essential criterion for establishing the physiological relevance of a protein kinase's action is the demonstration that phosphorylation of the site(s) influenced by that protein kinase in *vivo* occurs and is regulated in *vivo*. Using a rapid cryofixation method, we have demonstrated that activation of the nigrostriatal pathway increases the phosphorylation of striatal TH at serines 19, 31, and 40.

**Potential Protein Kinase/Second Messenger Systems**

Ser³⁰ Phosphorylation—CAM-protein kinase II is the only protein kinase yet known to phosphorylate Ser³⁰ (4), and it is present and activated in nerve terminals by depolarization (29, 30). Thus, a physiological role for activation of CAM-protein kinase II consequent to depolarization-dependent calcium influx seems likely. In perfused rat adrenal gland, the second messenger appears to be specifically that calcium derived from influx (as opposed to calcium mobilized from internal stores). Nicotine, which increases calcium influx without mobilizing intracellular calcium, selectively increases Ser⁴⁰ phosphorylation, whereas vasoactive intestinal polypeptide, which mobilizes intracellular calcium without increasing calcium influx, selectively increases Ser⁴⁰ phosphorylation (23, 31). In that both nicotine and vasoactive intestinal polypeptide increase catecholamine secretion via their abilities to increase intracellular calcium (31, 32), the CAM-protein kinase II-TH interaction appears to be compartmentally dis-
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Distinct from the secretory processes.

Ser<sup>60</sup> Phosphorylation—In contrast to Ser<sup>19</sup>, Ser<sup>60</sup> can be phosphorylated in vitro by a number of different protein kinases including CAM-protein kinase II, cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, protein kinase N, and S6 kinase (4, 33).<sup>a</sup> Although at least several of these protein kinases are present in nerve terminals and activated by depolarization, the promiscuity of Ser<sup>60</sup> as a phosphate acceptor makes it difficult to discern which of the possible protein kinase candidates is(are) actually responsible for the stimulation-dependent phosphorylation of Ser<sup>60</sup>. However, the involvement of CAM-protein kinase II or protein kinase C in Ser<sup>60</sup> phosphorylation seems unlikely. For example, in synaptosomes, essentially maximal increases in Ser<sup>60</sup> phosphorylation produced by elevated [K<sup>+</sup>], (presumably via CAM-protein kinase II) failed to influence Ser<sup>60</sup> phosphorylation (Table V). Similarly, treatment of synaptosomes for 5 min with a relatively high concentration (1 μM) of phorbol dibutyrate increased Ser<sup>60</sup> but not Ser<sup>19</sup> phosphorylation (Table V). Although longer treatment (15 min) did produce a small increase in Ser<sup>60</sup> phosphorylation, 4-α-phorbol dibutyrate did likewise (not presented), suggesting that some mechanism other than activation of protein kinase C was responsible for the Ser<sup>60</sup> phosphorylation.

Ser<sup>21</sup> Phosphorylation—Phorbol dibutyrate (but not 4-α-phorbol dibutyrate) increased Ser<sup>21</sup> phosphorylation; however, protein kinase C does not appear to be directly responsible for the phosphorylation of Ser<sup>21</sup>. In vitro, protein kinase C phosphorylates Ser<sup>60</sup> (4, 34). In fact, none of the more well-characterized protein kinases (4) nor a number of more recently described protein kinases<sup>b</sup> appears to phosphorylate Ser<sup>21</sup> in vivo. Pharmacological evidence to date suggests that increases in Ser<sup>21</sup> phosphorylation are associated with increases in inositol phospholipid turnover<sup>c</sup>, and efforts are being made to isolate and purify the protein kinase activity responsible for Ser<sup>21</sup> phosphorylation.

A bimodal distribution was observed in the in vivo studies with respect to the relative increases in Ser<sup>19</sup> versus Ser<sup>21</sup> phosphorylation produced by electrical stimulation (Table IV). Experiments performed in one laboratory resulted in a relatively larger increase in Ser<sup>21</sup> phosphorylation whereas those in the other resulted in a relatively larger increase in Ser<sup>19</sup> phosphorylation. Two observations suggest that the magnitude of Ser<sup>19</sup> phosphorylation is more directly influenced by the quantitative aspects of the stimulus than that of Ser<sup>21</sup>. For example, from Table V, the temporal course of Ser<sup>21</sup> phosphorylation in response to elevated [K<sup>+</sup>] was slower than that of Ser<sup>19</sup>. Second, in perfused rat adrenal, the magnitude of increase in Ser<sup>21</sup> phosphorylation in response to activation of the splanchic nerve is more dependent upon the stimulation parameters than is the increase in Ser<sup>19</sup> phosphorylation (28). Thus, it is possible that the stimulation conditions, although operationally matched between laboratories, may have produced different levels of activation of the medial forebrain bundle.

Ser<sup>8</sup> Phosphorylation—Vulliet and colleagues (35) have recently isolated the protein kinase activity from PC12 cells initially shown to phosphorylate Ser<sup>21</sup> in vitro and have coined the term “proline-directed protein kinase” to reflect an apparent requirement for the sequence -Xaa-Ser/Thr-Pro-Xaa-to confer substrate reactivity. The levels of this protein kinase activity are very low in brain and adrenal but high in PC12 cells (36). Consistent with this observation, the major difference between the pattern of multiple-site phosphorylation of TH in PC12 cells and corpus striatum is the relative phosphorylation of Ser<sup>8</sup>. In PC12 cells, ~30% of the <sup>32</sup>P incorporation into TH was on Ser<sup>8</sup> (10), whereas in corpus striatum Ser<sup>8</sup> phosphorylation accounted for less than 10% of the total (Fig. 2, A and B). Although it is not known whether the proline-directed protein kinase is responsible for Ser<sup>8</sup> phosphorylation in dopaminergic nerve terminals, the physiological relevance would be most given that Ser<sup>8</sup> phosphorylation was not influenced either by electrical stimulation in vivo or by depolarization of synaptosomes. There does, however, appear to be a reasonable rate of phosphate turnover on Ser<sup>8</sup> in that treatment of synaptosomes with okadaic acid increased <sup>32</sup>P incorporation into Ser<sup>8</sup> severalfold.

Relationship of Phosphorylation to Activity

The stimulation parameters used in the present study were chosen on the basis of their ability to increase TH activity and DA biosynthesis (14, 15), and the activation of TH was confirmed in one rat (not presented). Although it is not possible from the present data to determine the degree to which phosphorylation of each of the three sites would contribute to an increase in TH activity/DA biosynthesis, it is possible to adduce support for a contribution by phosphorylation at each of the different sites. (a) There is consensus that phosphorylation of Ser<sup>60</sup> increases TH activity, and a mechanism for the activation has recently been proposed (37). (b) Phosphorylation of Ser<sup>19</sup> can increase TH activity in the presence of an activator protein (38, 39), although an involvement of the activator remains to be demonstrated in situ. (c) Activation of TH can be associated with what appears to be a selective increase in Ser<sup>21</sup> phosphorylation in intact PC12 cells (40). However, given the data in Table V and elsewhere (10), this correlation would be strengthened considerably with inherently, as opposed to operationally, matched samples.

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Materials and Methods

MATERIALS: Male Sprague-Dawley rats (200-250 g) were obtained from Harlan Sprague-Dawley, Inc. Tyrosine hydroxylase (TH) antibodies were raised in rabbits by ICN Biochemicals. Affinity-purified sheep antibodies to catalytically active TH from a rat by affinity chromatography on tyrosine hydroxylase (TH) were used for immunoprecipitation. Other chemicals and reagents were as specified previously (30).

METHODS: Purified sheep antibodies to catalytically active TH from a rat by affinity chromatography on tyrosine hydroxylase (TH) were used for immunoprecipitation. Other chemicals and reagents were as specified previously (30).

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Multiple-site phosphorylation in vivo and in situ

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RESULTS

TH PROTEIN LEVELS IN CORPUS STRATUM. With few exceptions, previous estimates of the absolute tissue levels of TH were made by comparing enzyme activity in tissue homogenates to that of purified enzyme. However, differences in specific activities of purified preparations (from differences in stability, state of activation, and assay conditions) and the additional problem of endogenous inhibitors and activators in tissue homogenates have made estimates of actual tissue levels of TH based on activity measurements suspect. Thus, to establish the efficacy of the subcellular purification and to compare F3 labeling efficiency in the different neurotransmitter fractions, the absolute levels of TH protein were determined with an immunohistochemical assay standardized to purified rat TH protein levels (40) (Table I). Values for solubilized striatal samples ranged from 1.6 to 2.2 ng/µg protein (2.0 ± 0.1, mean ± S.E., n = 8). The levels of TH in homogenates and subcellular fractions of the corpus striatum are presented in Table I.

| TABLE I | Levels of TH in subcellular fractions of the rat corpus striatum. |
|---------|---------------------------------------------------------------|
| Subcellular Fraction | Protein* | Tyrosine Hydroxylase |
| | ug/µg wet weight | % of TH/µg protein |
| H | 90 | 100 | 2.0 |
| S1 | 75 | 98 | 2.4 |
| P1 | 12 | 5 | 0.8 |
| S2 | 39 | 51 | 2.4 |
| P2 | 32 | 50 | 2.8 |
| F3 | 5.2 | 14 | 4.7 |
| F4 | 4.4 | 10 | 4.0 |

*R one pair of striata weigh ~100 mg.

TH was relatively enriched in subcellular fractions containing synaptosomes. Synaptosomes purified on Percoll/sucrose gradients had the highest specific activity of TH (Table I). Importantly, TH immunoreactivity was present as a single band in all of the samples indicating that proteolysis was minimal. The similarity of TH levels and specific activity between S2 vs P2 was initially somewhat surprising. However, alterations in homogenization parameters did not substantially decrease TH levels in S2. Three potential sources for the TH in S2 are (A) the unlysed, dopaminergic axons and preterminal processes of the nigro-striatal projection, (B) dopaminergic nerve terminals that did not reseal and/or retain 100% of their contents, and (C) TH containing cell bodies (only 10-20/striatum) (47).

In terms of F3 labeling of TH, the specific radioactivity of TH (cpm [32P]/ng TH) was higher in P2 than in either F3 or F4 (p < 0.05). In turn, values for F4 were higher than for F3. Furthermore, potassium-stimulated increases in TH phosphorylation (% of control) were ordered P2 > F3 > F4; thus, the P2 fraction was chosen for the labeling studies.

QUANTITATION OF TH PHOSPHORYLATION. After in vivo labeling, two normalizing corrections of the data were required to compare F3 incorporation into TH from side to side in a single animal and (to a limited extent) across animals. (1) The primary correction was based on [32P] incorporation into total protein. Normalization to this value was necessary to correct for effects such as differences in the amount of TH protein, injected, the distance of the tissue from the cannula tip, and the relative labeling of ATP pools that could occur with each injection. The relative incorporation of [32P] into TH vs total protein was on the order of ~300 cpm into TH per 105 cpm into total protein. (2) Although conditions were established to provide quantitative immunoprecipitation (Fig. 1, right), the amount of TH protein in each immunoprecipitated sample was used to correct for sample-to-sample differences in the relative amount of TH (and/or efficacy of immunoprecipitation. In the experiments with synaptosomes, no corrections for total protein or TH protein levels were necessary. Sample-to-sample differences [32P] incorporation into total protein were typically small and, with the exception of samples treated with phosphatase or chelating acid, correction for this produced a modest decrease in variability.

Fig. 1 presents autoradiographs illustrating [32P] incorporation (left) into total protein (TOTAL) and into TH (TH/PI) and an example of the assay for TH levels (right).