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TYROSINE HYDROXYLASE ACTIVATION

COMPARISON OF *IN VITRO* PHOSPHORYLATION AND *IN VIVO* ADMINISTRATION OF HALOPERIDOL*

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Abstract—Rat striatal tyrosine hydroxylase (TH) was assayed 2 hr following treatment with 1 mg/kg haloperidol. TH activity in striata from haloperidol-treated rats (haloperidol TH) was increased significantly relative to control when assayed at pH 7.0, but not at pH 6.0, in the presence of 175 μM tetrahydrobiopterin (BH_4). TH was also phosphorylated *in vitro*, catalyzed by sufficient quantities of the catalytic subunit of bovine heart protein kinase to cause greater than 90% activation after 10 min. TH was activated by phosphorylation at both pH 6.0 and pH 7.0, but the activation was greater at pH 7.0. Haloperidol TH, activated relative to control TH at pH 7.0, was activated by phosphorylation, but there was no difference between haloperidol TH and control TH activity at either pH 6.0 or 7.0 following phosphorylation. Comparison of Lineweaver–Burk plots of nonphosphorylated and phosphorylated TH indicated that activation by phosphorylation was due to a 5-fold change in K_m for BH_4 and a 2-fold change in V_{max} at pH 7.0. Haloperidol TH kinetics were intermediate between those of nonphosphorylated and phosphorylated TH at pH 7.0. Analysis by Lineweaver–Burk, Hanes–Woolf, and Eadie–Scatchard plots suggested that the haloperidol TH kinetic data were the result of a mixture of two forms of the enzyme, with different affinities for cofactor. Theoretical calculations of TH activity of mixtures of nonphosphorylated and phosphorylated TH suggested that the haloperidol data could be explained by postulating a mixture of 25–35% phosphorylated TH molecules with 65–75% nonphosphorylated TH molecules. An hypothesis of the role of TH phosphorylation during conditions of increased neuronal firing rate, such as may occur with haloperidol treatment, is presented.

Tyrosine hydroxylase (tyrosine monoxygenase; EC 1.14.16.2) catalyzes the rate-limiting reaction in the biosynthesis of the catecholamines [1–3]. Cyclic AMP (cAMP)-dependent phosphorylating conditions have been shown to activate TH [4–6], by direct phosphorylation of the enzyme [7–13]. The mechanism of this activation is a subject of some controversy, with a number of investigators finding a change in the K_m of TH for cofactor upon phosphorylation [10–12], and others finding a change in V_{max} with no change in K_m upon phosphorylation of the purified enzyme [13]. Phosphorylation also causes a change in pH optimum of the enzyme, from pH 6, for the nonphosphorylated enzyme, toward the more basic range [12, 14, 15].

TH can be activated by nerve stimulation in preparations of hypogastric nerve and guinea pig vas deferens [16, 17]. This activation resembles that produced with enzyme from the same source in the presence of cAMP-dependent phosphorylating conditions. In striatal preparations, however, there appear to be subtle differences between depolarization and dibutyryl cAMP-induced activation of TH [15].

Neuroleptic treatment of rats *in vivo*, with drugs such as haloperidol, results in activation of neostriatal TH. This activation appears to be mediated by an increased affinity of striatal TH for its pteridine cofactor [18–20]. It has been speculated that phosphorylation may be the mechanism of TH activation following haloperidol administration [20]. Bahkit and Gibb [21] have shown that striatal TH activation under phosphorylating conditions and after haloperidol administration is pH dependent, but their kinetic data suggest that the mechanism of TH activation by phosphorylation may be distinct from that of haloperidol-induced activation. On the other hand, Pradhan *et al.* [22] have recently reported the results of similar experiments with quantitatively and qualitatively different results. Both sets of investigators used a synthetic pteridine cofactor in their studies, and in each case phosphorylating conditions were defined as addition of cAMP, Mg^{2+}, ATP, and a commercial preparation of cAMP-dependent protein kinase (in the case of Pradhan *et al.* [22]) to a TH assay, without preincubation of the phosphorylation mixture with the TH preparation. Under these circumstances, phosphorylation presumably was occurring while the assay was proceeding.

We sought to clarify the relationship between phosphorylation and haloperidol-mediated activation of TH. Previous studies have been limited by the inability to ensure complete phosphorylation of TH. We have investigated the kinetics of crude rat striatal TH activated maximally by a highly active, homogeneous preparation of the catalytic subunit of

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bovine heart cAMP-dependent protein kinase. Phosphorylation occurred during a 10-min preincubation to ensure that nonphosphorylated TH molecules would contribute little to TH activity during the initial phase of the TH assay. The effects of haloperidol administration on TH activity were also studied. TH activities were measured in the presence of the naturally occurring cofactor, tetrahydrobiopterin (BH_4), rather than with synthetic cofactor, as has been used by other investigators. The present report describes the results of these investigations.

MATERIALS AND METHODS

Materials. L-[1-^{14}C]Tyrosine was obtained from the New England Nuclear Corp., Gardena, CA. Hog kidney was acquired from a butcher's shop in Chinatown, San Francisco, CA. Sheep liver was obtained from Pel-Freeze, Rogers, AR, catalase from Worthington Biochemicals Corp., Freehold, NJ; biopterin from Regis Chemical Co., Morton Grove, IL; platinum oxide from K & K Biochemicals, Plainview, NY; and haloperidol from McNeil Laboratories, Fort Washington, PA. Other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, and were of the highest purity commercially available.

Methods. Biopterin was reduced to tetrahydrobiopterin by bubbling in hydrogen gas over platinum oxide catalyst by a modification of the method of Lloyd and Weiner [23]. The catalyst was removed by filtration, and the clear filtrate was aliquoted and stored under nitrogen at −70°. The concentration of BH_4 in the final solution was determined by measuring the absorption at 265 nm in 0.1 N HCl. The extinction coefficient under these conditions has been calculated to be 1.6×10^4 ($M^{-1}\,cm^{-1}$) [24, 25]. Stock solutions of tetrahydrobiopterin of 5–20 mM concentration were routinely prepared in this manner.

Male Sprague–Dawley rats (200–250 g) were injected intraperitoneally with 1 mg/kg of haloperidol dissolved in acidic saline (pH 4.5), or acidic saline alone. Average injection volume was 1 ml. Rats were stunned and immediately decapitated 2 hr after injection, and corpora striata were rapidly dissected and frozen on dry ice. Individual striata, weighing 20–25 mg, were homogenized in 0.3 ml of ice-cold 0.2 M sodium acetate buffer (pH 6.0), containing 0.2% Triton X-100. Homogenates were centrifuged at high speed for 5 min, and 25 μl samples of supernatant fluid were incubated under control or phosphorylating conditions for 10 min at 30° prior to assaying for 15 min for TH activity. Average protein concentration of the supernatant fractions was 15 mg/ml. Striatal TH activity from haloperidol-treated rats is referred to in the text as "haloperidol TH", while TH activity in saline-treated rat striata is referred to as "control TH".

cAMP-dependent protein kinase was prepared from bovine cardiac muscle by the method of Sugden *et al.* [26] through the second hydroxylapatite step. Protein kinase was assayed by the method of Witt and Roskoski [27]. The specific activity of this protein kinase preparation, which was homogeneous by sodium dodecylsulfate (SDS)–gel electrophoretic criteria, was found to be 4.4×10^5 pmoles phosphate incorporated into histone II-A per min per mg protein.

The rat striatal preparations were preincubated at 30° for 10 min with 700 μM ATP, 13 μM $MgCl_2$, and, in tubes in which phosphorylation occurred, 1–3 μg of the catalytic subunit heart protein kinase. The kinase was present in quantities sufficient to cause greater than 90% of maximum activation of TH within 10 min under these conditions. When γ-^{32}P-labeled ATP was used during the procedure, incorporation of label into protein was greater than 90% complete after 10 min. Total preincubation volume was 35 μl. The preincubation was followed by assay for TH activity.

TH was assayed by modification of the method of Waymire *et al.* [28]. A typical assay mixture contained 0.25 M morphilino-ethane sulfonate (MES), pH 6.0 or 7.0, 1300 units catalase, 2000 units sheep liver pteridine reductase, purified through the second ammonium sulfate step as described by Craine *et al.* [29], 1.2 mM NADPH, 12 or 60 μM L-[1-^{14}C]tyrosine, BH_4 at various concentrations, and TH sample, in a final volume of 80 μl. Final assay concentrations of the preincubation components were 5.6 mM $MgCl_2$ and 0.3 mM ATP. Experiments done at subsaturating concentrations of tyrosine were performed with 12 μM tyrosine. Kinetic experiments used 60 μM tyrosine, which was saturating under these conditions. BH_4 concentration in experiments performed at subsaturating concentration was 175 μM. The TH assay mixture was incubated at 30° for 15 min. The pH of representation reaction mixtures was measured with narrow range pH paper and found to be in the 0.1 pH unit of the intended pH in every case. The TH reaction was terminated by addition of 3-iodotyrosine (final concentration 3.2 mM) to inhibit the enzyme. Excess hog kidney L-aromatic amino acid decarboxylase, partially purified according to Waymire *et al.* [28], was added with pyridoxal phosphate (final concentration 0.13 mM) to bring the reaction volume to 150 μl. The pyridoxal phosphate was dissolved in potassium phosphate buffer (pH 8.0) to bring the concentration of this buffer in the assay to 0.083 M. Radioactive $^{14}CO_2$ was trapped by connecting assay tubes via thick-walled rubber tubing to counting vials containing Whatman 3 MM paper (1.7 × 7.1 cm) soaked in 0.3 ml β-phenylethylamine solution [20% (v/v) in methanol]. The reaction was continued for an additional 30 min at 30°. The assay was terminated by injection through the rubber tubing of 1 ml of 4% (v/v) perchloric acid in 2 mM Tiron. Radioactivity of the filter paper was determined by liquid scintillation counting following addition of scintillant. That the radioactive CO_2 formed was directly proportional to the amount of DOPA present at the commencement of decarboxylase incubation was verified by control experiments in which [1-^{14}C]DOPA was added prior to the decarboxylase step. TH activity is expressed as nmoles of DOPA formed per min per mg protein. All data points were derived from duplicate assays. The TH assay was linear with tissue concentration and time (up to 20 min) under the conditions studied.

Protein estimations were made by the method of Lowry *et al.* [30]. SDS–gel electrophoresis in Tris–

glycine buffers was performed in slab gels according to the procedures of Weber and Osborn [31]. Kinetic data were analyzed by the method of least squares, and statistical analysis employed Student's two-tailed *t*-test analysis.

RESULTS

TH from haloperidol-treated or control striata was activated significantly by phosphorylation when assayed at pH 6.0 (Fig. 1). The magnitude of the activation was greater in control than in haloperidol-treated striata, but the difference between activation of control and haloperidol-treated rat striatal TH was not found to be statistically significant for the number of rats used in the experiment (four; each striatum was assayed separately and the data were combined for each rat). Nonphosphorylated haloperidol TH was activated slightly over nonphosphorylated control TH, but this difference was also not found to be statistically significant.

Striatal TH was also assayed at pH 7.0, as shown in Fig. 2. At this pH, there was a 2-fold activation of TH in striatal extracts from haloperidol-treated rats relative to controls. The activation produced by haloperidol was the same whether TH activity was measured immediately after homogenization and centrifugation or 24 hr after being stored at 4° (data not shown), suggesting that little deactivation occurred at 4°. In addition, the magnitude of the activation produced by haloperidol did not change after either extensive dialysis or chromatography on Sephadex G-10. Incubation of the haloperidol-treated and control preparations under phosphorylating conditions caused activation in each case to the same level of TH activity, with no difference in activity between the haloperidol-phosphorylated and control-phosphorylated preparations. Thus, the "fold activation" of control TH was twice that of haloperidol TH under these phosphorylation and assay conditions. Comparison of the ordinates of Figs. 1 and 2 indicated that TH activity was markedly lower at pH 7.0 than at pH 6.0, but that the ratio of TH activity at pH 7.0 to TH activity at pH 6.0 was much greater for phosphorylated than for nonphosphorylated TH. TH from haloperidol-treated rat striata displayed a ratio of activities intermediate

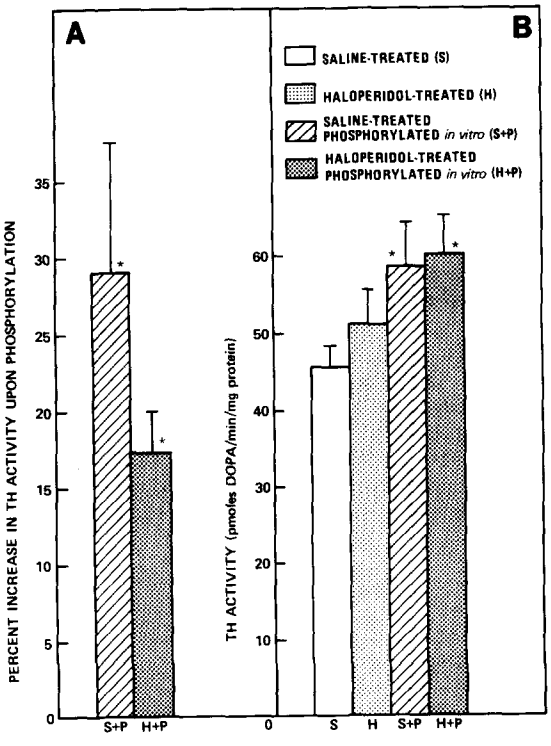

Fig. 1. Effect of phosphorylation on striatal TH from saline- or haloperidol-treated rats assayed at pH 6.0. (A) Percent increase in TH activity upon phosphorylation. (B) Mean TH activity; no differences are significant by Student's *t*-test. Each result is the mean ± S.E. of four rats; the striata of each rat were assayed separately in duplicate, and the results were combined for each rat. Compared are TH from saline-treated (S), haloperidol-treated (H), saline-treated phosphorylated (S + P), and haloperidol-treated phosphorylated (H + P) rat striata. Key: (*)$P < 0.05$ compared with nonphosphorylated enzyme by paired *t*-test. No other differences are significant.

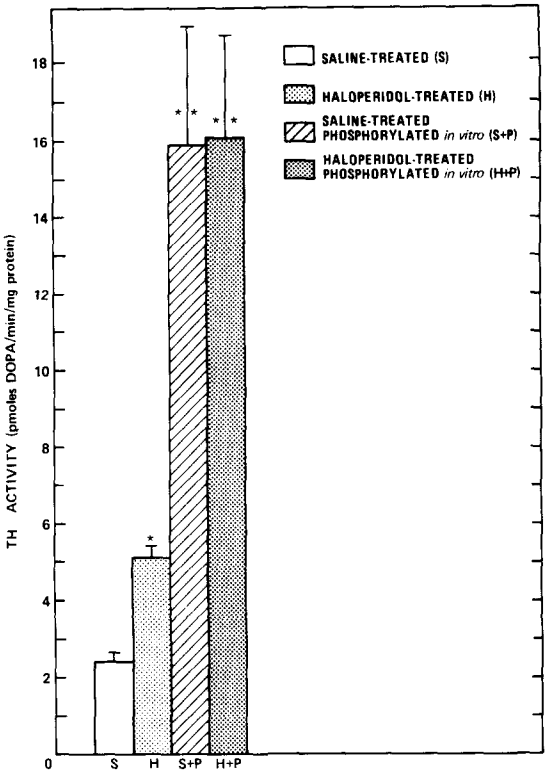

Fig. 2. Effect of phosphorylation on striatal TH from saline- or haloperidol-treated rats assayed at pH 7.0. Each result is the mean ± S.E. of four rats; the striata of each rat were assayed separately in duplicate, and the results were combined for each rat. Compared are TH from saline-treated (S), haloperidol-treated (H), saline-treated phosphorylated (S + P), and haloperidol-treated phosphorylated (H + P) rat striata. Key (*)$P < 0.005$ compared with saline-treated nonphosphorylated TH; and (**)$P < 0.001$ compared with nonphosphorylated TH. The difference between (H + P) and (S + P) is not statistically significant ($P > 0.05$).

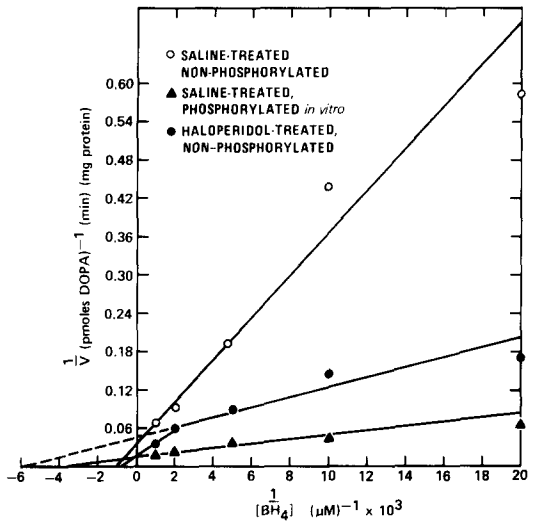

Fig. 3. Lineweaver–Burk plots of TH activity in rat striatal extracts at pH 7.0, varying the BH_4 concentration in the presence of 60 μM tyrosine. TH was phosphorylated as described in Materials and Methods. Data are plotted by the method of least squares analysis. The data are from a representative experiment. Compared are TH from saline-treated nonphosphorylated, saline-treated phosphorylated, and haloperidol-treated nonphosphorylated rat striata.

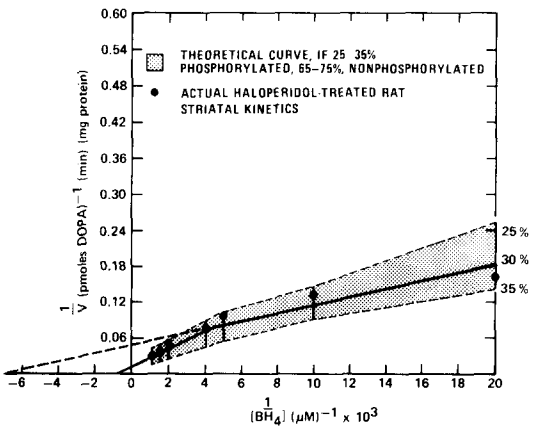

Fig. 4. Lineweaver–Burk plots of TH activity in rat striatal extracts at pH 7.0, varying the BH_4 concentration in the presence of 60 μM tyrosine. TH was phosphorylated as described in Materials and Methods. Data are plotted by the method of least squares analysis. Each data point is the average of four experiments. The shaded area represents the range of values expected from a mixture of 25–35% phosphorylated TH molecules, calculated as in Results. Compared are TH from saline-treated phosphorylated, and haloperidol-treated rat striata.

between that for phosphorylated and nonphosphorylated control TH. In all experiments, the fold activation produced by haloperidol treatment was 25–35% of the maximum activation produced by phosphorylation. Because of the accentuated difference between control and haloperidol TH activities at pH 7.0, as opposed to pH 6.0, kinetic experiments were performed at the higher pH value.

Kinetic experiments were performed on rat stiatal TH at pH 7.0, varying the BH_4 concentration in the presence of 60 μM tyrosine. TH from control rats with control (nonphosphorylating) preincubation, TH control rats with phosphorylating preincubation, and TH from haloperidol-treated rats with control preincubation were analyzed in this way. Each experiment was performed four times with striata from individual rats. The results of a typical experiment are graphed in Fig. 3 by the Lineweaver–Burk method. Activation of control enzyme by phosphorylation appeared to result from a large decrease in K_m for BH_4, and a 2-fold increase in V_{max}, when assayed at pH 7.0. The kinetics of the striatal TH from haloperidol-treated rats were intermediate between those of phosphorylated and nonphosphorylated TH from control striata. The data for haloperidol TH could be fitted to a straight line, but in all four experiments described here the data were best fitted to a biphasic curve, as plotted in Fig. 3. A composite of all the haloperidol kinetic data, at more cofactor concentrations than in Fig. 3, is shown in Fig. 4. Once again a biphasic curve was the best fit, as judged by comparison of correlation coefficients for the least squares lines. In the results shown in Fig. 4, the control TH kinetics, both phosphorylated and nonphosphorylated, resulted in correlation coefficients of greater than 0.95. For the haloperidol

Table 1. Kinetic parameters of TH activity at pH 7.0 in rat striatal extracts in the presence of 60 μM tyrosine*

	K_m (BH_4)	V_{max}
Saline-treated	1000 ± 135	19.8 ± 2.5
Saline-treated, phosphorylated *in vitro*	220 ± 30†	36.7 ± 4.0‡
Haloperidol-treated (data from 50–250 μM BH_4 points)	164 ± 32†, §	12.4 ± 1.4‡, ‖

* Data are the means of four experiments. Striatal extracts were preincubated under phosphorylating conditions in the presence or absence of 2 μg catalytic subunit of heart protein kinase. K_m (BH_4) is in μM. V_{max} units are pmoles DOPA formed per min per mg protein. Both K_m and V_{max} differences between saline and saline-phosphorylated TH were significant by Student's t-test.

† $P < 0.01$ compared with saline-treated, nonphosphorylated.

‡ $P < 0.05$ compared with saline-treated, nonphosphorylated.

§ $P > 0.05$ compared with saline-treated, phosphorylated *in vitro*.

‖ $P < 0.05$ compared with saline-treated, phosphorylated *in vitro*.

kinetics, the Pearson correlation coefficient for one line drawn through all the points was $r = 0.75$. On the other hand, that for a line through the lower substrate data points was much closer to unity, $r = 0.92$. In each of four experiments, similar correlation coefficients were observed. A summary of the kinetics results is shown in Table 1. The haloperidol data in the table are from the low BH_4 concentration data points (by the Lineweaver–Burk plotting method, as will be discussed later, there were relatively few high BH_4 concentration points, so the correlation coefficient for a line through these points is relatively less meaningful).

If the biphasic nature of the Lineweaver–Burk plots were representative of a mixture of two enzyme species, such as nonphosphorylated and phosphorylated TH, the K_m and V_{max} calculated from the low-substrate data points would be an approximation of the properties of the enzyme with higher affinity for BH_4 [32]. This model was tested, as will now be described. The equation used for Lineweaver–Burk analysis is:

$$V = (V_{max} \times BH_4)/(K_m + BH_4).$$

Therefore, for nonphosphorylated TH in rat striatal extract at pH 7.0 (using the data from Table 1):

$$V = (19.8 \times BH_4)/(1000 + BH_4).$$

For phosphorylated TH:

$$V = (36.7 \times BH_4)/(220 + BH_4).$$

From the data for nonphosphorylated and phosphorylated TH kinetics, theoretical data for a mixture of phosphorylated and nonphosphorylated TH molecules could be generated. If nonphosphorylated TH were converted to Y phosphorylated molecules, and $(1 - Y)$ remained nonphosphorylated, the velocity equation would be:

$$V \times Y \times (36.7 \times BH_4)/(220 + BH_4) + (1 - Y) \times (19.8 \times BH_4)/(1000 + BH_4).$$

The expected data from experiments performed under the conditions actually used, but assuming 25–35% activation by phosphorylation, are shown in the shaded area of Fig. 4. This range was chosen because experiments such as that shown in Fig. 2 resulted in haloperidol activation of TH which was constantly between 25 and 35% of the maximum stimulation of TH activity produced by phosphorylation *in vitro*. The range was in relatively good agreement with the actual haloperidol kinetic data, as shown. Referring to Table 1, the fact that the V_{max} of the haloperidol data extrapolated from the low substrate data points was 12.4, while that of completely phosphorylated TH was 36.7, suggests that approximately 33% of the haloperidol-treated TH was phosphorylated, in good agreement with the above conclusion.

The above results suggested that TH activation by haloperidol treatment and phosphorylation proceeded through similar mechanisms. However, the biphasic nature of the Lineweaver–Burk plots of haloperidol TH activity was subtle, and dependent on two or three data points at the high BH_4 concentration range. The Lineweaver–Burk method of plotting kinetic data, which gives disproportionate importance to data obtained at low substrate values, is often not the best for detecting mixtures of two enzymes which catalyze the same reaction but are present in different quantities and possess different kinetic properties [32]. A more sensitive method is the Eadie–Scatchard plot of V versus V/BH_4. The composite haloperidol data are plotted by the Eadie–Scatchard method in Fig. 5B. For comparison are included Eadie–Scatchard plots of nonphosphorylated and phosphorylated control TH, in Fig. 5A. A biphasic plot was noted in the case of haloperidol TH (Fig. 5B), but not for phosphorylated and nonphosphorylated TH (Fig. 5A). The same data were also plotted by the Hanes–Woolf method, in which BH_4/V is plotted versus BH_4. Again, biphasic curves were detected in the case of haloperidol TH (Fig. 6B), but not for nonphosphorylated or phosphorylated control TH (Fig. 6A). In Figs. 5B and 6B, theoretical curves generated for a mixture of 25–35% phosphorylated TH with 65–75% nonphosphorylated TH are shown as the shaded

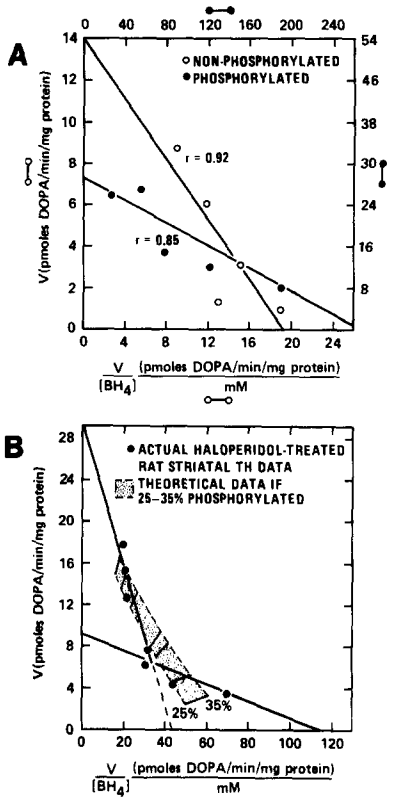

Fig. 5. Eadie–Scatchard plots of V versus V/S, for rat striatal TH at pH 7.0. BH_4 is the varied substrate. Data are replotted from Fig. 4. (A) Compared are TH from saline-treated and saline-treated phosphorylated rat striata. Each point is the mean from four experiments, plotted by the method of least squares; r values represent Pearson correlation coefficients. (B) Haloperidol-treated rat striatal data. Each point is the mean of four experiments. The shaded area represents theoretical data if 25–35% of TH molecules were phosphorylated, derived as described in Results. For each phase of the curve, data were plotted by the method of least squares. For both lines, $r > 0.95$.

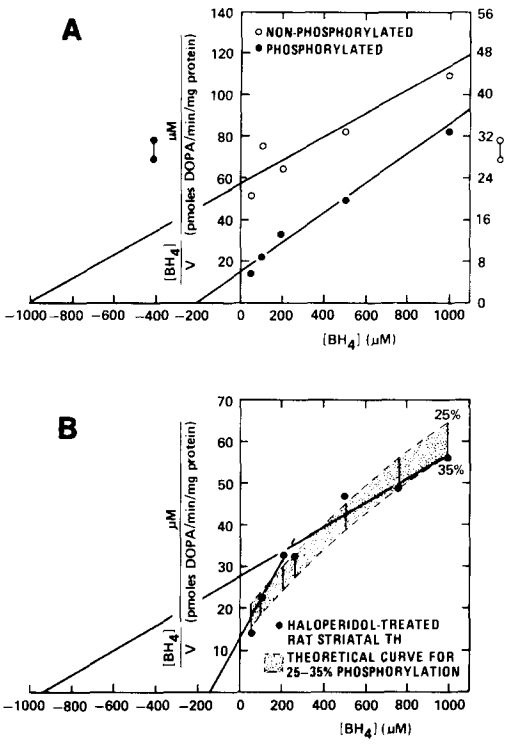

Fig. 6. Hanes–Woolf plots of S/V versus S, for rat striatal TH at pH 7.0. BH_4 is the varied substrate. Data are replotted from Fig. 4. (A) Compared are TH from saline-treated and saline-treated phosphorylated rat striata. Each data point is the mean from four experiments, plotted by the method of least squares. (B) Haloperidol-treated rat striatal data. Each data point is the mean of four experiments; $r > 0.95$ for all lines shown in this figure. Shaded area represents theoretical data if 25–35% of TH molecules were phosphorylated, derived as described in Results. For each phase of the curve, data were plotted by the method of least squares.

portions of the figures. These curves were generated in a manner analogous to that used in the derivation previously described and plotted in Fig. 4. In each case, the actual haloperidol data fit the theoretical construct relatively well.

DISCUSSION

Striatal TH from haloperidol-treated rats (haloperidol TH) was activated relative to striatal TH from saline-treated rats (control TH) when assayed at pH 7.0, but not at pH 6.0. Other investigators have found neuroleptic-induced activation of straital TH at pH 6 utilizing kinetic analysis [18–20], while recently it has been reported that there is little activation at this pH [21]. The present studies detected a small but not statistically significant increase in TH activity at pH 6 in the presence of 175 μM BH_4. This may reflect the fact that complete phosphorylation of control TH resulted in only 30% activation under the assay conditions employed. If haloperidol treatment causes 25–35% of the activation caused by phosphorylation (as suggested from the data at pH 7.0), then theoretically haloperidol TH ought to have been activated by 8–10% over control TH. Such activation was in fact observed (Fig. 1), but not at a statistically significant level, for which a greater sample size would be necessary. It is possible that significant changes would have been detected if kinetic analyses were performed at pH 6.0. However, the intent of these studies was to compare TH activation produced by phosphorylation and haloperidol treatment, and pH 7 was chosen to enhance differences between activated and nonactivated TH.

The striatal dissections contained about 7000 ng dopamine/g tissue (approximately 35 μM) [33]. The tissue was diluted 1 in 11 during homogenization (assuming homogeneous distribution of dopamine; in actuality, vesicular dopamine would pellet after homogenization), and a further dilution of 1 in 4 was made during the TH assay. Thus, dopamine was present in the TH assay at less than 1 μM final concentration. This dopamine concentration would be expected to have little effect on TH activity, as others [34] and we ourselves (data not shown) have found the K_i for dopamine of striatal TH to be of the order of 6 μM under conditions similar to those employed in the present investigations.

Phosphorylation of control striatal TH resulted in activation at both pH 6 and pH 7, but the relative activation was far greater at pH 7. In addition, 2-fold activation by haloperidol treatment was seen at pH 7. Complete *in vitro* phosphorylation of striatal TH from both control and haloperidol-treated rats resulted in no significant difference between control-phosphorylated and haloperidol-phosphorylated TH activity. This was consistent with the hypothesis that haloperidol treatment might be activating TH by phosphorylation. Haloperidol treatment consistently resulted in a "fold-activation" which was 25–35% of that caused by *in vitro* phosphorylation of TH.

Kinetic analysis of control TH and control-phosphorylated TH showed that phosphorylated TH was activated at pH 7 by a change in both K_m for BH_4 and V_{max} in this system. The K_m for tyrosine (approximately 10–15 μM) did not change after phosphorylation (M. A. Lazar and J. D. Barchas, unpublished observation). In the crude rat striatal preparation described here, the more dramatic change produced by phosphorylation was the approximately 5-fold decrease in affinity for pteridine cofactor.

TH from haloperidol-treated striata displayed biphasic kinetics when the data were analyzed by the Lineweaver–Burk, Eadie–Scatchard, or Hanes–Woolf methods. The data appeared consistent with the model that haloperidol administration activated TH by phosphorylation. Based on K_m and V_{max} values for phosphorylated and control TH in striatal extracts, theoretical calculations can be made for any mixture of nonphosphorylated and phosphorylated TH. The data from haloperidol-treated rats were consistent with a model suggesting that 25–35% of striatal TH molecules were phosphorylated. These estimates assume that few or no TH molecules are phosphorylated in the striatal preparations. This conclusion is reinforced by the fact that there was no hint of biphasicity when the kinetic data from control, nonphosphorylated TH was plotted by any of

the analytic procedures employed. The kinetic analyses are probably only sensitive enough to distinguish 10 or more percent phosphorylation of the total number of TH molecules.

It is apparent that if haloperidol treatment does result in activation of TH by phosphorylation, only a portion of the TH molecules are phosphorylated. This is in contrast to the all-or-nothing change in the state of the enzyme which must be concluded from the work by other investigators, as reflected by the monophasic Lineweaver–Burk plots they report [18–22]. It is possible, in the experiments reported here, that in all striatal TH-containing nerve terminals 25–35% of the enzyme molecules were phosphorylated after haloperidol treatment. Alternatively, since it is apparently a tetramer, of which each subunit may be phosphorylated, selective subunit phosphorylation may occur [8, 12]. Also consistent with the data would be complete phosphorylation of TH in 25–35% of the striatal TH-containing neurons. The model of TH activation by phosphorylation cannot distinguish among these possibilities from the data described in Results. Possibly, 25–35% of TH molecules are phosphorylated in each neuron, suggestive of great reserve on the part of the dopaminergic cell. Thus, further stimuli could increase TH activity further. If, on the other hand, 25–35% of TH-containing neurons in striatum are affected by haloperidol treatment, great selectivity of the haloperidol response would appear to be the case, since 65–75% of dopaminergic neurons presumably would not be responding to the haloperidol treatment. It is possible that higher doses of haloperidol would have caused increased activation of TH. However, it has been reported that the ED_{50} for haloperidol activation of TH is 0.05 mg/kg, with complete activation at doses greater than 0.5 mg/kg [35].

Bahkit and Gibb [21] have also compared TH activation by phosphorylation with that caused by haloperidol administration. They found that, as assay pH increases, the K_m for 6-MPH_4 of TH from haloperidol-treated rats decreased, whereas the K_m for 6-MPH_4 of control phosphorylated TH increased. The K_m for 6-MPH_4 of control TH changed little as pH increased. Largely on this basis, these investigators concluded that haloperidol caused activation of TH by a mechanism distinct from that of phosphorylation. On the other hand, Pradhan *et al.* [22] found that, as pH increased, the K_m for 6-MPH_4 of TH from haloperidol-treated rats increased slightly, whereas the K_m for 6-MPH_4 of control phosphorylated TH changed very little with increasing pH. The K_m for 6-MPH_4 of control TH increased dramatically as pH increased. Thus, as pH increased, both haloperidol TH and phosphorylated TH were activated over control TH, largely on the basis of lower K_m for cofactor. No qualitative differences between phosphorylation and haloperidol-induced activation of TH were reported. Thus, there are major discrepancies between the results of two apparently similar sets of experiments. In addition, Anagoste *et al.* [36] showed that *in vitro* dopamine synthesis was increased in striatal slices prepared from haloperidol-treated rats. The effect of cAMP on this system was not additive with that of haloperidol, and these authors suggested that the mechanisms may be the same. However, neither pH dependence studies nor kinetic analyses with various cofactor concentrations were performed. In contrast, Kapatos and Zigmond [37] found that the effects of haloperidol and cAMP on striatal synaptosomal TH were additive. Kinetic studies were not done because TH did not exhibit Michaelis–Menten kinetics in their system. These investigators also found no change in pH optimum after haloperidol treatment. Further complicating the interpretation of these data is the fact that each set of investigators added cAMP immediately prior to assay and, in all but one case [22], relied on endogenous kinase. Therefore, phosphorylation apparently was ongoing during the assays, which should have virtually ensured that the kinetics were measured on a mixture of phosphorylated and nonphosphorylated TH molecules. In addition, the kinetic studies assumed that all TH molecules were activated by neuroleptic treatment. It has been pointed out in this discussion that the nonlinearity of the Lineweaver–Burk plot of haloperidol TH activity is relatively subtle, compared with the nonlinearity revealed by alternative plotting methods, and this factor may be important in kinetic analysis of TH activation.

In summary, the activation of TH caused by haloperidol treatment is more pronounced at pH 7 than at pH 6, as is that caused by phosphorylation (Figs. 1 and 2). Complete phosphorylation of haloperidol-activated TH resulted in further activation of the enzyme, but the activity of haloperidol-phosphorylated TH was no different than that of control-phosphorylated TH at pH 6 or at pH 7. Kinetic analysis at pH 7 suggested that two species contributed to the TH activity in the striatal preparations from haloperidol-treated rats. The kinetic properties of the two populations of TH molecules were consistent with the hypothesis that the haloperidol-treated striatal preparations contained a mixture of nonphosphorylated and phosphorylated TH (Table 1). Quantitatively, the results were consistent with a model which postulates that 25–35% of TH molecules would be in phosphorylated form in striatal preparations from haloperidol-treated rats (Figs. 4–6). The data presented here constitute circumstantial evidence that haloperidol-induced activation of striatal TH is mediated by partial phosphorylation of the enzyme. Nevertheless, it is possible that haloperidol treatment causes, for example, limited proteolysis of TH, which has been shown to activate the enzyme [38], producing a form no longer responsive to phosphorylating conditions [39]. Absolute proof will require *in vivo* administration of $^{32}PO_4$ and specific immunoprecipitation of TH following haloperidol and saline treatments.

TH has been shown to be activated by nerve firing and depolarization in a number of systems [15–17, 20–22, 40–42]. Interestingly, *in vitro* preparations of guinea pig vas deferens appear to contain a mixture of two kinetic forms of TH which, upon repeated nerve stimulation, is converted to a single population of the form with higher affinity for cofactor [16]. Furthermore, rat adrenal TH consists of two forms, the percentages of which vary with the degree of

decapitation stress [43]. Thus, there is a precedent for two kinetic forms existing in the same preparation, although the present work detected only the lower affinity form in the absence of haloperidol treatment. The similarity between the mechanism of activation of TH by nerve stimulation and cAMP-dependent phosphorylation has been well described [17, 42, 43]. However, differences between phosphorylation and cAMP-induced activation have been described in rat striatal slices [15].

Haloperidol-induced activation of TH seems to require an intact striato-nigral pathway [44], and nerve firing is required for the activation [45]. The present work details the similarities between haloperidol-induced activation of TH, which may reflect increased dopaminergic neuronal firing, and *in vitro* phosphorylation of the enzyme.

It has also been shown that TH can be activated by a cAMP-independent protein kinase [46]. It is possible that stimulation-induced phosphorylation of TH is mediated by a cAMP-independent protein kinase. There are calcium-dependent protein kinases in the nerve terminal, which are activated during depolarization [47]. Calcium influx is necessary for release of neurotransmitter, and it has been postulated that the mechanism of calcium involvement may be by calcium-dependent phosphorylation [48]. The involvement of calcium in depolarization-induced activation of TH has been suggested by Salzman and Roth [49]. We hypothesize that calcium-dependent phosphorylation may play a role in activation of TH *in vivo*. If this were the case, the same stimulus which causes the release of neurotransmitter from the dopaminergic terminal would also increase the synthesis of dopamine. Since newly synthesized neurotransmitter is preferentially released from dopaminergic [50], as well as noradrenergic [51], terminals, activation of TH by phosphorylation induced by nerve firing may do more than merely replenish lost stores of catecholamine. Indeed, sustained release requires synthesis [51], and the coupling of synthesis and release through a common calcium-dependent pathway may enable the dopaminergic cell to maintain its function in the face of a variety of physiological conditions.

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