0026-895X/86/060561-09$02.00/0

MOLECULAR PHARMACOLOGY, 29:561-569

Dopamine Autoreceptor Regulation of the Kinetic State of Striatal Tyrosine Hydroxylase

KEVIN A. STRAIT and RONALD KUCZENSKI

Department of Pharmacology, Vanderbilt University, and Tennessee Neuropsychiatric Institute, Nashville, Tennessee 37217

Received January 2, 1986; Accepted March 13, 1986

SUMMARY

Tyrosine hydroxylase isolated from striatal synaptosomes exhibits biphasic Lineweaver-Burk kinetics for its tetrahydrobiopterin cofactor, consistent with multiple K_m forms of the enzyme. Incubation of striatal synaptosomes with forskolin (EC_{50} 0.45 μM) or dibutyryl cyclic AMP (EC_{50} 1.2 mM), results in activation of tyrosine hydroxylase, isolated from these synaptosomes via conversion of the enzyme to a single low K_m form (K_m 40 μM). The activation of synaptosomal tyrosine hydroxylase by forskolin or dibutyryl cyclic AMP is not additive and is similar to activation seen with cyclic AMP-dependent protein kinase phosphorylation of purified tyrosine hydroxylase. The addition of dopamine (IC_{50} 1.0 μM) (with nomifensine and pargyline) or apomorphine (IC_{50} 30 nM) to the synaptosomal incubation medium blocks the activation of tyrosine hydroxylase by forskolin. This effect of dopamine and apomorphine can in turn be blocked by preincubation of the synaptosomes with the dopamine receptor antagonist haloperidol (IC_{50} 30 nM and 4.5 nM, respectively) or chlorpromazine (IC_{50} 50 nM versus apomorphine). In contrast to the forskolin data above, dopamine failed to block the activation of tyrosine hydroxylase by dibutyryl cyclic AMP. Addition of dopamine to the tyrosine hydroxylase assay, in amounts equivalent to that carried over from the synaptosomal incubation with the tyrosine hydroxylase, had no effect on forskolin-activated enzyme. The observations that dopamine and apomorphine can block forskolin activation of tyrosine hydroxylase, that this blockade can in turn be prevented by preincubation with haloperidol or chlorpromazine, and that the amount of dopamine required for blockade of forskolin activation in synaptosomes has no effect on tyrosine hydroxylase when added to the enzyme assay constitute the first clear evidence of a presynaptic dopamine receptor (autoreceptor). This autoreceptor regulates the activity of tyrosine hydroxylase by preventing or reversing cyclic AMP-dependent activation of the enzyme, probably through a decrease in the phosphorylation state of tyrosine hydroxylase. Failure of dopamine to block dibutyryl cyclic AMP activation of tyrosine hydroxylase suggests that, if forskolin and dibutyryl cyclic AMP activate tyrosine hydroxylase through identical changes in phosphorylation state, then autoreceptor regulation of tyrosine hydroxylase must occur through a decrease in cyclic AMP levels.

TH (EC 1.14.16.1) is the rate-limiting enzyme in the synthesis of the catecholamines (1) and is involved in the regulation of catecholamine biosynthesis in a variety of tissues. The enzyme is subject to end-product feedback inhibition, and it has also become apparent that TH is susceptible to reversible modification of its kinetic properties. *In vivo* manipulations of nigrostriatal DA neuronal activity result in changes in the kinetic properties of striatal TH when assayed *in vitro* (2). The stability of such changes, through tissue preparation and enzyme isolation, suggests that changes in enzyme activity *in situ* may proceed through covalent modification. As an outgrowth of this observation, most recent efforts to elucidate potential *in vivo* regulatory effectors of the enzyme have focused on phosphorylation. A number of investigators have provided evidence that striatal TH is susceptible to activation under conditions favoring phosphorylation by a cyclic AMP-dependent protein kinase (3, 4). Direct incorporation of ^{32}P by cyclic AMP-dependent protein kinase into purified striatal TH has been noted (5, 6), and cyclic AMP-dependent phosphorylation alters the activity of TH by decreasing the K_m of the enzyme for its cofactor (7, 8).

Regulation of TH by cyclic AMP-dependent phosphorylation may also be operative *in situ* in striatal DA nerve terminals, since the enzyme in tissue slices and synaptosomes is activated by forskolin (8) (an activator of adenylate cyclase) and by db cAMP (9). Unlike TH in other tissues, the activity and phosphorylation state of striatal TH may be under the control of a presynaptic DA receptor (autoreceptor) regulating striatal DA synthesis. Carlsson *et al.* (10) provided the first evidence that DA synthesis in striatal nerve endings was decreased by an

This research has been supported by United States Public Health Service Grant DA-02676 and an agreement with the Tennessee Department of Mental Health and Retardation. K. A. S. is supported by National Institutes of Health Training Grant GM-07628.

ABBREVIATIONS: APO, apomorphine; BH_4, tetrahydrobiopterin; DA, dopamine; db cAMP, dibutyryl cyclic AMP; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HAL, haloperidol; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; KRB, modified Krebs-Ringer buffer; POPOP, *p*-bis[2-(5-phenyloxazolyl)]-benzene; TH, tyrosine hydroxylase; TMA, tetramethylammonium.

apparent DA autoreceptor. However, since TH activity was assayed *in situ,* via 3,4-dihydroxyphenylalanine accumulation after inhibition of 3,4-dihydroxyphenylalanine decarboxylase, it was impossible to determine whether the autoreceptor effect occurred directly on TH or indirectly through blockade of DA release causing increased foodback inhibition of TH activity. Since Carlsson's early work (10), other investigators have attempted to use DA synthesis, both *in vivo* and *in vitro* in tissue slices and synaptosomes, as a tool to characterize the DA autoreceptor (11–15). These studies have enjoyed limited success for several reasons. First, all measured the activity of TH *in situ* as reflected in rates of DA synthesis. As discussed previously, TH in nerve terminals is under the regulatory control of a variety of processes, some of which are direct (phosphorylation), and some indirect (blockade of DA release and consequent feedback inhibition). Second, the ability of DA agonists to cross cell membranes and inhibit TH activity directly and independent of a receptor confounds all *in situ* measures of DA synthesis. Finally, direct autoreceptor inhibition of TH would presumably work through inhibition or reversal of mechanisms which activate TH. Previous studies failed to employ activated TH.

The confounding variables encountered in previous studies on autoreceptors have been eliminated in the following series of experiments by using synaptosomal TH activated by cyclic AMP-dependent mechanisms. These activated synaptosomes were then exposed to DA agonists and antagonists. Following these exposures, TH was isolated from these synaptosomes allowing for the kinetic characterization of the enzyme. Isolation of TH eliminates any feedback or direct effects these DA agonists may have on TH activity. The data presented below provide the first conclusive evidence that a DA autoreceptor regulates the kinetic state of TH in striatal DA synaptosomes.

Materials and Methods

Materials used in this study were obtained from the following sources: DA hydrochloride, APO hydrochloride, db cAMP, HEPES, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, catalase, butyric acid, and Dowex 50WX 4-100, Sigma Chemical Co. (St. Louis, MO); platinum (IV) oxide, Aldrich; biopterin, Regis Chemical Co. (Morton Grove, IL); DTT and forskolin, Calbiochem-Behring Corp. (La Jolla, CA); L-[3,5-^{3}H]tyrosine, Research Products International. The following compounds were gifts: nomifensine, Hoechst-Roussel Pharmaceuticals; pargyline, Abbott Laboratories; HAL, McNeil Laboratories. All other reagents used were of the highest purity commercially available. BH_4 was prepared fresh in 10 mM HCl in the presence of 10 mg of PtO_2. The final concentration of BH_4 was determined spectrophotometrically (16).

Preparation of synaptosomes. Crude synaptosomes were prepared by a modification of the method of Gray and Whittaker (17). Male Sprague-Dawley rats (200–250 g) (Harlan Sprague-Dawley, Indianapolis, IN) were sacrificed by decapitation and the brains were removed and immediately placed in ice-cold saline followed by dissection on ice to remove bilateral striata. Striata were homogenized (100 mg/ml) in 0.32 M sucrose containing 50 μM EGTA, using 10 up-and-down strokes at 900 rpm in a glass-Teflon homogenizer (0.25-mm clearance). The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant was recentrifuged at 37,000 × *g* for 20 min to obtain the crude mitochondria pellet. The pellet was resuspended in 0.5 ml of 0.32 M sucrose containing 50 μM EGTA followed by a slow dilution (over 15 min) with 5 volumes of KRB containing 132 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 12 mM glucose, and 10 mM HEPES (pH 7.4). This suspension was then recentrifuged at 13,000 × *g* for 5 min and the resulting pellet was resuspended in KRB. The crude synaptosomal suspension was used in all subsequent studies.

Synaptosomal incubation. Synaptosomes were incubated at 37° for a total of 15 min, consisting of an initial 5-min preincubation, followed by addition of drugs and an additional 10-min incubation. All drugs were prepared in KRB except DA, APO, and HAL which were prepared in 10 mM HCl and diluted 1000-fold into KRB immediately before addition to the synaptosomal incubation. At the end of the 15-min incubation period, the incubation was stopped by addition of 5 volumes of cold KRB. The diluted synaptosomes were immediately centrifuged at 37,000 × *g* for 20 min and the supernatant was discarded. The pellet was resuspended in 0.15 M TMA chloride containing 10 mM TMA fluoride to inhibit phosphatase activity and 6 mM DTT to maintain TH activity. TMA chloride was used to produce an isotonic environment in which TH activity would not be affected by cations such as Na^+ and K^+ (18). Synaptosomes were lysed by sonication at 0° using a Bronson model 200 sonifier, equipped with a microtip. The lysed synaptosomes were centrifuged at 37,000 × *g* for 20 min. The supernatant was removed and used as the source of TH for subsequent assays of activity. Sonication under these conditions resulted in the solubilization of 85–90% of TH and 95% of cytoplasmic lactate dehydrogenase. Any changes in TH activity observed following manipulations of synaptosomes were maintained for at least 2 hr at 0° after synaptosomes were lysed.

Measurement of TH activity. TH activity was assayed using the ^{3}H-release method of Nagatsu *et al.* (19). L-[3,5-^{3}H]Tyrosine was diluted with unlabeled tyrosine to a specific activity of 1 mCi/mmol and purified by elution from Dowex 50WX 4-100 followed by lyophilization to dryness. The dried tyrosine was dissolved to a final concentration of 200 μM in 50% ETOH and stored in 1-ml aliquots at −70°. Immediately before use, the [^{3}H]tyrosine was blown to dryness under a stream of nitrogen and resuspended in water. The typical TH assay contained 0.14 M Tris maleate (pH 7.0), 2000 units of catalase, 0.1 mM ferrous sulfate, 35–700 μM BH_4, 6 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μg/ml of leupeptin. The incubation mixture was brought to 37° and 50 μl of enzyme was added. After a 2-min preincubation, L-[3,5^{-3}H]tyrosine (2 μM final) was added to initiate the reaction in a total assay volume of 350 μl. Identical incubations in the presence of 10^{-4} M 3-iodotyrosine served as the blank. The assay was stopped by addition of 100 μl of glacial acetic acid, and the ^3H_2O was separated from substrate on Dowex 50WX 4-100 columns. Radioactivity was determined in ACS (Amersham) enriched with 75 mg/liter of POPOP, on a Searle Analytic Mark III scintillation counter. In the absence of protease inhibitors, the assay was linear for only 4–8 min. When 2-mercaptoethanol was used in place of DTT, assays of TH at high BH_4 concentrations were linear for only 4–6 min. TH must be preincubated in the presence of BH_4, ferrous sulfate, and DTT for 2 min to avoid the appearance of a 45–60-sec lag in TH activity. Thus, in all experiments described, assays of both control and activated TH were linear for at least 20 min but an 8-min incubation was routinely used. Proteins were routinely determined by the method of Bradford (20) using gamma globulin as a standard. In experiments in which Lineweaver-Burk plots of TH activity versus BH_4 concentration were linear, K_m and V_{max} values were determined by a weighted linear regression analysis. Where appropriate, values were compared using paired or unpaired *t* tests.

Results

Activation of synaptosomal TH. Since it is logical to assume that an autoreceptor-mediated decrease in TH activity would be the result of inhibition or reversal of mechanisms which activate TH, synaptosomes were incubated with agents (forskolin, db cAMP) known to increase the activity of cAMP-dependent kinase to promote cAMP-dependent activation of TH. Representative Lineweaver-Burk plots of the activity of TH isolated from synaptosomes incubated in the presence of

forskolin are shown in Fig. 1. TH isolated from control (non-forskolin) synaptosomes exhibits nonlinear kinetics as a function of BH_4 concentration. This nonlinearity may reflect the presence of multiple K_m forms of TH for BH_4 (21, 22). Incubation of synaptosomes with increasing concentrations of forskolin altered the activity of subsequently isolated TH, with a threshold concentration near 15 nM and maximal activation at 15 μM. The change in activity appears to be the result of a shift of TH from a state exhibiting multiple K_m values for BH_4 to a state exhibiting a single low (K_m = 40 μM) K_m for BH_4. Nonlinearity of the control and submaximal forskolin-activated enzyme prevented accurate determinations of V_{max} except at a maximally effective forskolin concentration (15 μM forskolin, V_{max} = 22 pmol/mg of protein/min). The double reciprocal plots of enzyme maximally activated by forskolin pretreatment were linear to at least 17 μM BH_4 (data not shown). No change in the K_m for tyrosine (45 μM) was observed as a consequence of forskolin activation.

The concentration response curve for forskolin activation of synaptosomal TH is presented in Fig. 2. Synaptosomes were incubated with forskolin (15 nM–45 μM) for 10 min as described in Materials and Methods. It is apparent from Fig. 1 that the primary effect that forskolin preincubation has on TH occurs through a change in K_m for BH_4. To facilitate determinations of the forskolin concentration response curves, TH isolated from these synaptosomes was assayed for activity at a low BH_4 concentration (35 μM) since TH activity at low concentrations of BH_4 would be most reflective of a change in K_m for BH_4. Activity is expressed as percentage of activation of the control (non-forskolin) enzyme. Incubations of synaptosomes with forskolin resulted in a maximal TH activation 93% above control values, at 3 μM forskolin with an EC_{50} of 0.45 μM.

Incubation of synaptosomes in the presence of db cAMP also resulted in activation of TH subsequently isolated from these synaptosomes (Fig. 3). Representative Lineweaver-Burk plots of TH isolated from synaptosomes incubated in the absence of db cAMP or in the presence of submaximal db cAMP concentrations exhibit nonlinearity as a function of BH_4 concentration. Incubation of synaptosomes with db cAMP alters the activity of isolated TH with a threshold concentration near 10 μM and a maximal activation at 4 mM. The change in activity with db cAMP is the result of a shift in TH similar to that seen with forskolin, from an apparent multiple K_m population of enzyme to a single low K_m (K_m = 37 μM) form with a V_{max} (at 4 mM db cAMP) of 21 pmol/mg of protein/min. No change in K_m for tyrosine was observed. The concentration response curve for db cAMP activation of synaptosomal TH is presented in Fig. 4. TH from synaptosomes incubated with db cAMP was assayed at 35 μM BH_4. Maximal activation of TH with db cAMP results in an increase in activity of the enzyme 94% above control levels at 4 mM db cAMP, with an EC_{50} of 1.2 mM. Incubation of synaptosomes with maximally effective concentrations of forskolin (3 μM) and db cAMP (4 mM) resulted in activation of TH only to the same degree seen with either agent alone (data not shown). In all experiments in which TH was maximally activated by forskolin and/or db cAMP the range of V_{max} values obtained was 19–22 pmol/min/mg of protein, and the range of K_m values was 37–42 μM. Incubation of synaptosomes with 4.0 mM butyric acid had no effect on subsequently isolated TH activity (data not shown).

Inhibition of forskolin activation of TH activity. If a DA autoreceptor inhibits cAMP-dependent activation of TH, exposure of synaptosomes to DA receptor agonists should inhibit forskolin activation of the enzyme. The effects of DA and APO, as a function of concentration, on activation of TH in synaptosomes by 1.5 μM forskolin are presented in Fig. 5. The effects of DA on forskolin activation of synaptosomal TH were determined in the presence of 1 μM nomifensine to prevent the active uptake of DA into the synaptosome, and 10 μM pargyline to inhibit monoamine oxidase. DA in the concentration range 10 nM to 100 μM inhibited the ability of forskolin to activate TH. The IC_{50} for DA inhibition was 1 μM. Maximum inhibition of 84% of the forskolin activation was observed at DA concentrations of 10 μM and above. APO also inhibited the ability of forskolin to activate TH with an IC_{50} of 30 nM. Inhibition exhibited a plateau at 68% inhibition at APO concentrations near 0.1 μM. At higher concentrations of APO, a second component of inhibition was obtained which may reflect carryover of APO into the TH assay, since the level of TH activity at these APO concentrations was considerably less than that of control (non-forskolin) enzyme.

HAL blockade of DA and APO inhibition of forskolin activation. If the effects of DA and APO on TH activity are indeed mediated by a DA autoreceptor, the effects should be blocked by DA receptor antagonists. Synaptosomes were preincubated with HAL (0.1 nM–5μM) for 5 min at 37°, followed by

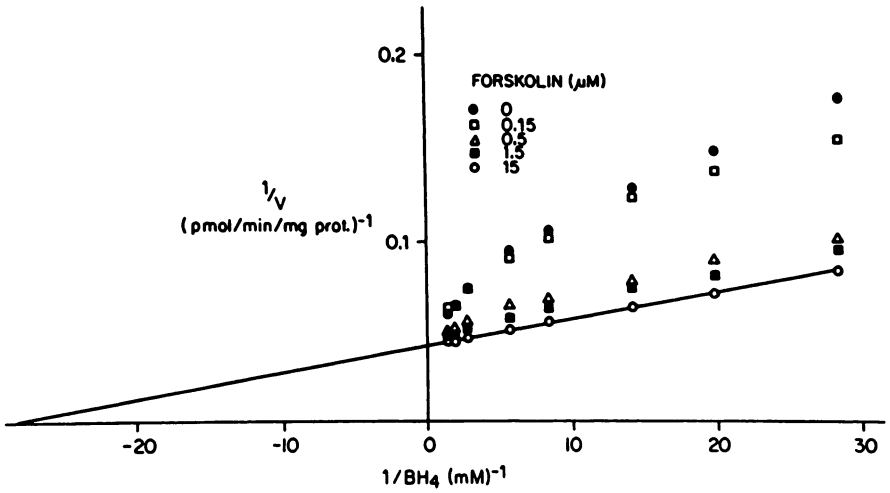

Fig. 1. Activity of TH isolated from striatal synaptosomes previously incubated with increasing concentrations of forskolin. Striatal synaptosomes were preincubated for 5 min at 37° followed immediately by a 10-min incubation in the absence (control) or presence of forskolin at the indicated concentrations. The incubation was stopped by addition of ice-cold KRB and centrifuged at 37,000 × *g* for 20 min to pellet synaptosomes. The pellet was resuspended, lysed by sonication, and recentrifuged. The resultant supernatant, containing isolated TH, was assayed at varying concentrations of BH_4 (35–700 μM) as described in Materials and Methods. Activity is expressed as pmol/mg of protein/min. Each *point* represents the mean of duplicate assays. This figure is a representative Lineweaver-Burk plot of four independent experiments which were essentially identical.

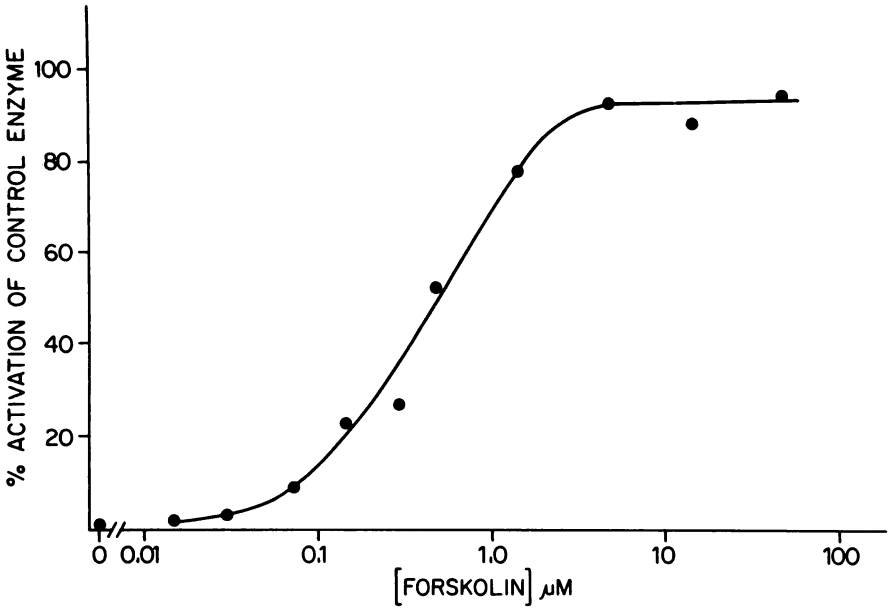

Fig. 2. Concentration response curve for activation of synaptosomal TH by forskolin. TH was isolated from synaptosomes previously incubated with varying concentrations of forskolin (15 nM–45 μM) (see Materials and Methods and legend to Fig. 1 for additional details). Isolated TH was assayed at a single BH_4 concentration (35 μM). Each *point* represents the mean of eight assays. Activity is expressed as percentage of activation above TH isolated from control synaptosomes incubated in the absence of forskolin. The data were accumulated from separate tissue preparations and forskolin incubations over a 2-day period.

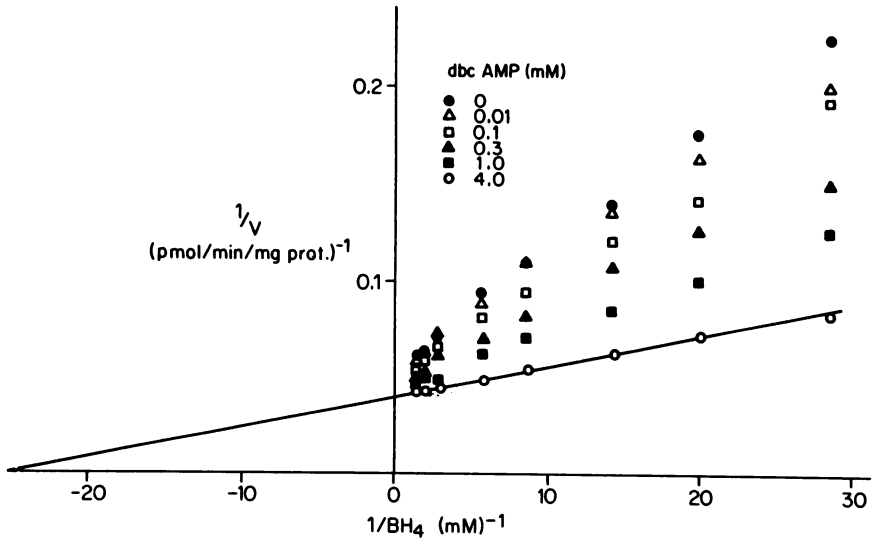

Fig. 3. Activity of TH from striatal synaptosomes previously incubated with increasing concentrations of db cAMP. Striatal synaptosomes were preincubated for 5 min at 37° followed immediately by a 10-min incubation in the absence (control) or presence of db cAMP at the indicated concentrations. The incubation was stopped by addition of ice-cold KRB and centrifuged at 37,000 × *g* for 20 min to pellet the synaptosomes. The pellet was resuspended, lysed by sonication, and recentrifuged. The resultant supernatant containing isolated TH was assayed at varying concentrations of BH_4 (35–700 μM) as described in Materials and Methods. Activity is expressed as pmol/mg of protein/min. Each *point* represents the mean of duplicate assays. This figure is a representative Lineweaver-Burk plot of three independent experiments which were essentially identical.

addition of 1.5 μM forskolin in the presence of DA (10 μM) or APO (0.25 μM). Fig. 6 presents the concentration response curves for HAL blockade of DA and APO inhibition. HAL exhibited IC_{50} values of 30 nM and 4.5 nM, respectively, for DA and APO blockade. Chlorpromazine was similarly shown to block the inhibition of forskolin activation by APO (IC_{50} = 50 nM) (data not shown).

Representative Lineweaver-Burk plots of TH isolated from synaptosomes incubated with forskolin, forskolin and DA, or forskolin, DA, and haloperidol are presented in Fig. 7. As in Figs. 1 and 3, control TH exhibits nonlinear BH_4 Lineweaver-Burk plots indicative of multiple K_m values. Addition of 3 μM forskolin shifts the curve to a single K_m form (K_m = 40 μM; V_{max} = 22 pmol/mg of protein/min). Incubation of synaptosomes with forskolin in the presence of DA (1 μM or 10 μM) results in inhibition of activation and a reinstatement of the nonlinear Lineweaver-Burk plot. Preincubation of synaptosomes with HAL prior to addition of forskolin and DA results in a blockade of the DA effect, resulting in a single low K_m form of the enzyme (K_m = 38 μM), similar to that seen with forslilin alone. DA (10 μM) or haloperidol (0.3 μM) when added to synaptosomes in the absence of forskolin had no effect on any portion of the nonlinear Lineweaver-Burk plot for isolated TH activity from these synaptosomes (Fig. 8). Likewise, preincubation of synaptosomes in the absence of forskolin, at APO concentrations up to 0.8 μM, failed to affect TH activity isolated from these synaptosomes.

Lack of inhibition of forskolin-stimulated TH by addition of DA directly to the enzyme assay. To determine whether the inhibition by DA of forskolin activation of TH reflected a direct effect of residual DA carried into the TH assay, levels of DA in the lysed synaptosomal enzyme preparation were determined by high pressure liquid chromatography following preincubation of synaptosomes with 10 μM DA, a maximally effective concentration for inhibiting forskolin activation. High pressure liquid chromatographic analysis indicated that 0.8 μM DA (final) was carried over from the synaptosomal incubation into the TH assay. Therefore, TH, activated by forskolin in the absence of DA, was assayed in the presence of 0.8 μM added DA, and the results are presented in Fig. 9

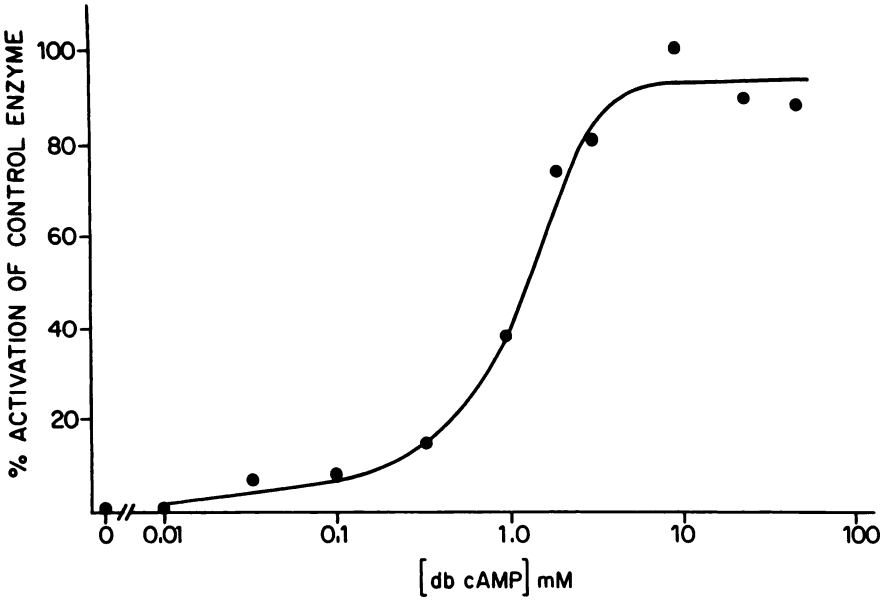

Fig. 4. Concentration response curve for activation of synaptosomal TH by db cAMP. TH was isolated from synaptosomes previously incubated with varying concentrations of db cAMP (10 μM–50 mM) (see Materials and Methods and legend to Fig. 1 for additional details). Isolated TH was assayed at a single BH_4 concentration (35 μM). Each *point* represents the mean of eight assays. Activity is expressed as percentage of activation above TH isolated from control synaptosomes incubated in the absence of db cAMP. The data were accumulated from separate tissue preparations and db cAMP incubations over a 2-day period.

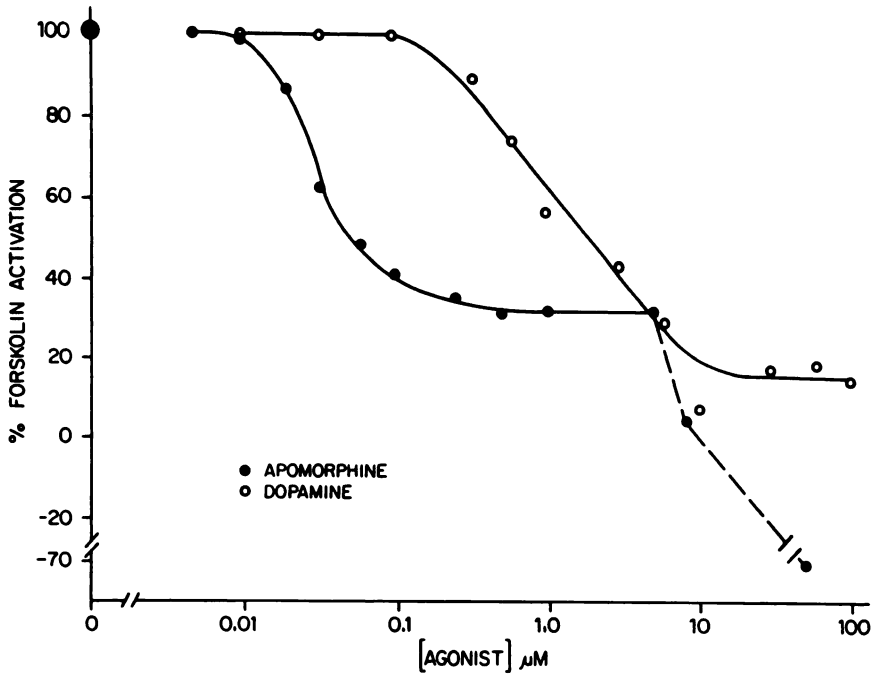

Fig. 5. Concentration response curves for inhibition by DA or APO of forskolin activation of synaptosomal TH. TH was isolated from synaptosomes incubated with 1.5 μM forskolin in the presence of DA (10 nM–100 μM) or APO (5 nM–50 μM). Synaptosomal incubations with DA were done in the presence of 1 μM nomifensine to prevent DA uptake and 10 μM pargyline to inhibit monoamine oxidase. TH was assayed at a single BH_4 concentration (35 μM). Each *point* represents the mean of eight assays. Activity is expressed as percentage of activation of TH isolated from synaptosomes incubated with 1.5 μM forskolin in the absence of DA or APO. Data were accumulated from separate tissue preparations and synaptosomal incubations over a 3-day period for each receptor agonist.

(*top*). In the absence of added DA, the K_m of TH for BH_4 was 41.3 ±1.1 μM, which increased slightly, but significantly (paired *t* test, $p < 0.02$, $N = 3$), to 46.3 ± 1.1 μM in the presence of 0.8 μM DA.

Lack of an inhibitory effect by DA of cAMP activation of synaptosomal TH. To determine whether db cAMP activation of TH in synaptosomes, like forskolin activation of TH in synaptosomes, could be inhibited by DA through the same autoreceptor-mediated mechanism, synaptosomes were incubated with db cAMP in the presence and absence of 10 μM DA, a maximally effective concentration of DA to inhibit forskolin activation of TH. The results are presented in Fig. 9 (*bottom*). TH was isolated and assayed for activity as previously described. TH activity from db cAMP synaptosomes had a K_m = 39.1 ± 0.8 μM whereas TH isolated from synaptosomes incubated with db cAMP in the presence of DA had a K_m = 45.3 ± 1.1 μM ($p < 0.04$, $N = 3$). Similarly, activation of synaptosomal TH by submaximally effective doses of db cAMP also showed no effect of DA agonist (data not shown).

Discussion

A number of investigators have suggested that a presynaptic DA receptor (autoreceptor) exists in striatum to regulate DA synthesis in DA nerve terminals. However, these studies utilized *in situ* DA synthesis as an index of autoreceptor function. Thus, influences from other neuronal projections, changes in intraneuronal DA pools, altered release of DA, and direct effects of DA agonists on TH itself could not be eliminated. The presence of these non-receptor-mediated influences on TH activity resulted in sufficient ambiguity to lead others to question the existence of autoreceptors on DA nerve terminals (see,

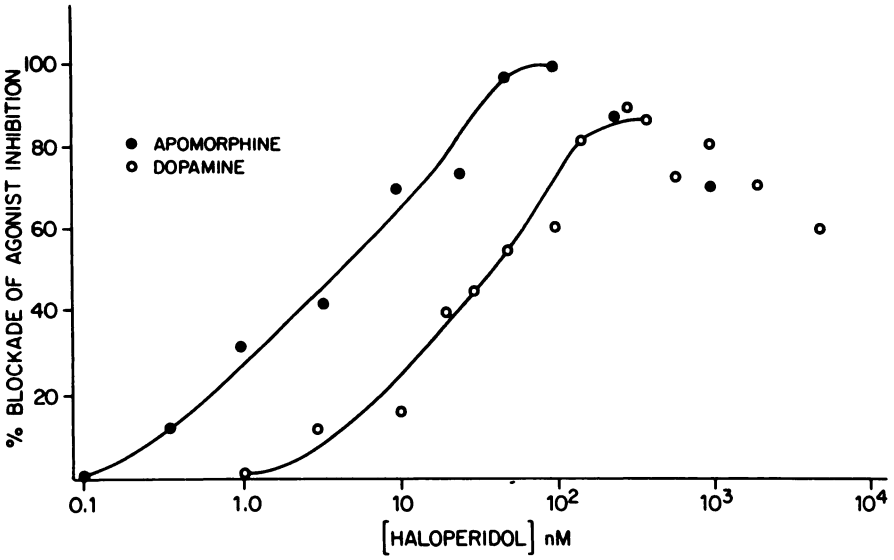

Fig. 6. Concentration response curves for HAL blockade of DA or APO inhibition of forskolin activation of synaptosomal TH. Synaptosomes were preincubated with HAL (0.1 nM–50 μM) 5 min at 37° followed by addition of 1.5 μM forskolin in the presence of DA (10 μM) or APO (0.25 μM) for an additional 10 min. Synaptosomal incubations with DA were done in the presence of 1 μM nomifensine to prevent uptake and 10 μM pargyline to inhibit monoamine oxidase. TH was isolated as described in Fig. 1 and assayed at 35 μM BH_4. Each *point* represents the mean of eight assays. Activity was expressed as percentage of blockade of DA or APO inhibition of forskolin activation of TH. The data were accumulated from separate tissue preparations and synaptosomal incubations over a 3-day period.

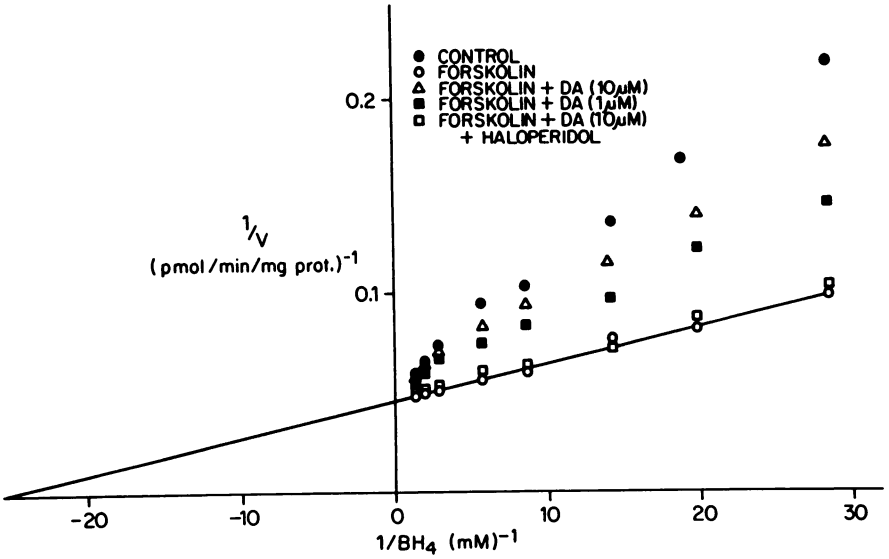

Fig. 7. Lineweaver-Burk plots of TH isolated from synaptosomes incubated with forskolin, forskolin plus DA, or forskolin plus DA and HAL. Synaptosomes were incubated with 3 μM forskolin, 3 μM forskolin and 10 μM DA, 3 μM forskolin and 1 μM DA, or 3 μM forskolin, 10 μM DA, and 0.3 μM HAL or in the absence of effectors (control), as described in Fig. 1 legend and Materials and Methods. Following isolation from synaptosomes, TH was assayed at various BH_4 concentrations (35–700 μM). Each *point* represents the mean of duplicate assays. This figure is a representative Lineweaver-Burk plot of three independent experiments which were essentially identical.

for example, Ref. 23). The results of the present studies provide the first conclusive evidence for a DA autoreceptor regulating DA synthesis in striatal synaptosomes. The data further indicate that this autoreceptor functions by preventing or reversing cyclic AMP-dependent mechanisms for activation of TH.

Incubation of striatal synaptosomes with forskolin or db cAMP activates TH subsequently isolated from these synaptosomes (Figs. 1–4). The activation of TH in both cases proceeds through a change in kinetic properties of the enzyme from a state of multiple K_m values for cofactor to a single low K_m state. Activation of synaptosomal TH by forskolin (Fig. 5), but not by db cAMP (Fig. 9, *bottom*), is prevented by addition of APO or DA with IC_{50} values of 30 nM and 1 μM, respectively. Prevention of TH activation by these DA agonists is accompanied by reversion of the kinetic properties of isolated TH to the multiple K_m state (Fig. 7). Preincubation of synaptosomes with the DA receptor antagonist HAL (Fig. 6) or chlorpromazine (data not shown) blocks the inhibition by DA agonists of forskolin activation of TH (Fig. 7).

A thorough review of the data presented in Figs. 7, 8, and 9 leads to the following conclusions. 1) The effects of DA agonists on TH activity are not the result of direct inhibition through carryover into the enzyme assay and, therefore, 2) the only plausible mechanism of the observed effects of DA agonists and antagonists on synaptosomal TH activity is through a presynaptic DA receptor (autoreceptor) able to regulate the kinetic state of TH. 3) DA autoreceptor-mediated regulation of the kinetic state of TH is probably the result of a decrease in cAMP-dependent phosphorylation via decreased cAMP levels.

The present studies were initiated with the hypothesis that autoreceptor regulation of DA synthesis through TH would occur by reversal or inhibition of mechanisms activating TH. In contrast, previous studies focused on potential autoreceptor effects on basal levels of DA synthesis in the absence of any activating influence. These studies yielded complete inhibition of synaptosomal DA synthesis via putative autoreceptor mechanisms, a finding which is inconsistent with the model for DA

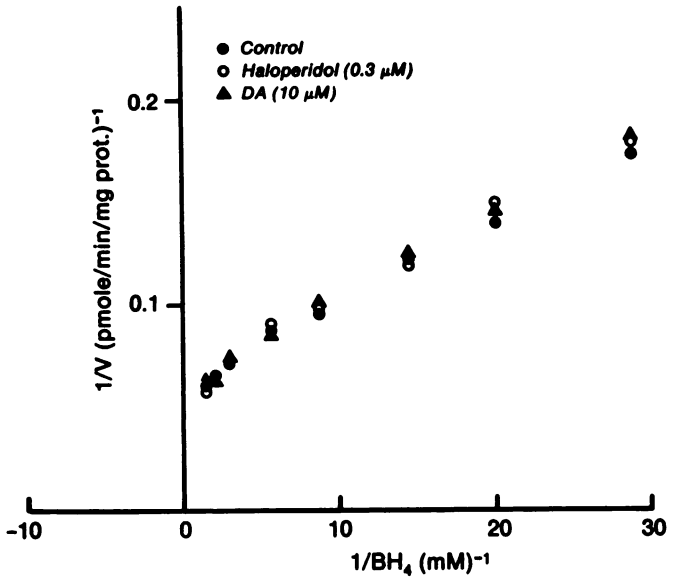

Fig. 8. Lineweaver-Burk plots of TH isolated from synaptosomes previously incubated in the presence or absence of DA or HAL under nonactivating conditions. Synaptosomes were incubated with 10 μM DA, 0.3 μM HAL, or in the absence of effectors (control), as described in the legend to Fig. 1 and in Materials and Methods. Following isolation from synaptosomes, TH was assayed at various BH_4 concentrations (35–700 μM). Each *point* represents the mean of duplicate assays. This figure is a representative Lineweaver-Burk plot of three independent experiments. In no case did preincubation with DA or HAL affect TH activity.

autoreceptor regulation of activated TH activity suggested by our data. Furthermore, the IC_{50} values for inhibition of *in situ* DA synthesis (1–10 μM) (11–14) by APO are 2 orders of magnitude greater than the IC_{50} for APO blockade of forskolin activation of TH (Fig. 5). Inhibition of DA synthesis by APO is similar to the secondary inhibition by APO of TH (Fig. 5) beyond basal levels of TH activity. We propose that both the inhibition of *in situ* DA synthesis and the secondary component of the APC effect depicted in Fig. 5 are the result of direct effects on TH by APO accumulated in the nerve ending. The primary evidence in studies of *in situ* DA synthesis for the effects of DA agonists occurring through an autoreceptor is that single concentrations of DA receptor antagonists shifted the concentration response curves for agonist inhibition of synthesis to the right. The use of a single concentration of antagonist leaves open to question whether the antagonist-induced shift is due to DA autoreceptor blockade or the result of nonspecific effects on the striatal nerve terminals. In synaptosomes, nonspecific effects of HAL became apparent at concentrations above 100 nM (Fig. 6) and become substantial at the μM concentrations of HAL employed to shift the agonist response curve in DA synthesis studies.

It is reasonable to assume that DA autoreceptor regulation of TH activity operates through inhibition or reversal of mechanisms which activate TH, not through inhibition or basal activity. Therefore, prior to characterization of the DA autoreceptor effects on TH activity, mechanisms for activating synaptosomal TH were examined. Purified preparations of striatal TH have been shown to be susceptible to cAMP-dependent phosphorylation (5, 6). Concomitantly, the enzyme is activated through a decrease in K_m for cofactor (6, 7). For these reasons, synaptosomes were incubated with forskolin or db cAMP, and isolated TH was then characterized with respect to its kinetic response to BH_4. Both forskolin and db cAMP incubations resulted in identical change in the kinetic properties of TH in terms of K_m for cofactor and maximal activation (Figs. 1–4). Furthermore, addition of both forskolin and db cAMP at maximally effective concentrations resulted in TH activation no greater than that observed in the presence of either agent alone, suggesting that both effects were working through identical mechanisms. Since the shift in K_m for cofactor of TH isolated from incubations of synaptosomes in the presence of forskolin or db cAMP is identical to the change in K_m of purified TH for cofactor following cAMP-dependent phosphorylation, it is reasonable to conclude that activation of TH

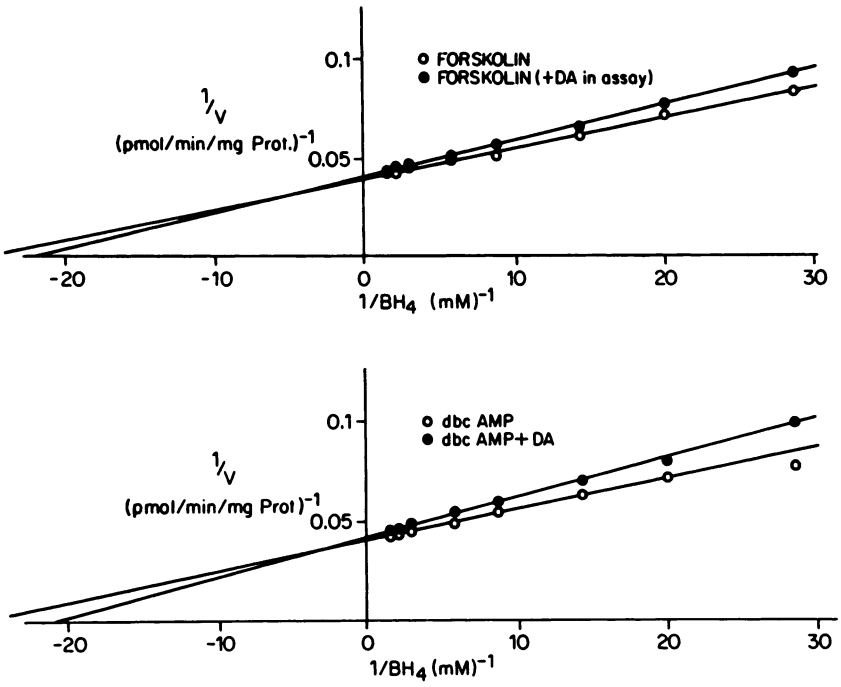

Fig. 9. Direct inhibition in the TH assay by DA of forskolin-activated enzyme (*top*) compared to synaptosomal TH activation by db cAMP in the presence of DA (*bottom*). *Top.* Synaptosomes were incubated with 3 μM forskolin as described in Fig. 1. TH isolated from these synaptosomes was assayed for activity in the absence or presence of 0.8 μM DA added directly into the assay mix. Activity was measured at various BH_4 concentrations (35–700 μM). Each *point* represents the mean of duplicate assays. *Bottom.* Synaptosomes were incubated with 4.0 mM db cAMP in the absence or presence of 10 μM DA. TH isolated from these synaptosomes was assayed for activity in the absence of added DA by varying the BH_4 concentration (35–700 μM). Each *point* represents the mean of duplicate assays. The data presented in both *top* and *bottom* are representative of three independent experiments which were essentially identical.

by forskolin or db cAMP proceeds through cAMP-dependent phosphorylation.

Activity of TH isolated from control synaptosomes exhibits nonlinear Lineweaver-Burk plots indicative of either negative cooperativity or multiple enzyme forms with different K_m values for cofactor. Negative cooperativity does not appear to be a characteristic of TH, since purified rat striatal TH exhibits simple Michaelis-Menten kinetics for BH_4 in both the nonphosphorylated and cAMP-dependent phosphorylation states (6). The nonlinearity in Lineweaver-Burk plots of TH for cofactor has been suggested to be the result of partial phosphorylation of TH in a number of other systems (21, 22). Thus, the nonlinear kinetics of TH from striatal synaptosomes would appear to reflect the presence of a small fraction of phosphorylated TH.

The ability of DA and APO to prevent or reverse forskolin activation of TH and reinstate nonlinear BH_4 kinetics (Fig. 7) presumably reflects inhibition of cAMP-dependent phosphorylation of the enzyme. The data of Vrana and Roskoski (22) are indirectly consistent with this suggestion. Those authors noted that TH in striatal extracts exhibited nonlinear Lineweaver-Burk plots for cofactor which become linear, with a single low K_m, after addition of cAMP and ATP. Further incubation after cessation of phosphorylating conditions resulted in a time-dependent loss of activation and reinstatement of nonlinearity. The readdition of cAMP and ATP again activated TH and reinstated linear Lineweaver-Burk kinetics. Thus, phosphorylation/dephosphorylation/rephosphorylation promotes changes in the kinetic properties of TH identical to those observed following synaptosomal autoreceptor manipulation.

Preincubation of synaptosomes with HAL blocked the ability of both DA and APO to inhibit forskolin stimulation of TH activity (Fig. 6). Theoretically, equieffective concentrations of DA or APO would be blocked to the same extent by HAL to yield identical HAL concentration response curves. However, we used high, not necessarily equieffective concentrations of DA and APO to maximally inhibit forskolin stimulation. Thus, differences in the relative effective concentrations of the DA agonists utilized results in the different IC_{50} values for the DA antagonists.

The ability of HAL and chlorpromazine, at nM concentrations, to prevent APO and DA inhibition of forskolin activation of TH provides strong evidence in favor of a receptor-mediated effect on TH. Equally compelling evidence for an autoreceptor is derived from the nature of the effect of DA and APO on TH activity. DA and APO promote nonlinear Lineweaver-Burk plots of TH activity versus BH_4 concentration, an effect which is inconsistent with competitive inhibition by DA or APO of TH. Addition of DA directly to the TH assay in amounts equal to the amount carried over (Fig. 9, *top*) had little effect on TH activity. Finally, DA was not able to inhibit db cAMP activation of TH (Fig. 9, *bottom*) which is inconsistent with a direct effect on TH.

The latter observation also provides some insight into the mechanism of DA autoreceptor function. Inhibition or reversal of cAMP-dependent activation of TH probably occurs through one of four mechanisms: inhibition of adenylate cyclase, activation of phosphodiesterase, inhibition of cAMP-dependent kinase, or activation of a phosphatase. The failure of DA to inhibit db cAMP activation of synaptosomal TH argues against the third and fourth alternatives and suggests that the DA autoreceptor functions to inhibit the activation of TH by modulating cAMP levels. However, in the absence of direct measures of cAMP levels, alternative explanations of autoreceptor functioning cannot be ruled out entirely.

In the absence of a reliable measure of DA autoreceptor function, pharmacological characterization of this DA receptor has been minimal. However, the little evidence available from the literature suggests that the autoreceptor may resemble the D_2 receptor (24). Based on studies of the prototypical D_2 receptor from rat pituitary (25), the D_2 receptor appears to be negatively coupled to adenylate cyclase (26, 27). Similar findings have recently been reported in striatum (28). Our data, indicating DA autoreceptor regulation of TH activity through changes in cAMP levels, although not conclusive, are consistent with the mechanism of a D_2 receptor. The fact that the relative potencies of APO and DA, as well as HAL and chlorpromazine, are similar to the D_2 receptor suggests that the autoreceptor regulating TH activity in striatal terminals could be a presynaptic D_2 receptor.

There is evidence to suggest DA autoreceptor regulation of DA release from striatal DA nerve terminals (29, 30). Several possibilities exist for a potential relationship between autoreceptor regulation of TH activity and DA release. A single autoreceptor may function to regulate both DA synthesis and DA release through a common mechanism (for example, cyclic AMP levels), or two distinct autoreceptors may function to regulate each phenomenon separately. Alternatively, autoreceptor regulation of DA release may occur as an indirect consequence of autoreceptor regulation of TH activity, through diminished availability of newly synthesized DA. Additional experimentation will be necessary to distinguish these possibilities.

Finally, autoreceptor regulation of the activation of TH in striatal DA nerve terminals suggests the possibility of similar regulatory mechanisms on other catecholaminergic nerve terminals. The existence of presynaptic α_2-receptors on noradrenergic nerve terminals which are negatively coupled to adenylate cyclase (31) would fit the proposed model for the regulation of striatal TH by diminished cyclic AMP levels. Verification of presynaptic autoreceptor regulation of TH activity in other catecholaminergic systems will require measures of TH kinetic properties and receptor characterization similar to those employed above.

References

1. Levitt, M., S. Spector, A. Sjoerdsma, and S. Udenfriend. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea pig heart. *J. Pharmacol. Exp. Ther.* **148**:1–8 (1965).
2. Zivkovic, B., A. Guidotti, and E. Costa. Effects of neuroleptics on striatal tyrosine hydroxylase: changes in affinity for the pteridine cofactor. *Mol. Pharmacol.* **10**:727–735 (1974).
3. Harris, J. E., R. J. Baldessarini, V. H. Morgenroth, and R. H. Roth. Activation of cyclic 3′:5′-adenosine monophosphate of tyrosine hydroxylase in the rat brain. *Proc. Natl. Acad. Sci. USA* **72**:789–793 (1975).
4. Lovenberg, W., E. A. Bruckwick, and I. Hanbauer. ATP, cyclic AMP, and magnesium increase the affinity of rat striatal tyrosine hydroxylase for its cofactor. *Proc. Natl. Acad. Sci. USA* **72**:2955–2958 (1975).
5. Edelman, A. M., J. D. Raese, M. A. Lazar, and J. D. Barchas. Tyrosine hydroxylase from brain: phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. *J. Pharmacol. Exp. Ther.* **216**:647–653 (1981).
6. Richtand, N. M., T. Inagami, K. Misono, and R. Kuczenski. Purification and characterization of rat striatal tyrosine hydroxylase: comparison of the activation by cyclic AMP-dependent phosphorylation and other effectors. *J. Biol. Chem.* **260**:8465–8473 (1985).
7. Lazar, M. A., A. J. Lockfeld, R. J. W. Truscott, and J. D. Barchas. Tyrosine hydroxylase from bovine striatum: catalytic properties of the phosphorylated

and non-phosphorylated forms of the purified enzyme. *J. Neurochem.* **39**:409–422 (1982).
8. El Mestikawy, S., J. Glowinski, and M. Hamon. Tyrosine hydroxylase activation in depolarized dopaminergic terminals—involvement of Ca^{2+}-dependent phosphorylation. *Nature* (*Lond.*) **302**:830–832 (1983).
9. Goldstein, M., R. L. Bronaugh, B. Ebstein, and C. Roberge. Stimulation of tyrosine hydroxylase activity by cyclic AMP in synaptosomes and in soluble striatal enzyme preparations. *Brain Res.* **109**:563–574 (1976).
10. Carlsson, A., W. Kehr, M. Lindquist, T. Magnusson, and C. V. Atack. Regulation of monoamine metabolism in the central nervous system. *Pharmacol. Rev.* **24**:371–384 (1972).
11. Haubrich, D. R., and A. B. Pflueger. The autoreceptor control of dopamine synthesis. An *in vitro* and *in vivo* comparison of dopamine agonists. *Mol. Pharmacol.* **21**:114–120 (1982).
12. McMillin, B. A. Striatal synaptosomal tyrosine hydroxylase activity. A model system for study of presynaptic dopamine receptors. *Biochem. Pharmacol.* **31**:2643–2647 (1982).
13. Bitran, M., and G. Bustos. On the mechanism of presynaptic autoreceptor-mediated inhibition of transmitter synthesis in dopaminergic nerve terminals. *Biochem. Pharmacol.* **31**:2851–2860 (1982).
14. Westfall, T. C., L. Naes, and C. Paul. Relative potency of dopamine agonists on autoreceptor function in various brain regions of the rat. *J. Pharmacol. Exp. Ther.* **224**:199–205 (1983).
15. Roth, R. H. Dopamine autoreceptors: pharmacology function and comparison with postsynaptic dopamine receptors. *Commun. Psychopharmacol.* **3**:429–445 (1979).
16. Bailey, S. W., and J. E. Ayling. Separation and properties of the 6-diastereoisomers of L-erythro-tetrahydrobioptrin and their reactivities with phenylalanine hydroxylase. *J. Biol. Chem.* **253**:1598–1605 (1978).
17. Gray, G. G., and V. P. Whittaker. The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* **96**:78–88 (1962).
18. Kuczenski, R. Monovalent cations and striatal tyrosine hydroxylase. *J. Neurochem.* **37**:681–686 (1981).
19. Nagatsu, T., M. Levitt, and S. Udenfriend. A rapid and simple radioassay for tyrosine hydroxylase activity. *Anal. Biochem.* **9**:122–126 (1964).
20. Bradford, M. A. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
21. Andrews, D. W., T. A. Langan, and N. Weiner. Evidence for the involvement of a cyclic AMP-dependent protein kinase in the activation of soluble tyrosine hydroxylase from rat striatum. *Proc. Natl. Acad. Sci. USA* **80**:2097–2101 (1983).
22. Vrana, K. E., and R. Roskoski. Tyrosine hydroxylase inactivation following cAMP-dependent phosphorylation activation. *J. Neurochem.* **40**:1692–1700 (1983).
23. Laduron, P. M. Lack of direct evidence for adrenergic and dopaminergic autoreceptors. *Trends Pharmacol. Sci.* **5**:459–461 (1984).
24. Leff, S. E., and I. Creese. Dopamine receptors re-explained. *Trends Pharmacol. Sci.* **4**:463–467 (1983).
25. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).
26. Enjalbert, A., and J. Bockaert. Pharmacological characterization of the D-2 dopamine receptor negatively coupled with adenylate cyclase in rat anterior pituitary. *Mol. Pharmacol.* **23**:576–584 (1983).
27. Munemura, M., T. E. Cote, K. Tsuruta, R. L. Eskay, and J. W. Kebabian. The dopamine receptor in the intermediate lobe of the rat pituitary gland: pharmacological characterization. *Endocrinology* **107**:1676–1683 (1980).
28. Kamal, L. A., S. Arbilla, and S. Z. Langer. Presynaptic modulation of the release of dopamine from the rabbit caudate nucleus: differences between electrical stimulation, amphetamine and tyramine. *J. Pharmacol. Exp. Ther.* **216**:592–598 (1981).
29. Onali, P., M. C. Olianas, and G. L. Gessa. Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum. *Mol. Pharmacol.* **28**:138–145 (1985).
30. Reimann, W., A. Zumstein, R. Jackisch, K. Starke, and G. Hertting. Effect of extracellular dopamine on the release of dopamine in the rabbit caudate nucleus: evidence for a dopaminergic feedback inhibition. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **306**:53–60 (1979).
31. Langer, S. Z. Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.* **32**:337–362 (1980).

Send reprint requests to: Dr. Ronald Kuczenski, Department of Psychiatry (M-003), University of California, San Diego, La Jolla, CA 92093.