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Presynaptic Regulation of Tyrosine Hydroxylase Activity in Rat Striatal Synaptosomes by Dopamine Analogs

W. G. WAGGONER, JOHN MCDERMED AND H. J. LEIGHTON

Departments of Pharmacology and Organic Chemistry, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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SUMMARY

WAGGONER, W. G., J. MCDERMED AND H. J. LEIGHTON. Presynaptic regulation of tyrosine hydroxylase activity in rat striatal synaptosomes by dopamine analogs. *Mol. Pharmacol.* **18:** 91-99 (1980).

Tyrosine hydroxylase activity in rat striatal synaptosomes was inhibited by four different dopamine agonists: dopamine (DA) (IC_{50} = 0.2 μM), apomorphine (APO) (IC_{50} = 0.1 μM), 6,7-dihydroxy-2-aminotetralin (ADTN) (IC_{50} = 0.2 μM), and 7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin (7HDPT) (IC_{50} = 1.3 μM). The inhibitory activities of DA and ADTN were antagonized by the addition of the dopamine uptake inhibitor benztropine but not by the addition of the dopamine receptor antagonist fluphenazine. The inhibitory activity of APO was marginally antagonized by the addition of either benztropine or fluphenazine. The inhibitory activity of 7HDPT was antagonized by fluphenazine and other neuroleptics but not by benztropine. In contrast to the other agonists, 7HDPT did not inhibit soluble tyrosine hydroxylase assayed in the presence of the synthetic cofactor, 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$). The inhibition of synaptosomal tyrosine hydroxylase by 7HDPT was also antagonized by $DMPH_4$, dibutyryl cAMP, and $FeSO_4$ in the presence of β-mercaptoethanol but not by ATP, GTP, cAMP, or dibutyryl cGMP or by high concentrations (11 mM) of calcium. Incubation of synaptosomes with concentrations of 7HDPT which inhibited synaptosomal tyrosine hydroxylation by greater than 50% did not cause a significant change in the V_{max} of tyrosine hydroxylase or the K_m of tyrosine hydroxylase for the synthetic cofactor $DMPH_4$. It is concluded that 7HDPT is a valuable agent for studying presynaptic mechanisms of tyrosine hydroxylase regulation in the rat striatum since its activity appears to be mediated primarily through presynaptic plasma membrane dopamine receptors. The mechanism by which presynaptic dopamine receptors may regulate tyrosine hydroxylase activity is discussed.

INTRODUCTION

The rate-limiting enzyme in the synthesis of dopamine in the rat striatum is tyrosine hydroxylase (EC 1.14.16.2; TH[1]) which requires tyrosine, molecular oxygen, ferrous ion, and a reduced pterin cofactor for activity. As originally proposed by Nagatsu *et al.* (1), end-product feedback regulation of TH by catecholamines is considered a major mechanism for control of synthesis of dopamine (2). Kinetic studies have shown that catecholamines inhibit adrenal TH by competing with the pterin cofactor for binding to the enzyme (3). More recent data have suggested additional regulatory mechanisms involving allosteric changes in TH mediated by activation of plasma membrane dopamine receptors (4). These allosteric changes in TH have been characterized primarily by changes in the K_m of TH for synthetic pterin cofactors (4), although changes in V_{max} (5), K_m for tyrosine (5), and K_i for dopamine (6) have been reported.

The localization of the dopamine receptors responsible for the allosteric changes in rat striatal TH is uncertain. It has been proposed that allosteric changes in TH are mediated through presynaptic (6) as well as postsynaptic dopamine receptors (4, 7). Indeed, dopamine receptors have been demonstrated to occur on striatal presynaptic dopaminergic nerve terminals, striatal postsynaptic nondopaminergic neurons, nondopaminergic afferents to striatum, nondopaminergic afferents to substantia nigra, and nigral dopaminergic cell bodies (for review, see Ref. 8). Any of these dopamine receptors may be involved in

[1] Abbreviations used: TH, tyrosine hydroxylase; cAMP, cyclic adenosine 3′:5′-monophosphate; db-cAMP, N^6,$O^{2'}$-dibutyryladenosine-3′,5′-cyclic phosphate; db-cGMP, N^6,$O^{2'}$-dibutyrylguanosine-3′,5′-cyclic phosphate; IBMX, 3-isobutyl-1-methyl xanthine; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterine; $6MPH_4$, DL-6-methyl-5,6,7,8-tetrahydropterine; DA, dopamine; APO, (−)-apomorphine; ADTN, (±)-6,7-dihydroxy-2-aminotetralin; 7HDPT, (±)-7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; dpm, disintegrations per minute.

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the regulatory functions of nigrostriatal dopaminergic neurons.

The biochemical link between dopaminergic receptor activation and TH activity is confusing. In the rat striatum, dopamine agonists increase cAMP production (9), cAMP and various analogs of cAMP increase TH activity (10, 11), but dopamine agonists decrease TH activity (12). Thus, the major biochemical assessments of dopaminergic activity, increased levels of cAMP and inhibition of tyrosine hydroxylase, are apparently not functioning in concert.

The objectives of the present investigation were to study the effects of selected dopaminergic agents on tyrosine hydroxylase activity in synaptosomes prepared from striatal tissue in an effort to assess the necessity of intact neuronal feedback loops for inhibition of TH activity and to examine more fully previous observations on the nature of the presynaptic regulation of TH. Results from these studies demonstrate that presynaptic regulation of TH at the receptor level can occur in synaptosomes, but that the mechanism of this effect does not necessarily involve stable allosteric changes in TH.

MATERIALS AND METHODS

Preparation of crude synaptosomal fraction. Male Sprague–Dawley rats (Charles River Breeding Laboratories) were killed by decapitation and the corpora striata were dissected out and placed on ice. The P_2 fraction (containing synaptosomes, myelin, and mitochondria) was prepared essentially according to Gray and Whittaker (13). Tissues were homogenized in 12 vol of 0.01 M Tris–HCl, pH 7.4, containing 0.32 M sucrose in a Teflon–glass homogenizer and centrifuged at 1000*g* for 15 min to remove nuclei and unbroken cells. The resulting supernate was centrifuged at 20,000*g* for 20 min to sediment the P_2 fraction.

Purification of ^{3}H-tyrosine and TH assay. TH was measured by a modification of the tritium release method (14). L-3,5-^{3}H-Tyrosine (45 Ci/mmol) in 2% ethanol was passed through a 0.6 × 2-cm column of alumina previously equilibrated with 0.01 M Tris–acetate, pH 8.3. The effluent was acidified with acetic acid to give a 0.2 N final concentration of acetic acid and poured onto a 0.6 × 1.5-cm column of Dowex 50W (4% cross-linked, dry mesh 200–400) which had been equilibrated in 0.2 N acetic acid. The column was washed with 50 ml of 0.2 N acetic acid and the tyrosine was eluted with 3 to 5 ml of 2.0 N HCl. The tyrosine was isolated by freeze-drying, redissolved in 2% ethanol, and stored at 4°C. On the days of tyrosine hydroxylase assay, an aliquot of the purified tyrosine was freeze-dried and redissolved in the appropriate buffer. TH reactions were carried out at 37°C in 10 × 75-mm disposable glass culture tubes. Following incubation, the reaction mixtures were immediately frozen by plunging the reaction tubes into dry ice–ethanol. The ^3H_2O produced by the TH reaction was isolated from the reaction mixture by a sublimation–condensation method (Fig. 1), rather than by column chromatography. A preliminary description of the assay method has been reported previously (15).

TH assay in synaptosomes. Synaptosomal TH activity was measured after resuspending the P_2 fraction in a physiological buffer containing: Tris–HCl, pH 7.4, 50 mM; NaCl, 125 mM; KCl, 5 mM; $CaCl_2$, 1 mM; $MgCl_2$, 1 mM; and glucose, 10 mM. A 50-μl aliquot of the resuspended P_2 fraction was added at 0°C to 150 μl of the physiological buffer containing various drugs and L-3,5-^{3}H-tyrosine (45 Ci/mmol). Final protein and tyrosine concentrations were 1.0 mg/ml and 0.5 μM, respectively. Reaction mixtures were then incubated at 37°C for 15 min and quick-frozen prior to the isolation of ^3H_2O. Control ^3H_2O formation ranged between 40,000 and 75,000 dpm/200 μl assay; boiled blanks ranged between 1200 and 4000 dpm/200 μl assay. Reactions were linear with respect to time up to 15 min and with respect to protein up to 1.0 mg/ml.

The inhibition of synaptosomal TH by various drugs is expressed as the percentage inhibition of control ^3H_2O formation according to the formula:

$$\% \text{ Inhibition} = 100\% - [(\text{dpm}_{\text{drug-treated sample}} - \text{dpm}_{\text{blank}})/(\text{dpm}_{\text{control}} - \text{dpm}_{\text{blank}})] \times 100\%.$$

In assays where the effects of TH inhibitors were studied in the presence of other drugs (e.g., antagonists), the "$\text{dpm}_{\text{control}}$" term in this equation was replaced by the dpm produced in the presence of the antagonist.

Kinetic studies in synaptosomal lysates. Synaptosomes were lysed by homogenization in 10 vol of a hypotonic solution of 0.05 M Tris–acetate, pH 6.0, and centrifuged at 40,000*g* for 30 min. The supernate, averaging 1.2 mg protein/ml, which contained soluble TH was incubated with Tris–acetate, pH 6.0 (50 mM), β-mercaptoethanol (20 mM), ferrous sulfate (0.1 mM), L-3,5-^{3}H-tyrosine (1 Ci/mmol) (20 μM), and variable concentrations of pteridine cofactors for 20 min at 37°C. These reactions were also stopped by freezing in dry ice–ethanol and ^3H_2O was isolated. Samples were assayed within 90 min of synaptosomal lysis. The reactions were linear with time up to 20 min and with protein up to 2.0 mg/ml. Velocity was expressed as picomoles ^3H_2O formed per minute per milligram protein. Protein was determined by the Coomassie brilliant blue method (16). K_m and V_{max} were determined using a computer analysis developed by Cleland (17).

Uptake studies in synaptosomes. The uptake of tyrosine into synaptosomes was determined under the conditions used to assay TH in synaptosomes. The P_2 fraction was incubated at 37°C in the physiological buffer with 0.5 μM L-3,5-^{3}H-tyrosine and aliquots were removed at 1, 2.5, 5, 7.5, 10, 12.5, and 15 min. Synaptosomes were separated from the medium by filtration on a 0.65-μm Millipore filter and washing with two 10-ml volumes of 0.32 M sucrose at 0°C. Millipore filters were dissolved in 1.0 ml of ethylene glycol monomethylether for 1 h at room temperature and mixed with 10 ml of Aquasol-2 for liquid scintillation counting.

Release studies in synaptosomes. For the study of DA release from synaptosomes, the striatal P_2 fraction was resuspended to a final protein concentration of 10 mg/ml in physiological buffer containing 20 mM β-mercaptoethanol and 10^{-5} M pargyline. The mixture was incubated with 10^{-7} M ^{3}H-dopamine (25 Ci/mmol) for 15 min at

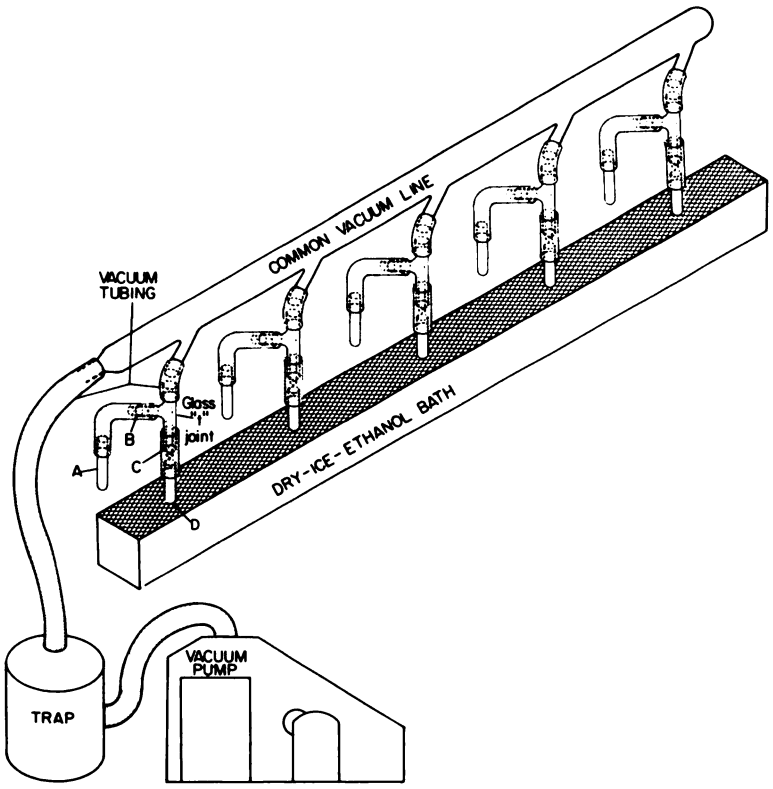

FIG. 1. *Diagrammatical representation of the apparatus used to isolate ^3H_2O by sublimation-condensation*

Frozen reaction mixtures (150–200 μl) containing ^3H_2O and ^{3}H-tyrosine in 10 × 75-mm disposable glass tubes (A) were attached to the apparatus as shown. Tygon tubing (⅛-in. i.d.), which fits tightly into the glass t-joint at point B, directs ^3H_2O vapors into the 10 × 75-mm disposable glass collection tube (D). A clearance of about 1 mm between tygon and glass at point C ensures complete evacuation of the reaction mixture. Following a 15- to 20-min period of evacuation, 50-μl aliquots of ^3H_2O were taken for scintillation counting. The actual apparatus will accommodate 30 reaction tubes.

37°C and then diluted with 10 vol of cold buffer to stop the uptake of labeled dopamine. The P_2 fraction was isolated by centrifugation (20,000*g*, 20 min) and washed by rehomogenization in 100 vol of buffer to remove loosely bound ^{3}H. After recentrifugation the wash was discarded and the labeled P_2 fraction was resuspended in buffer to a final protein concentration of 1 mg/ml. Then 1-ml aliquots were incubated at 37°C for 5 min with and without various drugs. Following incubation, the mixture was chilled on ice and centrifuged (20,000*g*, 10 min). An aliquot of the supernate, containing ^{3}H-DA and its metabolites released from the synaptosomal pellet, was taken for liquid scintillation counting. Total dpm for 1.0 ml of the synaptosomal suspension ranged between 70,000 and 120,000. Control synaptosomes incubated at 0 or 37°C released approximately 20–25 and 40–50%, respectively, of the total ^{3}H in the sample. In comparison, the maximum release (the amount of ^{3}H-amine in the final supernate after incubation of the labeled synaptosomes at 37°C with potent releasing agents such as 10^{-4} M tyramine or 10^{-3} M DA) was approximately 80–90% of the total ^{3}H-amine in the sample.

Materials. L-3,5-^{3}H-tyrosine (45 Ci/mmol) was purchased from Amersham Searle. Dihydroxyphenylethylamine, 3,4-[*ethyl*-2-^{3}H(N)]dopamine (25 Ci/mmol) was purchased from New England Nuclear. Catalase, db-cAMP, db-cGMP, cAMP, IBMX, Dowex 50 WX4-400, and atropine sulfate were purchased from Sigma. L-Tyrosine, dopamine hydrochloride, $DMPH_4$ hydrochloride, $6MPH_4$ dihydrochloride, and alumina were purchased from Calbiochem-Behring. Coomassie brilliant blue protein assay reagent was purchased from Biorad. The following compounds were kindly donated by the producers: fluphenazine hydrochloride (Squibb), (−)-apomorphine hydrochloride (Merck), haloperidol (Janssen), propranolol hydrochloride and the enantiomers of butaclamol hydrochloride (Ayerst), and phentolamine hydrochloride (Ciba). Millipore filters (DAWP) 02500 of size 0.65 μm, 25 mm in diameter, were purchased from the Millipore Corp. The racemic 2-aminotetralins were prepared as previously described (18).

RESULTS

Effects of dopamine analogs on synaptosomal TH. Incubation of striatal synaptosomes with dopamine (DA), (−)-apomorphine (APO), (±)-6,7-dihydroxy-2-aminotetralin (ADTN), or (±)-7-hydroxy-*N*,*N*-di-n-propyl aminotetralin (7HDPT) resulted in a concentration-dependent inhibition of synaptosomal TH activity (Fig. 2). The respective IC_{50} values for DA, APO, and ADTN were 2.4×10^{-7}, 1.1×10^{-7}, and 2.2×10^{-7} M. The half-maximal inhibition seen with 7HDPT occurred at 1.3×10^{-6} M. In contrast to DA, APO, and ADTN, treatment with 7HDPT produced only a partial inhibition to synaptosomal TH activity, making a relative assessment of IC_{50} in comparison to the other agents of uncertain

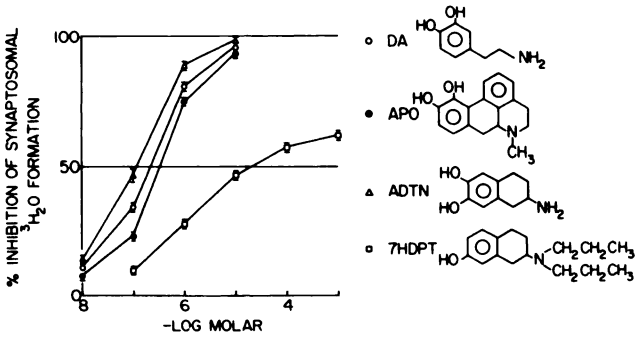

FIG. 2. *Inhibition of synaptosomal tyrosine hydroxylase by apomorphine (APO), dopamine (DA), 6,7-dihydroxy-2-aminotetralin (ADTN), and 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7HDPT)*

All reaction components were combined at 0°C and then incubated at 37°C for 15 min. Data are represented as the mean ± SEM of three or four determinations. Control = 63,300 dpm/200 μl assay. Percentage inhibitions were calculated as described in Materials and Methods.

meaning. The maximum inhibition with 7HDPT (10^{-3} M) was approximately 60–75%, whereas with the other agents a complete inhibition of TH activity was noted at high concentrations.

To further define the similarities and differences among these agents, their inhibitory effects on synaptosomal TH were compared in the presence of the DA uptake blocking drug benztropine (5×10^{-6} M), the dopamine antagonist/neuroleptic fluphenazine (10^{-6} M), and a combination of fluphenazine and benztropine. As shown in Fig. 3, panel A, inhibition with APO was marginally antagonized by benztropine, fluphenazine, or a combination of these agents. The inhibition of TH activity with DA presented a somewhat different picture (Panel B) in that benztropine shifted the inhibition curve to the right in a parallel fashion. Fluphenazine alone did not significantly affect the DA inhibition, and the combination of fluphenazine and benztropine was equivalent to the effect of benztropine alone. The effect of these blocking agents on the ADTN inhibition was qualitatively different (Panel C) from that observed for APO and DA. In this case, the combination of benztropine and fluphenazine produced a greater parallel shift to the right than benztropine alone. Again fluphenazine alone did not antagonize the ADTN inhibition of TH activity. In clear distinction to APO, DA, and ADTN, the inhibition with 7HDPT (Panel D) was not antagonized by benztropine, but was antagonized by fluphenazine. The combination of fluphenazine and benztropine gave the same inhibition as fluphenazine alone (53% reversal of the 10^{-6} M 7HDPT activity). The inhibition of synaptosomal TH by 7HDPT was also antagonized by 10^{-6} M concentrations of haloperidol (71% reversal of the 10^{-6} M 7HDPT activity), chlorpromazine (29% reversal of the 10^{-6} M 7HDPT activity), and (+)-butaclamol (44% reversal of the 10^{-6} M 7HDPT activity) but not by 10^{-6} M (−)-butaclamol. Additionally, 7HDPT activity was not antagonized by 10^{-6} M concentrations of atropine, propranolol, or phentolamine.

Direct effects of DA analogs on soluble TH. The direct effects of DA, APO, ADTN, and 7HDPT on TH were determined in hypotonic striatal homogenates. DA, APO, and ADTN were found to inhibit soluble TH in a concentration-dependent manner (Fig. 4). No inhibition of TH activity was noted with 7HDPT even at 10^{-3} M, indicating that 7HDPT does not compete with the synthetic cofactor ($DMPH_4$) for a binding site on TH or directly inhibit the enzyme in any other manner.

Dopamine release studies. The possibility that 7HDPT could release DA from vesicular stores into the cytoplasm of the synaptosome and thereby inhibit TH activity by end-product inhibition was studied by assessing the propensity of tyramine to inhibit synaptosomal TH activity. In agreement with other reports (22), treatment with tyramine, an established indirect-acting sympathomimetic, caused a significant inhibition of synaptosomal TH (Fig. 5). However, in contrast to 7HDPT, the tyramine activity was not antagonized by fluphenazine (10^{-6} M). Additionally, 7HDPT was more effective than tyramine in inhibiting synaptosomal TH but much less effective than tyramine in causing the release of ^{3}H-DA and ^{3}H-DA metabolites from synaptosomes (Fig. 5).

Uptake studies. In order to determine if 7HDPT inhibits TH by blocking the uptake of the radioactive substrate, the effects of 7HDPT (10^{-5} M) on tyrosine

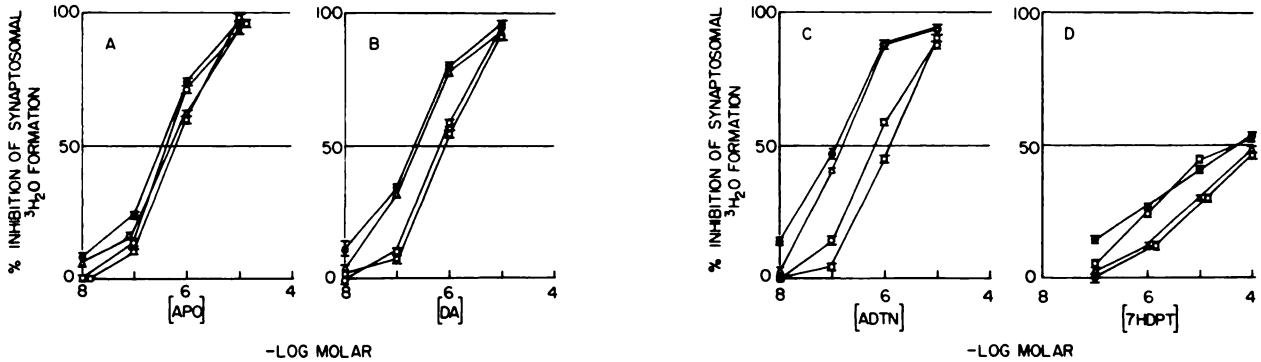

FIG. 3. *Inhibition of synaptosomal tyrosine hydroxylase by APO (A), DA (B), ADTN (C), and 7HDPT (D)*

Assays were performed in the presence of control buffer (●); the dopamine receptor antagonist fluphenazine, 10^{-6} M (△); the dopamine uptake blocker benztropine, 5×10^{-6} M (○); or fluphenazine, 10^{-6} M, + benztropine, 5×10^{-6} M (□). Synaptosomes were added to a mixture of other reaction components at 0°C and then incubated at 37°C for 15 min. Data are represented as the mean ±SEM of three or four determinations. Control = 65,800 dpm/200 μl assay. Treatment of synaptosomes with fluphenazine, benztropine, or a combination of these agents during the assay of TH produced activities that were 104, 105, and 107% of control, respectively. Percentage inhibitions were corrected for these activity changes as described in Materials and Methods.

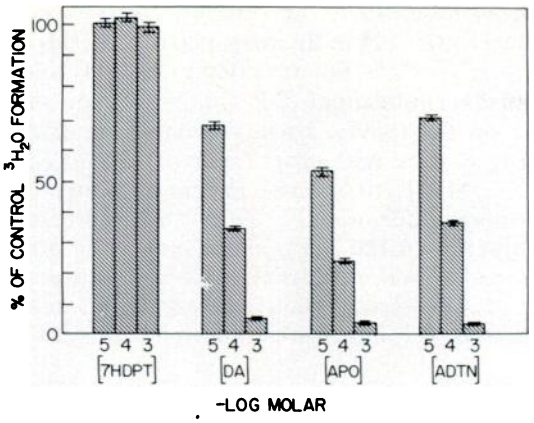

FIG. 4. *Effects of 7HDPT, DA, APO, and ADTN on soluble tyrosine hydroxylase*

Normal rat striata were homogenized in 10 vol of 0.05 M Tris-acetate, pH 6.0, and centrifuged at 40,000*g* for 20 min. The resulting supernate containing soluble tyrosine hydroxylase was assayed with 0.3 mM $DMPH_4$, 20 mM β-mercaptoethanol, 0.43 mM $FeSO_4$, 20 μM tyrosine, 0.05 M Tris-acetate, pH 6.0, with various concentrations of the indicated DA agonists. Data are represented as percentage of untreated control ± SEM for three or four determinations. Control activity was 16.8 pmol/min/mg protein.

uptake were determined. A concentration of 7HDPT (10^{-5} M), which consistently produced a 45–50% inhibition in TH activity, did not block tyrosine uptake over the 15-min time course routinely used in the assay for TH activity.

Kinetic analysis of tyrosine hydroxylase from synaptosomes treated with 7HDPT and APO. Synaptosomes were incubated in physiological buffer at 37°C with or without 7HDPT for 15 min, lysed, and centrifuged, and the resulting supernate, containing soluble TH, was studied for changes in V_{max} and K_m of the enzyme for the synthetic cofactors $6MPH_4$ and $DMPH_4$. As shown in Fig. 6, the K_m of TH for $DMPH_4$ (1.57 ± 0.15 mM) and V_{max} (1050 ± 70 pmol/min/mg protein) in lysates from

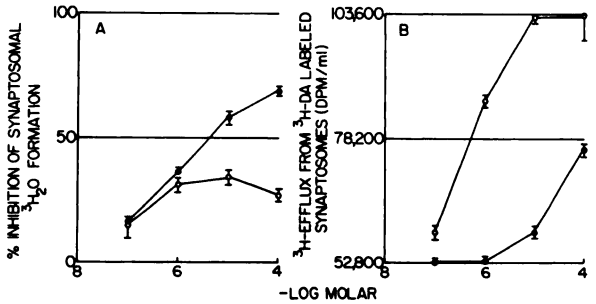

FIG. 5. *Effects of 7HDPT* (●) *and tyramine* (○) *on rat striatal synaptosomes*

(A) Inhibition of tyrosine hydroxylase. Data are represented as the mean ± SEM of three or four determinations. Control = 75,300 dpm/200 μl assay. Percentage inhibitions were calculated as described in Materials and Methods. (B) Release of ^{3}H-DA and metabolites from synaptosomes prelabeled with ^{3}H-DA. Synaptosomes prelabled with ^{3}H-DA according to Materials and Methods were incubated with 7HDPT or tyramine at 37°C for 5 min and an aliquot of the 20,000*g* supernate was analyzed for released ^{3}H. Untreated control release was 52,800 dpm/ml of supernate. Total radioactivity in 1 ml of the synaptosomal suspension = 114,100 dpm. Data are expressed as the mean ± SEM and are representative of four separate experiments.

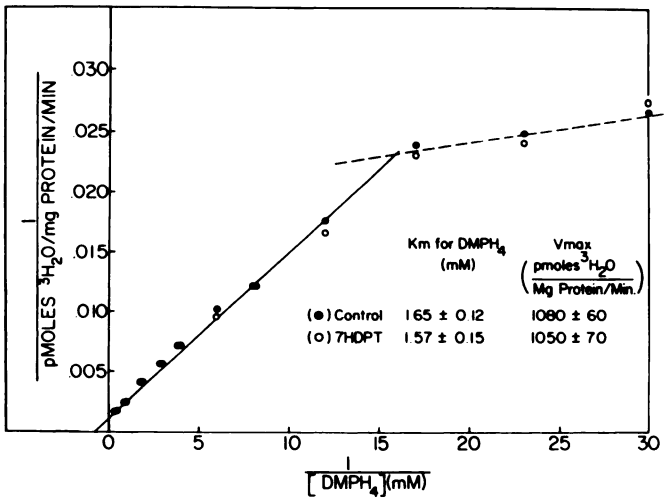

FIG. 6. *Effect of 7HDPT on the kinetics of tyrosine hydroxylase*

Synaptosomes were incubated for 15 min at 37°C in physiological buffer (●) or in physiological buffer + 10^{-5} M 7HDPT (○). Following incubation, the synaptosomes were resedimented (20,000*g*, 20 min), lysed, and recentrifuged (40,000*g*, 30 min) to obtain soluble TH which was assayed according to Materials and Methods at variable concentrations of $DMPH_4$. The V_{max} and K_m of tyrosine hydroxylase for $DMPH_4$ were calculated according to the method of Cleland (17), using the values obtained from $DMPH_4$ concentrations between 0.059 and 1.0 mM. These data are typical of several experiments.

7HDPT-treated synaptosomes were not significantly different from control K_m (1.65 ± 0.12 mM) and V_{max} (1080 ± 60 pmol/min/mg protein) values. This point was exhaustively studied using a variety of assay conditions (e.g., $DMPH_4$ as cofactor; 2-(*N*-morphilino)ethane sulfate, pH 6.0, as buffer; 0.05 M tris-acetate, pH 6.5, as buffer; 5 mM NaF, 20 mM sodium phosphate, pH 6.0, as buffer; 10^{-4} M DA added to assay; sheep liver dihydropterine reductase/catalase/NADPH as cofactor regenerating system; lysis of synaptosomes in distilled H_2O or 0.05 M Tris-acetate, pH 6.0, containing 0.2% Triton X-100; 100 μM tyrosine; and dialyzing lysates for 4 h against assay buffer to remove low molecular weight components). Under none of these assay conditions could a change in enzyme activity be seen following 7HDPT treatment. Additionally, incubation of synaptosomes with 10^{-5} M APO (a concentration which completely inhibits synaptosomal TH activity) did not produce a change in the activity of soluble TH.

In agreement with other workers (11), the standard assay used in these studies was able to detect the increases in soluble striatal TH activity when soluble TH was assayed under phosphorylating conditions. For instance, in the presence of components necessary for protein phosphorylation (10 mM $MgCl_2$, 0.5 mM IBMX, 0.1 mM EGTA, 0.1 mM cAMP, 0.5 mM ATP), the activity of soluble TH from isotonically lysed striatum ([$DMPH_4$] = 0.083 mM) was increased from 9.6 ± 0.5 to 39.9 ± 1.1 pmol/min/mg protein. This activation corresponded to a fivefold change in K_m for $DMPH_4$ (from 0.51 ± 0.06 to 0.10 ± 0.01 mM) and no change in V_{max} (74.7 pmol/min/mg protein).

Effects of $DMPH_4$ and other reducing agents on the inhibition of synaptosomal TH by DA and 7HDPT. Incubation of synaptosomes with synthetic cofactor

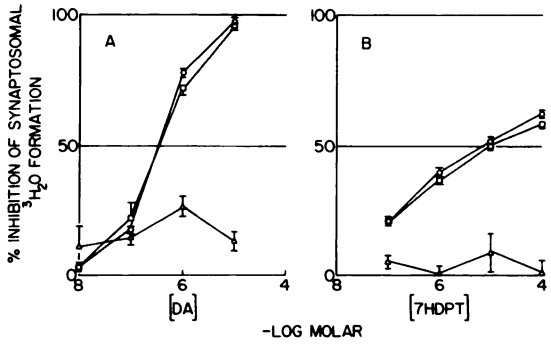

FIG. 7. *Effects of DMPH$_4$, 10 mM, on the inhibition of synaptosomal tyrosine hydroxylase by DA (A) or 7HDPT (B)*

Assays were performed in the presence of control buffer (○), 5000 U/ml catalase (□), or 5000 U/ml catalase + 10 mM DMPH$_4$ (△). Data are represented as the mean ± SEM of three or four determinations. Control (no catalase) = 56,500 dpm/200 μl assay. DMPH$_4$ in the presence of catalase produced a TH activity which was 44% of control. Synaptosomal TH activity in the presence of catalase alone was 66% of control. Percentage inhibitions were corrected for these activity changes as described in Materials and Methods. (Similar antagonism of 7HDPT and DA activities by 10 mM DMPH$_4$ was noted in the presence of 15,000 U/ml catalase which provided complete protection of synaptosomal TH activity from the inhibitory effects of 10 mM DMPH$_4$.)

DMPH$_4$ (10 mM) was carried out in the presence of 5000 U/ml catalase because DMPH$_4$ alone at concentrations higher than 10^{-5} M produced significant inhibition of synaptosomal TH. Although catalase alone at this concentration inhibited synaptosomal TH by 34%, it had no effect on the relative inhibitory activities of 7HDPT and DA (Fig. 7). In the presence of catalase, concentrations of DMPH$_4$ (10 mM) required to antagonize DA inhibition of synaptosomal TH also antagonized 7HDPT activity (Fig. 7), whereas lower concentrations of DMPH$_4$ produced only a marginal antagonism of DA and 7HDPT activities. DMPH$_4$ was considered to be acting intrasynaptosomally because lysis of synaptosomes by homogenization in 0.2% Triton X-100 greatly reduced (by 60%) the amount of ^3H_2O produced under these assay conditions. Additionally, synaptosomal TH activity in the presence of 10 mM DMPH$_4$ was abolished by 1.0 mM L-tryptophan, which is an inhibitor of L-tyrosine uptake but not an inhibitor of TH. Inhibition by L-tryptophan of synaptosomal TH in the presence of DMPH$_4$ indicates that the DMPH$_4$ is not producing ^3H_2O by interacting with extrasynaptosomal T.H. and that any effects by DMPH$_4$ on TH activity are intrasynaptosomal. Additionally, in agreement with other reports (20), low concentrations of DMPH$_4$ (0.03 mM) produced a significant increase in synaptosomal TH (26%) in the presence of β-mercaptoethanol (20 mM), again indicating an intrasynaptosomal action of DMPH$_4$. (Even in the presence of 20 mM β-mercaptoethanol, concentrations of DMPH$_4$ greater than 0.1 mM were inhibitory to synaptosomal TH.)

The inhibition of synaptosomal TH by 7HDPT was also antagonized by 0.1 mM ferrous sulfate in the presence of 20 mM β-mercaptoethanol but not by β-mercaptoethanol (20 mM) alone. Ferrous ion was considered to be acting intrasynaptosomally because synaptosomes preincubated with ferrous sulfate and washed prior to TH assay still lacked the normal sensitivity to 7HDPT.

Effects of cyclic nucleotides on the inhibition of TH by dopamine analogs. Incubation of striatal synaptosomes with 1.0 mM db-cAMP or 1.0 mM IBMX or a combination of these agents resulted in a small consistent increase (10–30%) in TH activity. The combination of these agents markedly antagonized the inhibitory effects of 7HDPT and APO while only slightly antagonizing the inhibitory effects of ADTN and DA (Fig. 8). IBMX, 1.0 mM, or db-cAMP, 1.0 mM, alone also antagonized the inhibitory effects of 7HDPT; however, 1.0 mM concentrations of adenosine, ATP, GTP, cAMP, db-cGMP, or cGMP did not affect the inhibition of TH activity noted with 7HDPT.

If synaptosomes were incubated with db-cAMP (3 mM) for 10 min and washed prior to assay to remove db-cAMP, a significant antagonism of 7HDPT activity was still observed (43% reversal of the 10^{-6} M 7HDPT activity). Assay of these preincubated washed synaptosomes in the presence of 3 mM db-cAMP produced a slightly greater antagonism of 7HDPT activity (60% reversal of the 10^{-6} M 7HDPT activity).

Incubation of synaptosomes for 15 min at 37°C with 3 mM db-cAMP in the presence of 1 mM IBMX consistently increased the activity of soluble TH obtained from lysed

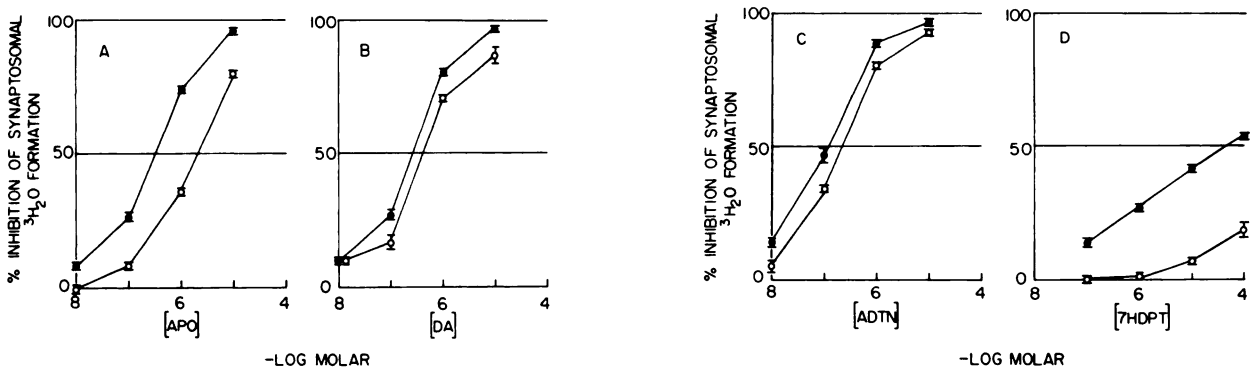

FIG. 8. *Effects of 1 mM db-cAMP in the presence of 1 mM IBMX* (○) *compared to control* (●) *on the inhibition of synaptosomal tyrosine hydroxylase by APO (A), DA (B), ADTN (C), or 7HDPT (D)*

Data are represented as the mean ±SEM of three or four determinations. Control = 71,800 dpm/200 μl assay. The combination of IBMX and db-cAMP produced a TH activity which was 113% of control. Percentage inhibitions were corrected for this activity change as described in Materials and Methods.

synaptosomes (from 7.2 ± 0.9 to 11.0 ± 1.5 pmol/min/mg protein in the presence of 0.05 mM $DMPH_4$). This activation corresponded to a decrease in the K_m of soluble TH for $DMPH_4$ from 1.1 ± 0.2 to 0.6 ± 0.1 mM (mean ± SEM of three experiments). Lysis and assay in the presence of 5 mM NaF, 20 mM sodium phosphate, pH 6.0 (to protect TH from dephosphorylation via phosphatase activity), did not potentiate the cyclic nucleotide effect. When db-cAMP and IBMX were added to soluble TH from lysed control synaptosomes, no effect on soluble TH activity was noted, indicating that the db-cAMP + IBMX effect requires synaptosomal integrity.

The effects of Ca^{2+} on the inhibition of synaptosomal TH by dopamine agonists. Since calcium ion has been shown to antagonize the effects of presynaptic receptor activation in peripheral tissues (21) and to change TH activities in brain preparations (6), the effects of $CaCl_2$ on rat striatal synaptosomes were investigated. Increasing the calcium concentrations of the synaptosomal reaction mixture from 1 to 11 mM did not significantly change the activity of synaptosomal TH, nor did it antagonize the activity of 7HDPT, ADTN, DA, or APO. In addition, 7HDPT activity is not antagonized by raising the calcium concentrations from 1 to 11 mM even under depolarizing conditions (isotonic 55 mM KCl).

DISCUSSION

A major problem in assessing the contribution of presynaptic dopamine receptors to the regulation of TH activity is that dopamine agonists which are potent inhibitors of synaptosomal TH activity contain a catechol moiety in their structure. If these agonists can gain entry into dopaminergic nerve terminals (via uptake or diffusion), they may inhibit TH by competing with the required reduced pterin cofactor for a common binding site on the enzyme (3). In support of this hypothesis, dopamine agonists without catecholamine moieties have minimal effects on synaptosomal TH (22, 23). As shown in the Results, DA, APO, and ADTN contain a catecholamine in their structure and inhibit soluble TH activity. Additionally, these agents are capable of entering adrenergic nerve endings (24–26).

Assessment of the relative receptor-mediated and intraneuronal activity of these dopamine agonists usually requires the use of an uptake blocking agent (e.g., benztropine); the assumption being made is that a complete block of uptake results from treatment with the blocking agent and that no diffusion of the agonists into the nerve ending occurs. Because adrenergic nerve endings are very efficient at concentrating catecholamines, less than a complete block of uptake may not be sufficient to prevent intraneuronal inhibition of TH activity. Additionally, a lipid soluble agonist like apomorphine is very difficult to study because diffusion into the nerve ending cannot easily be retarded.

An ideal agent for the study of receptor-mediated inhibition of TH would be either an agent which could not enter the nerve ending by active transport or diffusion or an agent which is completely devoid of any direct interactions with TH. This latter requirement is fulfilled by the noncatechol tetralin, 7HDPT, which has been previously reported to exhibit behavioral dopaminergic effects *in vivo* (27). Data in support of a dopaminergic plasma membrane receptor mechanism for 7HDPT are summarized as follows: (i) 7HDPT inhibition of synaptosomal TH is blocked by a variety of dopamine receptor antagonists including (+)-butaclamol but not (−)-butaclamol; (ii) 7HDPT inhibition is not blocked by comparable concentrations of other receptor antagonists (atropine, phentolamine, propranolol); (iii) 7HDPT inhibition is not blocked by benztropine; (iv) 7HDPT does not block tyrosine uptake into synaptosomes; (v) 7HDPT does not inhibit soluble TH at concentrations as high at 10^{-3} M; and (vi) 7HDPT has been shown[2] to displace both ^{3}H-DA ($IC_{50} = 4 \times 10^{-7}$) and ^{3}H spiroperidol ($IC_{50} = 3 \times 10^{-6}$) from rat striatal membranes. Additionally, an indirect intraneuronal effect of 7HDPT in releasing endogenous DA from vesicular stores has been ruled out as the major mechanism of action by comparative studies with tyramine (Fig. 5).

From Fig. 5 it can be seen that release of ^{3}H-DA and inhibition of TH are not well correlated. Although high concentrations of 7HDPT produced some release of ^{3}H-DA, this effect was small compared to the effect of tyramine at comparable concentrations. In contrast, the maximum inhibition of TH by 7HDPT was greater than the maximum inhibition by tyramine. Significant inhibition of synaptosomal TH occurs at concentrations of 7HDPT (10^{-6} M) which cause very little, if any, ^{3}H-DA efflux. The mechanisms of action of tyramine and 7HDPT are further contrasted by the fact that 7HDPT but not tyramine inhibition of TH is antagonized by fluphenazine. Thus, the mechanism of synaptosomal TH inhibition by 7HDPT appears to be mediated primarily via a dopaminergic receptor, although at high 7HDPT concentrations an indirect effect via vesicular release of DA cannot be entirely ruled out.

In contrast to 7HDPT, DA appears to inhibit synaptosomal TH primarily by an intraneuronal mechanism because its activity is blocked by benztropine and because it inhibits soluble TH. Fluphenazine treatment was without effect on the DA inhibition except at concentrations of DA well below the K_m for DA uptake. The observed antagonism with fluphenazine is probably due to the weak uptake blocking properties of fluphenazine (28). However, the data may reflect a presynaptic dopaminergic mechanism which is overridden at higher concentrations by intraneuronal inhibition.

Inhibition of TH by ADTN and APO, unlike that by DA, appears to result from a combination of intraneuronal and plasma membrane receptor effects. ADTN appears to inhibit TH in a manner very similar to that of DA, because its activity is blocked by benztropine but not by fluphenazine and because it is also an inhibitor of soluble TH. However, in the presence of benztropine, ADTN activity is additionally antagonized by fluphenazine, suggesting dopamine agonist activity. APO is similar to 7HDPT in that its activity in synaptosomes is antagonized by neuroleptics. However, it is distinguished from 7HDPT in that a complete inhibition of TH activity occurs at high concentration. The complete inhibition of TH activity may reflect internal competition with the endogenous cofactor for binding to TH and may result

[2] Ferris, R. M., personal communication.

from the penetration of APO into synaptosomes, owing to its lipid solubility.

In another respect, APO and 7HDPT are similar. The inhibitory activity of both agents is antagonized by db-cAMP in the presence of IBMX. The antagonistic activity of db-cAMP in the presence of IBMX is greater against 7HDPT than against APO and is very slight against DA and ADTN (Fig. 8). Thus, this antagonism may further distinguish DA receptor mechanisms of TH inhibition from intraneuronal mechanisms. Because db-cGMP is ineffective as an antagonist of the 7HDPT activity, it is concluded that the cAMP moiety but not the butyrate or cGMP moieties is responsible for the antagonism.

The mechanism by which cAMP derivatives antagonize the presynaptic receptor-mediated inhibition of striatal TH requires further study. The observed antagonism of 7HDPT by db-cAMP may reflect allosteric activation of tyrosine hydroxylase, activation of a supporting reductase system for TH, or direct blockade of the DA receptor-mediated events leading to the observed inhibition of TH. The concept that cyclic nucleotides produce stable changes in the TH system is supported by the observation that the antagonism of 7HDPT by db-cAMP remains even when the db-cAMP has been removed by washing. The increase in soluble TH activity obtained from lysed synaptosomes which had been preincubated with db-cAMP + IBMX indicates that allosteric changes in TH may be connected with altered levels of cyclic nucleotides in dopaminergic nerve terminals. The opposite effects of db-cAMP and 7HDPT on synaptosomal TH activity as well as the antagonism of the 7HDPT effect by db-cAMP suggest that the inhibition of TH by presynaptic dopamine receptor activation is not mediated by increased intraneuronal levels of cAMP. This is in agreement with the concept that striatal postsynaptic dopaminergic receptors are linked to adenylate cyclase but striatal presynaptic dopaminergic receptors are not cyclase linked (8). However, in view of the modulatory influence of db-cAMP on synaptosomal TH, the existence of additional presynaptic receptors, linked to adenylate cyclase, cannot be ruled out.

Presynaptic DA receptor inhibition of TH activity does not necessarily require allosteric changes in TH. No evidence could be found suggesting that presynaptic DA receptor activation by 7HDPT produced a stable kinetic change in TH. Concentrations of 7HDPT which consistently inhibited synaptosomal TH by 50% did not produce a change in the V_{max} or the K_m of TH for synthetic cofactors $DMPH_4$ or $6MPH_4$ under any of the assay conditions employed. Furthermore, concentrations of APO which consistently inhibited >95% of the synaptosomal TH activity did not produce stable changes in the V_{max} or K_m of TH for $DMPH_4$. Therefore, the DA receptors regulating the observed kinetic changes in TH activity do not appear to be located on the presynaptic dopaminergic nerve terminals of striatal synaptosomes. However, this study does not address the possibility that presynaptic dopaminergic receptor stimulation could attenuate depolarization-induced changes in TH activity which may occur *in vivo* or under certain *in vitro* depolarizing conditions. For example, during the regular depolarization of neurons *in vivo*, the presynaptic DA receptors may have additional functions, such as the regulation of Ca^{2+} influx (21), which in turn may produce kinetic alterations of TH (6).

As others have noted (29), curvilinear kinetics were frequently obtained, making the assessment of K_m and V_{max} somewhat arbitrary. That is, the K_m may vary with the concentration range of cofactor used to construct the double reciprocal plot. The nonlinear reciprocal plots may result from a mixture of enzymes with different K_m's (32) or from TH activity changes during the assay which are a function of cofactor concentration. Regarding the mechanism of 7HDPT action, the important point is that the reciprocal plots of soluble tyrosine hydroxylase activities from both control and 7HDPT-treated synaptosomes were not significantly different even at low cofactor concentrations, indicating no apparent change in the V_{max} or K_m of the enzyme. The K_m for $DMPH_4$ of TH from lysed synaptosomes obtained in these measurements was of the order of 1.1 to 1.6 mM, indicating that the enzyme was predominantly in the inactivated state in agreement with published reports (4, 5). Because this enzyme was already in an inactive state, it is not surprising that a further increase in the K_m did not occur upon the treatment of synaptosomes with the DA agonists 7HDPT and APO.

Because 7HDPT does not compete with $DMPH_4$ binding to TH, as noted in the studies with soluble TH (Fig. 4), the antagonism of the DA receptor-mediated inhibition of synaptosomal TH by the synthetic cofactor $DMPH_4$ implies that presynaptic DA receptors somehow modulate the cofactor utilization by TH. However, kinetic analysis of TH following presynaptic receptor stimulation (Fig. 6) indicates that an allosteric change in TH is unlikely. Therefore presynaptic DA receptor activation could be inhibiting TH either by lowering reduced cofactor levels or by uncoupling the TH reaction by causing the oxidation of a reduced intermediate of TH. The antagonism of 7HDPT activity by other reducing agents (Fe^{2+} in the presence of β-mercaptoethanol) supports this hypothesis. Indeed, there may be additional reducing systems involved in TH regulation besides the pterin cofactor reductase system (30).

Finally, the characteristics of the dopaminergic inhibition of TH differ in several aspects from the presynaptic mechanisms involved in inhibition of the stimulated release of neurotransmitter. Presynaptic receptor inhibition of neurotransmitter release is inversely proportional to the extracellular Ca^{2+} concentration and requires a depolarization stimulus (e.g., electrical, KCl, veratridine) to be observed (21). The neuroleptic-sensitive inhibition of TH with 7HDPT occurs in the absence of a depolarization stimulus and, as noted in the Results, is insensitive to changes in the extracellular Ca^{2+} (1–11 mM). These observations suggest that regulation of calcium influx is not a part of the process by which presynaptic dopamine receptors regulate TH activity in synaptosomal systems. However, in physiological situations where neurons are frequently depolarized, Ca^{2+} influx may play a role in the presynaptic control of TH.

In conclusion, presynaptic DA receptor regulation of TH has been demonstrated and characterized by the use

of the dopamine agonist, 7HDPT. In contrast to other dopamine agonists (DA, APO, ADTN) which can inhibit TH directly, 7HDPT seems to inhibit synaptosomal TH activity primarily via a presynaptic plasma membrane DA receptor. Activation of this receptor by 7HDPT does not produce stable allosteric changes in TH but appears to modify a supporting system for TH such as the pterin reductase system. In contrast to the activation of postsynaptic striatal DA receptors, the activation of the presynaptic DA receptor regulating TH does not involve increased levels of cAMP. In contrast to presynaptic receptors regulating neurotransmitter release, the presynaptic DA receptors regulating TH are not affected by changes in extracellular calcium concentration. These data suggest that two types of DA receptor mechanism exist (cAMP dependent and cAMP independent) and, similarly, that two types of presynpatic autoreceptor mechanisms exist (Ca^{2+} dependent and Ca^{2+} independent).

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Send reprint requests to: H. J. Leighton, Department of Pharmacology, Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, N.C. 27709.