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EFFECT OF CLONIDINE ON THE ACTIVITY OF TRYPTOPHAN HYDROXYLASE FROM RAT BRAINSTEM FOLLOWING *IN VIVO* OR *IN VITRO* TREATMENT

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Abstract—*In vivo* administration of clonidine hydrochloride (Catapres) via tail vein injection produced a rapid increase in brainstem tryptophan hydroxylase activity assayed *in vitro* under subsaturating conditions of reduced pterin cofactor, $6MPH_4$. Enzyme activity returned to and remained at control levels about 60 min after treatment with low doses of clonidine (5 μg/kg). However, with higher doses of the drug (15 μg/kg), enzyme activity fell to below control levels for about an hour. Incubation of brainstem slices with clonidine also produced a dose-dependent increase in enzyme activity. The increase in enzyme activity appears to be mediated indirectly since it was abolished when brain catecholamine levels were depleted by pretreatment with 6-hydroxydopamine 8 days prior to clonidine treatment. The kinetic properties of tryptophan hydroxylase prepared 25 and 90 min after clonidine administration indicate that the initial increase and subsequent decrease in enzyme activity seen under these conditions may be due to changes in apparent V_{max} of the enzyme.

The antihypertensive drug clonidine (2-[(2,6-di-chlorophenyl)amino]-2-imidazolidine) is thought to mediate its depressor action through activation of central presynaptic α_2-adrenergic receptors [1, 2]. Biochemical and electrophysiological studies indicate that it exerts effects on central noradrenergic neurons [3]. Thus, systemically or iontophoretically administered clonidine depresses the spontaneous firing of noradrenergic neurons in the locus coeruleus [4, 5], and this inhibitory action of the drug is also observed in slice preparations [6]. It is moreover paralleled by a reduction of brain NE‖ turnover [7, 8] and of levels of the NE metabolite 3-methoxy-4-hydroxyphenyl glycol [9]. Besides exerting effects on noradrenergic neurons, clonidine also influences the activity of serotonergic neurons, presumably as a result of the noradrenergic input these neurons receive [10, 11]. Thus, systemically administered clonidine suppresses the firing of 5-HT neurons in the dorsal raphe nucleus of the midbran [4] and this interaction is antagonized both by the indirectly acting amine *d*-amphetamine and by 6-OHDA pretreatment to destroy CA neurons [4]. Consistent with its depressant action on the firing of 5-HT neurons, clonidine also reduces 5-HT synthesis and turnover in whole brain [7, 9, 12, 13].

In view of these observations and the evidence that both 5-HT synthesis [14–18] and the activity of the rate-limiting enzyme in 5-HT synthesis, tryptophan hydroxylase [19–23], are influenced by neuronal firing or potassium depolarization, we decided to examine whether clonidine treatment influences tryptophan hydroxylase activity. In this paper, we present data showing that clonidine given acutely *in vivo* has a biphasic action on the activity of tryptophan hydroxylase that is observed *in vitro*. A preliminary report of some of this work was presented in 1981 [24].

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: dithiothreitol (DTT) (A grade), nicotinamide adenine dinucleotide phosphate reduced, (NADPH) A grade, DL-6-methyl-5,6,7,8-tetrahydropterin HCl (DL-$6MPH_4$) and bovine liver catalase in water containing 0.5% thymol (1.57×10^6 I.U./ml at 30°) from Calbiochem-Behring, San Diego, CA; Tris (hydroxymethyl) amino methane (enzyme and buffer grade, ultra pure) and both D- and L-tryptophan from Schwarz Mann, Orangeburg, NY; bovine serum albumin and 5-hydroxytryptophan (5-HTP) from the Sigma Chemical Co., St. Louis, MO; analytical grade concentrated HCl, analytical grade, 70%, perchloric acid and methanol (Chrom AR) for HPLC from Mallinckrodt, St. Louis, MO; Sephadex G-25 (coarse type) from Pharmacia, Piscataway, NJ; disodium ethylene diaminetetraacetate (EDTA) and absolute ethanol from Fisher Scientific Products, Fairlawn,

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‖ Abbreviations: NE, norepinephrine; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindoleacetic acid; DL-$6MPH_4$, DL-6-methyl-5,6,7,8-tetrahydropterin HCl; 6-OHDA, 6-hydroxydopamine; CA, catecholamine; PFPA, pentafluoropropionyl anhydride; brocresine, 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate, NSD 1055; Tris, Tris (hydroxymethyl) amino methane; DTT, dithiothreitol; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; and HPLC-EC, high performance liquid chromatography with electrochemical detection.

NJ; 6-hydroxydopamine (6-OHDA) from the Regis Chemical Co., Morton Grove, IL; pentafluoropropionyl anhydride (PFPA) from the Pierce Chemical Co., Rockford, IL; propionic anhydride and boric acid gel from the Aldrich Chemical Co., Milwaukee, WI; ethyl acetate, benzene and pyridine from the J. T. Baker Chemical Co., Phillipsburg, NJ; and deuterated NE and 5-HT standards from MSD Isotopes, Merck & Co., Inc., St. Louis, MO. Clonidine hydrochloride (Catapres) was a gift from Boehringer Ingelheim, Ridgefield, CT; and brocresine (3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate) was donated by Lederle Laboratories, Pearl River, NY.

All other reagents were of the highest grade available commercially.

Dissection of brain tissue. Male Sprague–Dawley rats (Flow Laboratories, Rockville, MD), weighing 150–350 g but matched for weight within an experimental group to within ±30 g, were killed by decapitation. The cerebellum was cut away, the cerebral hemispheres were pushed back, and a cut was made just behind the level of the anterior commissure. This preparation, consisting of diencephalon (i.e. thalamus and hypothalamus), midbrain and medulla pons, is equivalent to the brainstem minus cerebellum and is referred to as simply "brainstem" in the paper for brevity. This preparation served as the source of tryptophan hydroxylase. In some experiments, cerebral cortices were also saved for determination of 5-HIAA levels by HPLC with EC detection.

Preparation and incubation of brainstem slices. The freshly dissected ice-cold brainstem tissue was weighed and then cut into 250 μm slices with a tissue chopper (model MT-2, Dupont-Sorvall, Newtown, CT). Each sliced brainstem was scooped into a centrifuge tube containing 5 ml of ice-cold incubation medium of the following composition: NaCl, 150 mM; KCl, 6 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; glucose, 10 mM; and Tris acetate buffer, pH 7.4, 10 mM, to which various clonidine concentrations were added. The slices were incubated at 25° for 10 min, and the medium was bubbled with 100% oxygen throughout. At the end of the incubation, the slices were separated from the medium by gentle centrifugation (12,000 *g* for 5 min) in a refrigerated centrifuge (RC-5, Dupont-Sorvall), and were then used to prepare the tryptophan hydroxylase extract.

Drug administration. Clonidine hydrochloride (dissolved in 0.9% saline) or 0.9% saline in the case of controls was administered via tail vein injection. Unanesthetized rats were restrained in a rat holder (Narco Biosystems, Houston, TX), placed over a warming pad kept at 37° to produce vasodilatation. Two-minute exposure to the pad produced adequate vasodilatation. Saline or clonidine was administered immediately, and the animal was then removed from the holder and kept in its cage until killed by decapitation.

In the experiments with the neurotoxin, 6-OHDA [25], rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and mounted in a small animal stereotaxic apparatus (David Kopf Instruments, Tujuna, CA). The incisor bar was elevated 5 mm. The 6-OHDA HBr was dissolved (10 mg/ml free base) in 0.9% saline containing 0.01% ascorbate, and kept on ice, protected from light, until needed. Burr holes were drilled unilaterally 1.5 mm lateral to Bregma. The bevelled tip of a 5-cm length of 26-gauge stainless steel tubing, attached to 20-gauge PE tubing (30 cm) and a 50-μl Hamilton syringe, filled with either 6-OHDA or vehicle, was lowered 4.2 mm below the surface of the dura, and 20 μl of the drug or vehicle was infused over a 1-min period. Movement of a bubble introduced into the PE tubing permitted the infusion rate to be monitored. The cannula was left in place for a further 2 min, and was then removed and the scalp sutured with wound clips. Animals were given 40,000 units of procaine penicillin G and dihydrostreptomycin (Combiotic, Pfizer, New York, NY) intramuscularly and were allowed 8 days for recovery prior to use in experiments.

The extent of norepinephrine depletion induced by this treatment was determined by gas chromographic mass spectrometry, on aliquots of the same brainstem homogenates used to prepare the tryptophan hydroxylase extracts.

Gas chromatographic-mass spectrometric determination of NE and 5-HT in 6-OHDA-treated rats and their vehicle-treated controls. For 5-HT determinations [26], 50 ng of 5-hydroxytryptamine-$\alpha d_2, \beta d_2$ was added to aliquots (100 μl) of brainstem homogenate, and these were then diluted to 0.5 ml with 0.4 N $HClO_4$, rehomogenized, and centrifuged at 16,000 *g* for 10 min. The supernatant fractions were transferred to clean tubes, and 0.1 ml saturated Na_2CO_3, 10 μl pyridine, and 50 μl propionic anhydride were added and mixed thoroughly. The propionyl derivative was extracted into 1.0 ml of ethyl acetate, and this was transferred to a clean tube and blown to dryness under nitrogen. The second derivatization step was carried out by heating the residue to 65° for 15–30 min in the presence of 200 μl PFPA. The material obtained after evaporation under N_2 was redissolved in 0.3 ml benzene and washed thoroughly with 0.3 ml phosphate buffer (0.05 M, pH 8.0). The benzene layer was removed to a clean glass vial (Pierce, Reacti-vials), blown dry, and finally redissolved in 50 μl of dry ethyl acetate. Samples (usually 5–10 μl) were run on a Finnigan 3200 gas chromatograph-mass spectrometer (GC-MS) using the following conditions: injector 250° (isothermal); oven 200–220°; column (1.8 M × 2 mm i.d.) 3% OV-17 on Gas-Chrom Q; ion monitors set at *m/z* 332, 336, 360, 364.

For the assay of NE [27] 10 ng of norepinephrine-$\alpha d_2, \beta d_1$ was added to 200-μl aliquots of brainstem homogenate, and these were then rehomogenized in 2.8 ml of 0.05 M $HClO_4$, 100 μl of 0.1 M EDTA, and 100 μl of 1.0 M sodium metabisulfite. After centrifugation, the supernatant fraction was removed and 1.0 ml of 1 M ammonium phosphate, pH 7.5, was added and thoroughly mixed. This solution was passed through a column of activated boric acid gel [27] and washed sequentially with 1.0 ml of ammonium phosphate, pH 7.5, 60 ml of distilled water, and 2 ml of 0.1 M acetic acid in methanol. NE was eluted with 1.3 M acetic acid in methanol. The eluate was blown dry under nitrogen and then reacted overnight with 0.5 ml of 2 M HCl in ethanol.

After drying under N_2, this product was reacted with PFPA (0.2 ml) for 30 min at 70°. Unreacted PFPA was blown off with N_2, and the final product was dissolved in 50 μl of dry ethyl acetate containing 0.5% PFPA. An aliquot of this (usually 5 μl) was injected onto a 3% OV-1 column (1.8 M × 2 mm i.d.) (injector 250°, oven 160° isothermal) with ion monitors set at *m/z* 431, 432, 459 and 460. Column life was lengthened by baking the column at 250° overnight after each use. This eliminated residual PFPA prior to storage of the column.

For both 5-HT and NE measurements, standard curves were run in parallel with the tissue samples. The quantity of deuterated monoamines in the standard samples matched that in the tissue samples.

Preparation of tryptophan hydroxylase. Freshly dissected, weighed brainstem or pelleted brainstem slices were homogenized with 0.05 M Tris acetate buffer, pH 7.4 (1:1.5, w/v), using a Kontes Duall glass homogenizer (Kontes Scientific, Vineland, NJ), clearance 0.01 cm, and the homogenate was centrifuged at 18,000 rpm (39,000 *g*) for 30 min in a refrigerated centrifuge (RC-5, Dupont-Sorvall) at 4°. DTT, in a final concentration of 2 mM, was added to the resulting supernatant fraction, which was then passed over a Sephadex G-25 (course type) column (22 × 1.3 cm) equilibrated at 7° with 0.05 M Tris acetate, pH 7.4, containing 2 mM DTT, to remove small molecules which raise the fluorescence of the blanks. The pink-colored protein fraction which emerged in the void volume was collected and assayed immediately or frozen at −20° and assayed the following day. In experiments involving 6-OHDA pretreatment, the enzyme homogenate was prepared in 2 vol. of the acetate buffer with a Tissumizer (45 sec at setting 50) (Tekmar, Cincinnati, OH) instead of the glass homogenizer.

Tryptophan hydroxylase assay. Tryptophan hydroxylase was assayed *in vitro* by the method of Friedman *et al.* [28] with minor modifications [19, 21]. The reaction was carried out in a final volume of 300 μl which contained in μmoles: Tris acetate, pH 7.4, 12; NADPH, 0.06; $6MPH_4$, 0.015 (subsaturating concentration) in 0.005 N HCl; DTT, 0.12; brocresine, 0.04; catalase, 1000 units; and the enzyme supernatant fraction. After a 5-min preincubation period at 37° in a shaking water bath (model 50 Precision Scientific, Chicago, IL), the reaction was initiated by the addition of 0.06 μmoles L-tryptophan; the blanks received D-tryptophan. The mixture was incubated for different times (up to 20 min maximum), and the reaction was stopped by the addition of 30 μl of 70% perchloric acid; the protein was spun down in a Beckman Microfuge (model B Beckman Instrument, Palo Alto, CA) using 1.5-ml disposable plastic centrifuge tubes. The 5-HTP formed in the reaction was quantitated by fluorescence with a Farrand model 801 spectrofluorometer (Farrand Optical, Valhalla, NY) (excitation 305 nm, emission 535 nm) after addition of 50 μl of concentrated HCl to 200 μl of the clear supernatant fraction in a 0.5-ml quartz cuvette.

In all experiments the assay was checked for linearity with both time of incubation (linear up to 25 min) and protein content (linear between 80 and 500 μg protein). Protein was determined on each enzyme preparation by the method of Lowry *et al.* [29] using bovine serum albumin as the standard. Results are expressed as pmoles 5-HTP formed per mg protein per min, or as percent of control enzyme activity ± standard error of the mean.

Determination of tissue 5-HIAA levels by HPLC-EC. Cerebral cortices from experimental and control rats were homogenized with a Tissumizer for 45 sec at setting 50 in 1.2 ml of 10% perchloric acid. Aliquots (20 μl) of clear supernatant fraction obtained after centrifugation at 4° for 15 min at 40,000 *g* were chromatographed on an Altex 3 μm ultrasphere ODS column (4.6 mm i.d. × 7.5 cm) using an ammonium acetate buffer, 0.35 M, pH 4.6, in 5% methanol at 1.2 ml/min [30]. 5-HIAA content was determined with external standard and expressed in ng 5-HIAA per g wet weight fresh tissue ± S.E.M. The liquid chromatograph with electrochemical detector was model LC304 from Bioanalytical Systems (West Lafayette, IN) with a glassy carbon electrode. The oxidation potential was set at 0.65 V.

RESULTS

Effect of acute clonidine pretreatment on tryptophan hydroxylase activity. Different doses of clonidine·HCl, given via tail vein injection to conscious rats, were examined for their effect on the activity of tryptophan hydroxylase isolated from the brainstem at different times after drug administration and assayed under subsaturating concentrations of cofactor. Control animals received saline injections. The results are summarized in Fig. 1 and indicate that enzyme activity increased significantly 25 min after treatment with doses of the drug from 1 to 20 μg/kg (expressed as μg clonidine·HCl), and remained elevated 45 min later. However, 90 min after drug administration enzyme activity remained above control levels only at the lowest dose of drug tested (0.5 μg/kg); it had returned to control levels at 2 μg/kg and fallen below control levels at the higher doses of drug tested (10, 15 and 20 μg/kg). Two hours later enzyme activity did not differ from control levels at 0.5, 1 and 2 μg/kg clonidine·HCl, but remained depressed at all the higher doses.

A more detailed time course of the effect of intravenously administered clonidine on tryptophan hydroxylase activity was followed at 5 and 15 μg/kg (Fig. 2). Brainstem enzyme activity was elevated above control levels for about 45 min with both doses. It had returned to control values 1 hr after the higher of the two doses (15 μg/kg) and 90 min after the lower dose (5 μg/kg); it remained at control level at all subsequent time points examined (3, 4, and 5 hr) after the low dose of clonidine. However, 90 min after administration of the higher dose, enzyme activity had fallen below control levels and remained depressed at 2 hr. Three hours after drug administration it returned to control levels where it remained at 4 and 5 hr. Thus, the higher doses of clonidine tested (10–20 μg/kg) appear to produce a biphasic effect on tryptophan hydroxylase activity, an initial increase in activity, followed by a significant depression of activity and then a return to control levels.

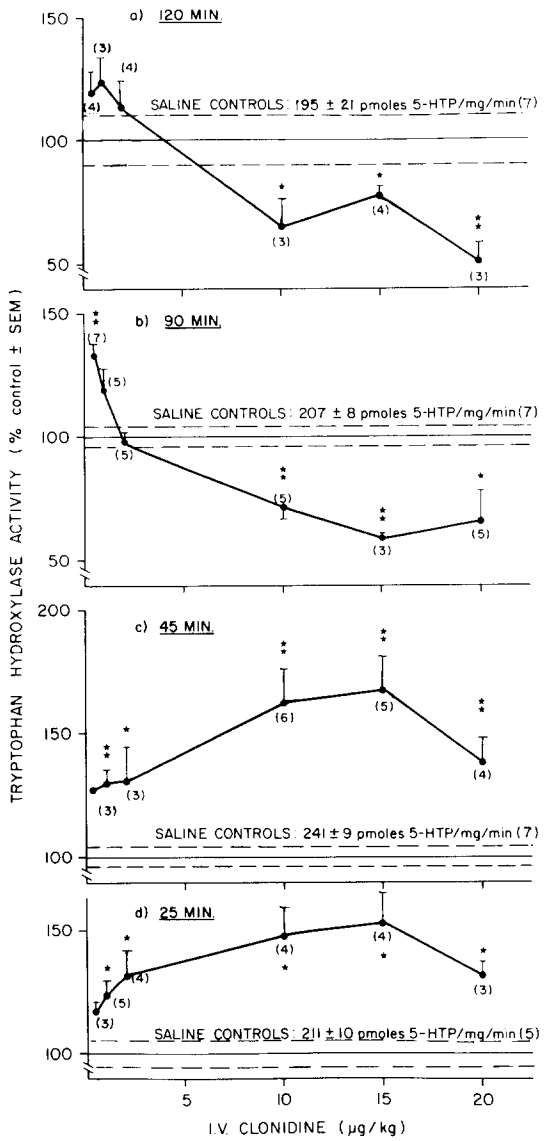

Fig. 1. Effect of increasing doses of clonidine·HCl on brainstem tryptophan hydroxylase activity at various times after injection. Rats were given clonidine·HCl (0.5 to 20 μg/kg, i.v.) or saline and killed 25, 45, 90 or 120 min later. The results are expressed as percent of control ± the standard error of the mean. The numbers of animals tested are given in parentheses. Significance of the difference between enzyme activity from drug- versus saline-treated animals was determined by Student's *t*-test. Key: (**) $P < 0.005$ and (*) $P < 0.02$. Other values were not significantly different.

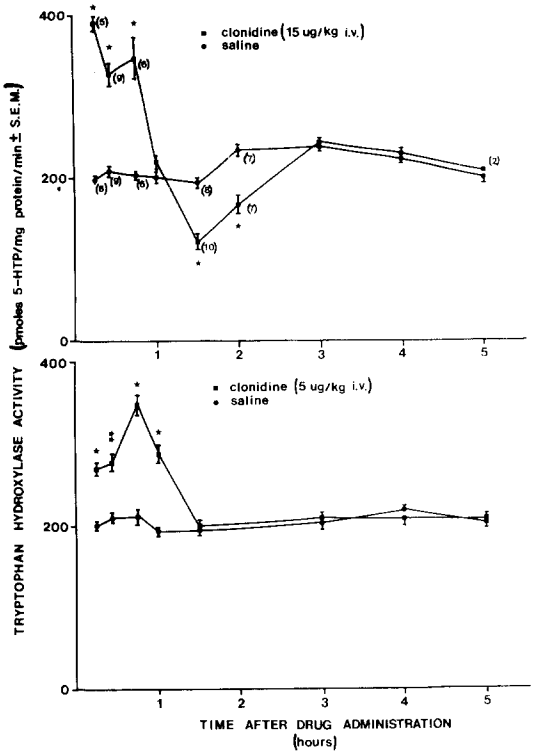

Fig. 2. Effect of i.v. clonidine·HCl (5 or 15 μg/kg, i.v.) on brainstem tryptophan hydroxylase activity at various times after tail vein injection. The results are expressed in pmoles 5-HTP formed per mg protein per min ± standard error of the mean. The number of animals tested at each dose and time point was three unless otherwise indicated. Significance of difference between enzyme activity from drug- versus saline-treated animals was determined by Student's *t*-test. Key: (*) $P < 0.001$ and (**) $P < 0.005$. Other values were not significantly different.

Effect of intravenous clonidine on the kinetic properties of tryptophan hydroxylase. The changes in enzyme activity produced by clonidine at 25 (increased activity) or 90 (decreased activity) min after i.v. injection appear to be due primarily to changes in the apparent V_{max} of tryptophan hydroxylase. Pretreatment with clonidine (15 μg/kg, i.v.) for 25 min increased the apparent V_{max} of tryptophan hydroxylase significantly (30%), compared with enzyme from saline-treated animals. This was true for values of V_{max} obtained from experiments in which tryptophan concentration was varied in the presence of 300 μM $6MPH_4$ or in which the $6MPH_4$ concentration was varied in the presence of 500 μM L-tryptophan. No change in apparent K_m for substrate tryptophan was observed. However there was a very small *increase* in K_m for cofactor after clonidine treatment that was significant at the $P < 0.05$ level (Table 1). After a 90-min pretreatment with clonidine (15 μg/kg, i.v.) the apparent V_{max} of tryptophan hydroxylase was slightly lower than that of brainstem enzyme from saline-injected animals, but the values of the apparent K_m for substrate and artificial cofactor were both unchanged. Figure 3 shows Lineweaver–Burk plots of data from individual experiments after 25 and 90 min of clonidine or saline treatment.

Effect of clonidine on tryptophan hydroxylase activity in brainstem slices. Exposure of 250 μm slices of rat brainstem to various concentrations of clonidine for 10 min at 26° produced a concentration-dependent increase in the activity of tryptophan hydroxylase prepared from the slices. This increase in activity was significantly higher than controls at 500 nM clonidine (135% of controls) and reached a maximum at 100 μM clonidine (203% of controls). In no case did enzyme activity fall below control

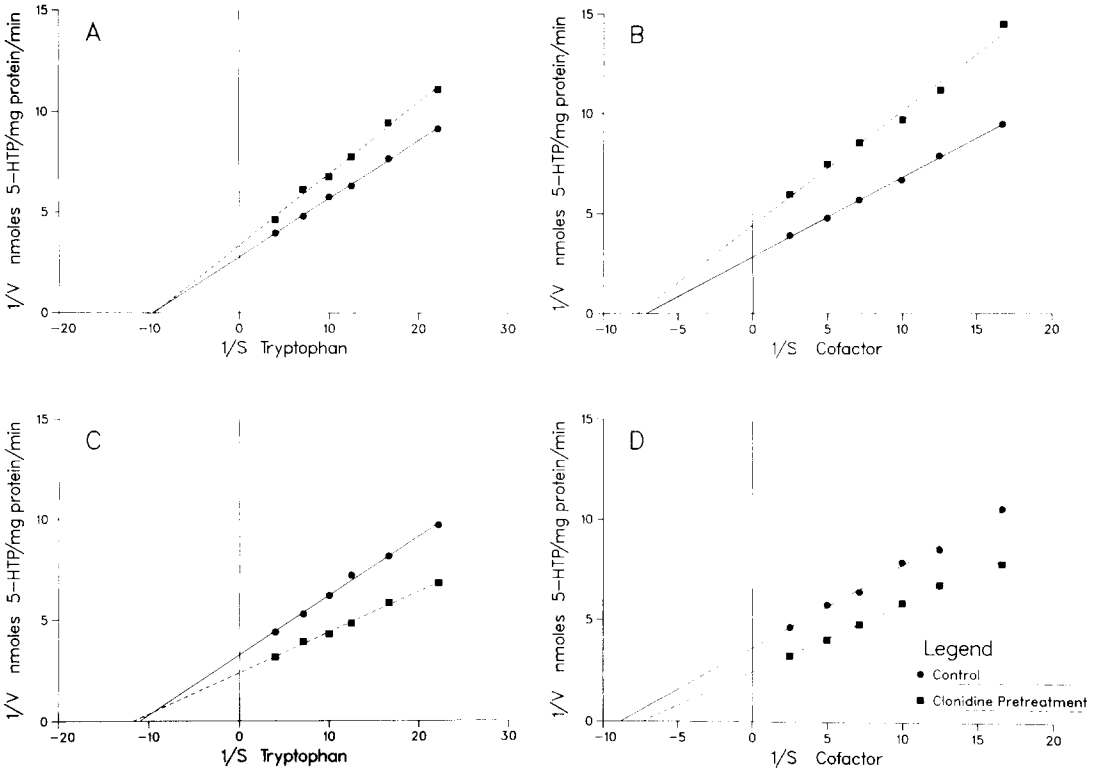

Fig. 3. Effect of clonidine on the kinetic properties of tryptophan hydroxylase. Results of two experiments involving clonidine · HCl treatment of 90 (panels A and B) and 25 (panels C and D) min duration (15 μg/kg, i.v.) or saline control. In each experiment brainstems were pooled from three drug-treated and three saline-treated rats, and the low speed supernatant fractions were assayed in duplicate in the presence of various concentrations of L-tryptophan and 300 μM cofactor, $6MPH_4$ (panels A and C) or various cofactor concentrations and 500 μM L-tryptophan (panels B and D). Abscissa: reciprocal of substrate or cofactor concentration, $[mM]^{-1}$; ordinate: $1/V$ in $[nmoles\ 5\text{-}HTP/mg\ protein/min]^{-1}$. Individual values of K_m and V_{max} from four to five experiments following 25- and 90-min drug treatments were determined by linear regression and averaged. These data ± standard error of the mean are summarized in Table 1.

Table 1. Effect of clonidine · HCl (15 μg/kg) on the kinetic properties of tryptophan hydroxylase 25 or 90 min after intravenous injection*

	Tryptophan†			$6MPH_4$‡		
	K_m	V_{max}	N	K_m	V_{max}	N
25 min						
Control	91 ± 6	313 ± 8	4	132 ± 6	315 ± 11	5
Clonidine	89 ± 7	423 ± 17	4	152 ± 5	447 ± 11	5
	NS	$P < 0.005$		$P < 0.05$	$P < 0.001$	
90 min						
Control	92 ± 7	345 ± 11	4	120 ± 4	288 ± 13	4
Clonidine	102 ± 4	290 ± 6	4	123 ± 11	226 ± 12	4
	NS	$P < 0.02$		NS	$P < 0.02$	

* In each experiment, enzyme was prepared from brainstems pooled from three saline- or clonidine-treated rats and was assayed in duplicate or triplicate at six different concentrations of substrate or cofactor. K_m and V_{max} values were determined by linear regression for each experiment, and the values were averaged and expressed as mean ± S.E.M. V_{max} is expressed as pmoles 5-HTP per mg protein per min; K_m is given in micromolar concentrations. Significance of the differences between enzyme from saline- and clonidine-injected animals was determined by Student's t-test. NS = not significant. N = number of individual experiments.

† Tryptophan concentration was varied in the presence of 300 μM $6MPH_4$.

‡ $6MPH_4$ concentration was varied in the presence of 500 μM L-tryptophan.

values (Fig. 4). Addition of these same concentrations of clonidine directly to the assay medium containing control enzyme had no effect on enzyme activity, thereby ruling out a direct effect of the drug on the enzyme (data not shown).

Effect of 6-OHDA pretreatment on the clonidine-mediated increase in tryptophan hydroxylase following acute in vivo *or* in vitro *administration.* The increase in the activity of brainstem tryptophan hydroxylase observed 25 min after intravenous clonidine (15 μg/kg) administration was blocked completely if the rats had been pretreated with 6-OHDA intracerebroventricularly 8 days prior to the clonidine administration. The increase in enzyme activity seen after incubation of brainstem slices with 100 μM clonidine was likewise blocked under these conditions (Table 2). Brainstem NE and 5-HT levels were assayed by gas chromatography-mass spectrometry on samples of brainstem homogenates used to prepare the tryptophan hydroxylase extract (Table 2). NE levels were depleted approximately 60% by 6-OHDA in saline- and clonidine-treated groups versus their respective vehicle-pretreated saline and clonidine controls. The fact that block of the clonidine-induced increase in enzyme activity was complete, even though amine depletion was incomplete, may be explained by the very much greater sensitivity of noradrenergic nerve terminals to the neurotoxic actions of 6-OHDA compared with cell bodies [25] coupled with the presumably essential role of the terminals as final relay for any noradrenergically mediated actions of clonidine on 5-HT neurons. It should be noted that the twitching reflex that occurs in untreated rats upon decapitation was abolished in all the 6-OHDA-treated animals [31]. There was a modest decline in brainstem 5-HT levels in the 6-OHDA-treated rats which probably represents neurotoxin-induced damage to some 5-HT neurons. However, it was not accompanied by any alteration in tryptophan hydroxylase activity compared with vehicle-treated, saline-injected controls.

Effect of clonidine on the levels of 5-HIAA in cerebral cortices in control and 6-OHDA-treated rats. There was a significant increase in cortical 5-HIAA levels in response to i.v. clonidine versus i.v. saline in control vehicle-treated rats. However, no effect of clonidine was observed on cortical 5-HIAA levels in the 6-OHDA-treated animals (Table 3).

Table 2. Effect of intracerebroventricular (ICV) pretreatment with 6-hydroxydopamine on the increase in tryptophan hydroxylase activity induced by clonidine given *in vivo* or *in vitro**

	Tryptophan hydroxylase activity (pmoles 5-HTP/mg protein/min)	
	ICV pretreatment	
	Vehicle	6-OHDA (200 μg)
(A) *In vivo* treatment:		
Saline (control)	177 ± 2 (5)	173 ± 5 (6)
Clonidine (15 μg/kg, 25 min)	314 ± 14† (6)	168 ± 5 (8)
(B) *In vitro:* slices		
Control	209 ± 2 (3)	210 ± 5 (3)
Clonidine 10^{-4} M	346 ± 5† (3)	210 ± 7 (3)

	Brainstem amine levels (ng/g fresh tissue)					
	NE			5-HT		
	ICV pretreatment			ICV pretreatment		
	Vehicle	6-OHDA	% Change	Vehicle	6-OHDA	% Change
(C) *In vivo* treatment:						
Saline (control)	367 ± 59 (5)	160 ± 11‡ (4)	−56	514 ± 26 (5)	433 ± 29¶ (6)	−16
Clonidine (15 μg/kg, 25 min)	407 ± 49 (5)	140 ± 20§ (8)	−65	560 ± 48 (6)	410 ± 31‖ (8)	−27

* Values are means ± S.E.M.; the number of animals is given in parentheses. Conditions for experiments A, B, and C are as follows. (A) 6-OHDA (200 μg free base dissolved in 20 μl of 0.9% saline containing 0.01% ascorbic acid) was injected into the right lateral ventricle under sodium pentobarbital anesthesia; controls received vehicle (20 μl of 0.9% saline containing 0.01% ascorbate). Eight days later pretreated rats were given i.v. clonidine in 0.9% saline (15 μg/kg) or i.v. saline and were killed 25 min later for determination of tryptophan hydroxylase activity in low speed supernatant extracts of brainstem. Brainstem 5-HT and NE levels (C) and cortical 5-HIAA (Table 3) levels were measured in the same rats, as described under Methods. (B) Brainstem slices (250 μm) from another group of 6-OHDA- or vehicle-pretreated rats were incubated for 10 min at 26° in the presence of 10^{-4} M clonidine. No amine determinations were made for this experiment. (C) 5-HT and NE levels were determined by GC-MS in aliquots of brainstem homogenate from rats treated with i.v. clonidine or i.v. saline (A) as described under Methods.

† Significantly different from saline-treated control values, $P < 0.001$.

‡–‖ Significantly different from vehicle-treated controls: ‡ $P < 0.02$, § $P < 0.005$ and ‖ $P < 0.05$.

¶ Not significant.

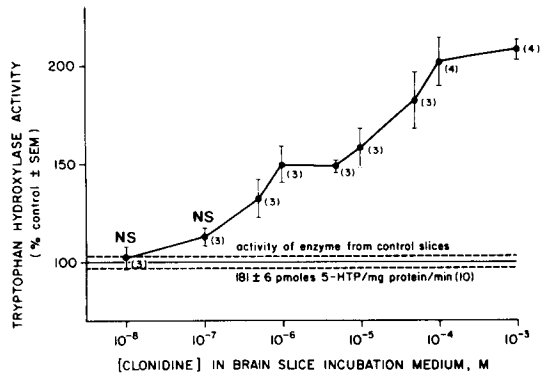

Fig. 4. Effect of various concentrations of clonidine·HCl on the activity of tryptophan hydroxylase from brainstem slices. Brainstem was removed, and 250 μM slices were incubated for 10 min at 26° in the presence of various concentrations of clonidine·HCl. The results are expressed as percent of control activity ± the standard error of the mean. Significance of the difference between clonidine-treated versus control slices was determined by Student's *t*-test. NS indicates not significantly different from controls. Other values: $P < 0.005$.

DISCUSSION

The results presented here were entirely unexpected and show that acute i.v. administration of clonidine produces an initial, rapid increase in brainstem tryptophan hydroxylase activity followed by a return to control levels about 90 min later, at low doses, or a depression of enzyme activity with doses above 10 μg/kg. In view of the relationship between enhanced 5-HT neuronal activity and activation of tryptophan hydroxylase [22, 23], it seemed logical that a decrease in the spontaneous rate of firing of 5-HT neurones which is known to reduce 5-HT synthesis *in vivo* [16] might decrease the activity of tryptophan hydroxylase detected *in vivo*. The reason why clonidine administration actually increased tryptophan hydroxylase activity at a time when dorsal raphe firing is reported to be suppressed (15 min after i.v. clonidine) is not clear. The recording studies on clonidine were, however, only made in the dorsal raphe and, even though there appears to be a CA input to all raphe nuclei [10], there is no evidence that the serotonergic perikarya present in these areas are all influenced in the same manner by clonidine. It is also possible that the responsiveness of 5-HT neurons in dorsal raphe to drugs under chloral hydrate anesthesia, which was used in the recording studies, may not be identical to that in unanesthetized animals [32] which were used in these studies. However, enzyme activity was still increased by i.v. clonidine in chloral hydrate anesthetized animals (L. B. Weekley and M. C. Boadle-Biber, unpublished observations). Our finding that 5-HIAA levels in cortices were increased 25 min after i.v. clonidine is consistent with enhanced firing of 5-HT neurons in the midbrain raphe but does not establish that this has, in fact, occurred. The earlier biochemical studies [7, 9, 12, 13] in which both 5-HIAA levels and 5-HT synthesis and turnover were found to be depressed by clonidine at longer time intervals (45 min–3 hr) also correlates with the secondary fall in tryptophan hydroxylase activity reported here at 90–120 min with >10 μg/kg, i.v., clonidine. The inhibition of dorsal raphe neuronal firing which Svensson *et al.* observed after i.v. clonidine (see Ref. 4) was of much briefer duration.

The short-lived increase in enzyme activity does not appear to involve a direct interaction of the drug with the enzyme, or with the 5-HT neurons themselves, for it is abolished after pretreatment of rats with 6-OHDA, thereby indicating that a CA-containing innervation is required. The experiments with brainstem slices suggest further that the presence of the CA-containing terminals (separated from their perikarya) are sufficient to mediate the clonidine-induced increase in tryptophan hydroxylase activity. One possibility is that clonidine alters baseline release of CA from terminals present in the slices, and that it is this interaction that triggers the change in tryptophan hydroxylase activity. The observation points to the involvement of the NE

Table 3. Effect of clonidine on cortical 5-HIAA levels with and without 6-OHDA pretreatment*

	Cortical 5-HIAA levels (ng/g fresh tissue)	
	ICV pretreatment	
	Vehicle	6-OHDA (200 μg)
In vivo treatment		
Saline (control)	298 ± 21 (5)	336 ± 20 (6)
Clonidine, (15 μg/kg, 25 min)	550 ± 44† (6)	334 ± 17 (8)

* 5-HIAA levels were determined by HPLC-EC in cerebral cortex removed from the 6-OHDA- and vehicle-pretreated rats on which brainstem tryptophan hydroxylase activity (Table 2A) was measured. Saline or clonidine was given intravenously 8 days after ICV pretreatment with vehicle or 6-OHDA. Each value is the mean ± S.E.M.; the number of animals is given in parentheses.

† Significantly different from saline-treated controls, $P < 0.001$.

terminals that innervate the raphe nuclei in mediating this action of clonidine on tryptophan hydroxylase. It also appears to rule out any direct postsynaptic actions of the drug (α_1) since they should persist after 6-OHDA treatment.

A recent publication by Conway and Jarrott [33] indicates that a single dose of clonidine (15 μg/kg) has a half-life of about 60 min in the brain and that clonidine levels in brain peak within 3 min. Enzyme activity at this dose also rises rapidly by 15 min and then declines over the next 45 min. Thus, the rise and fall in enzyme activity may simply reflect the rapid rise and fall in brain clonidine concentration. Conway and Jarrott also state that metabolites of clonidine are produced after acute drug administration but did not identify them. Whether such metabolites contribute to the action of the drug on tryptophan hydroxylase is not known.

The study of the kinetic properties of tryptophan hydroxylase at 25 and 90 min after i.v. administration of clonidine (15 μg/kg) revealed that the changes in enzyme activity may be accounted for by a modest increase or decrease in apparent V_{max}, respectively, without any marked changes in the affinity of the enzyme for its substrate or for the artificial reduced pterin cofactor $6MPH_4$.

Numerous other instances exist of treatments that alter the kinetic properties of tryptophan hydroxylase. For example, soluble preparations of the enzyme can be activated by exposure to phospholipids, detergents, and calcium-activated proteases, or incubation under phosphorylating conditions [34–37]. *In vivo*, depletion of brain tryptophan levels, either by experimentally induced diabetes or acute chlorimipramine administration, has been found to increase the apparent V_{max} of the enzyme without altering the affinity for substrate or cofactor [38–40], although the results with diabetic rats have been challenged [41]. Such an interaction seems unlikely to account for the present results, however, since Reinhard and Roth [9] reported no change in brain tryptophan levels after i.v. administration of clonidine. Recently, we found a small increase in V_{max} in cortical enzyme preparations in response to *in vivo* electrical stimulation (M. C. Boadle-Biber, J. Johannessen, N. Narasimhachari and T-H. Phan, to be published), and we and others also found a V_{max} change in response to potassium depolarization of brain slices in earlier studies [19, 20]. These findings would also be consistent with the interpretation that the clonidine treatment increased 5-HT neuronal activity. There is, however, no previous report of a treatment that can lower the V_{max} of tryptophan hydroxylase below that of control enzyme. However, if enzyme activity is regulated by phosphorylation by calcium-calmodulin dependent protein kinase, as has been suggested [36], then one would predict its activity to increase in response to neuronal firing when intraneuronal free calcium levels increase [42] and to fall when impulse flow is suppressed and intraneuronal free calcium levels fall. Since 5-HT neurons fire tonically, it is conceivable that the enzyme is in a partially activated state in untreated control animals, and that this could be reversed by treatments that block impulse flow. Whether such a mechanism could account for the decrease in tryptophan hydroxylase activity 90 min after acute i.v. clonidine is unknown. Furthermore, it does not resolve the discrepancy between the electrophysiological studies [4] and our biochemical results.

The results presented here suggest that clonidine interacted indirectly with 5-HT neurons in a way that altered the activity of the rate-limiting enzyme in 5-HT synthesis, tryptophan hydroxylase. This effect was unexpected in view of the reported suppressant actions of clonidine on 5-HT neuronal firing, and it requires further pharmacological characterization, e.g. with α_1 and α_2 antagonists and agonists. Whether interactions of clonidine with 5-HT neurons are involved in the expression of the hypotensive action or sedative side effects of the drug is not known, but it would not be unreasonable in view of the evidence that 5-HT neurons participate in sleep mechanisms and in blood pressure regulation [43], and in the action of other hypotensive drugs [44].

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