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INHIBITION OF TRYPTOPHAN HYDROXYLASE BY BENSERAZIDE AND OTHER CATECHOLS

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Abstract—Tryptophan hydroxylase (L-tryptophan, tetrahydropteridine:oxygen oxidoreductase [5-hydroxylating]; EC 1.14.16.4; TPH), the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin, was inhibited directly by benserazide, an inhibitor of aromatic-L-amino-acid decarboxylase (3,4-dihydroxy-L-phenylalanine carboxy-lyase; EC 4.1.1.28; AAAD). Benserazide was a competitive inhibitor for the pterin cofactor tetrahydrobiopterin and an uncompetitive inhibitor for the substrate tryptophan. NSD 1015, another decarboxylase inhibitor, did not directly inhibit TPH. Other compounds with catechol moieties in their structures such as 3,4-dihydroxyphenylalanine (DOPA), dopamine, apomorphine, and SKF 38393 were also found to be potent inhibitors of TPH. These results indicate that drugs or neurotransmitters with catechol structures directly inhibit the activity of TPH and add to a growing body of evidence indicating that endogenous dopamine can exert untoward effects on serotonin neurons, including inhibition of TPH. Furthermore, the use of decarboxylase inhibitors to cause the accumulation of 5-hydroxytryptophan as an *in vivo* measure of TPH activity could be problematic, particularly when drugs with catechol structures or dopamine-releasing compounds are also administered.

Inhibitors of aromatic-L-amino-acid decarboxylase (3,4-dihydroxy-L-phenylalanine carboxy-lyase; EC 4.1.1.28; AAAD) have a number of applications in neurochemical studies. By inhibiting the conversion of L-DOPA to dopamine (DA) or that of 5-hydroxytryptophan (5-HTP) to serotonin (5-HT), AAAD inhibitors cause the hydroxylated amino acids to accumulate, and the extent of their accumulation either *in vitro* or *in vivo* is used as a measure of tyrosine or tryptophan hydroxylation respectively. While studying the regulation of tryptophan hydroxylase (L-tryptophan, tetrahydropteridine:oxygen oxidoreductase [5-hydroxylating]; EC 1.14.16.4; TPH), the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter 5-HT, we observed in HPLC assays that 5-HTP was not converted to 5-HT *in vitro*. In addition, certain AAAD inhibitors appeared to inhibit TPH. Recently, Reinhard and Shearin [1] reported that the AAAD inhibitor benserazide (Ro 4-4602) inhibited tyrosine hydroxylase (L-tyrosine, tetrahydropteridine:oxygen oxidoreductase [3-hydroxylating]; EC 1.14.16.2; TH), leading these investigators to caution the use of AAAD inhibitors in the *in vivo* assay of tyrosine hydroxylation. Our results indicate that benserazide, and a number of other compounds with catechol moieties in their structures, are also relatively potent inhibitors of TPH.

MATERIALS AND METHODS

Benserazide was provided by Hoffmann-LaRoche (Nutley, NJ) and 3-hydroxylbenzyl hydrazine (NSD 1015), dopamine, and 3,4-dihydroxyphenylalanine (DOPA) were purchased from the Sigma Chemical Co. (St. Louis, MO). Apomorphine and SKF 38393 were purchased from Research Biochemicals Inc. (Natick, MA). Tetrahydrobiopterin was obtained from Dr. B. Shircks (Jona, Switzerland). All other chemicals were of the highest grade commercially available. The DOPA was free of DA contamination as determined by HPLC with electrochemical detection.

TPH activity was determined in extracts of rat brain mesencephalic tegmentum. This brain area contains the dorsal and median raphe nuclei, the cell bodies of origin of most forebrain 5-HT projections [2] and is very high in TPH activity [3]. Rats were decapitated and dissected brains were frozen and stored at −135° until assayed. Frozen tegmenta were weighed and homogenized in 4 vol. of 0.05 M Tris buffer, pH 7.4, containing 1 mM dithiothreitol and 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA). Homogenates were centrifuged at 40,000 *g* and the resulting supernatant was desalted on a 0.8 × 18 cm column of Sephadex G-25 equilibrated with the same homogenizing buffer. The desalted extracts were used as the source of TPH activity without further purification. Protein was determined by the method of Bradford [4].

The activity of TPH was determined as previously described [5] with the following modifications for HPLC. Approximately 30 μg of protein was added to tubes containing various concentrations of potential TPH inhibitors (see below), and TPH reactions were initiated with the addition of tryptophan and tetrahydrobiopterin (BH_4). Each assay tube contained the following substituents (in

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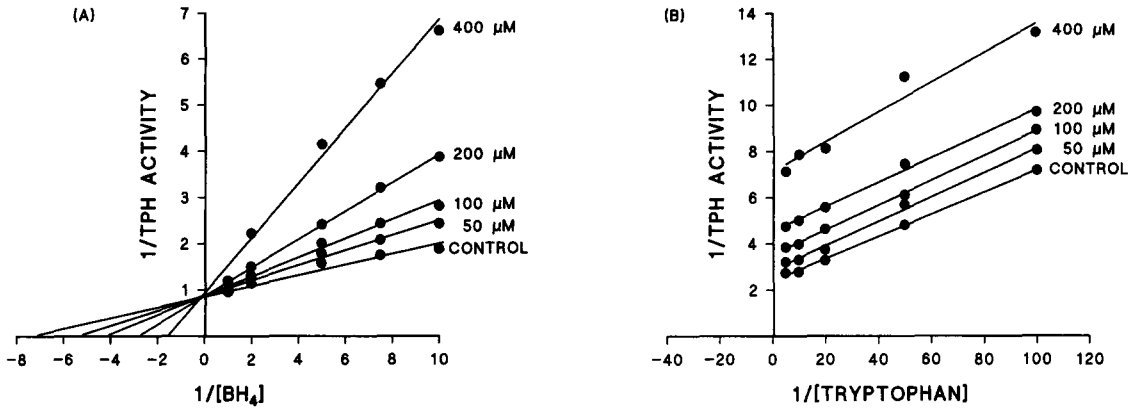

Fig. 1. Inhibition of tryptophan hydroxylase by benserazide. Kinetic properties of TPH were determined by (A) varying BH_4 from 10 to 1000 μM at a fixed tryptophan concentration of 250 μM, and (B) varying tryptophan from 10 to 400 μM at a fixed BH_4 concentration of 200 μM. The concentrations of benserazide are adjacent to the appropriate regression line. TPH activity is the reciprocal of the ng of 5-HTP produced in the 10-min assay.

the indicated final concentrations) in a volume of 100 μL: 0.05 M Tris buffer, pH 7.4, 1 mM EGTA, 15 μg catalase, and various concentrations of tryptophan and BH_4. Tubes were incubated at 37° for 10 min after which reactions were terminated by the addition of 10 μL of 6 N $HClO_4$. Precipitated protein was removed by centrifugation in a Beckman Minifuge B for 5 min, and all samples were then diluted 1:10 in 0.01 N HCl, 5 mM EDTA, and 0.1% (w/v) ascorbic acid. The amount of 5-HTP formed was determined by HPLC with fluorescence detection as previously described [6]. Tubes not containing tryptophan or BH_4 served as blanks. Potential inhibitors of TPH were prepared in 0.01% (w/v) ascorbic acid and controls included the solvent as well.

The kinetic properties of TPH were determined by varying tryptophan over a concentration range of 10–400 μM at a constant BH_4 concentration of 200 μM. BH_4 was varied from 10 to 1000 μM at a constant tryptophan concentration of 250 μM. Enzyme kinetic data were analyzed as described in Segal [7].

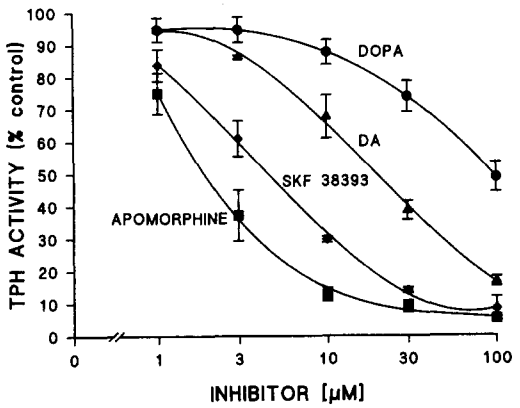

Fig. 2. Inhibition of tryptophan hydroxylase by catechols. TPH activity was determined in the presence of the indicated inhibitors (concentrations plotted on a logarithmic scale) using a BH_4 concentration of 100 μM and a tryptophan concentration of 250 μM. The data are expressed as percent of control (no inhibitor) and are the means ± SEM of four experiments. Control TPH activity was 0.65 ± 0.03 nmol 5-HTP/mg protein in 10 min.

RESULTS

Tegmental extracts did not contain 5-HTP, 5-HT, or 5-hydroxyindoleacetic acid (5-HIAA) after desalting. Incubation of extracts in the presence of tryptophan and BH_4 led to the production of 5-HTP without the appearance of 5-HT or 5-HIAA. Thus, the TPH assay conditions did not promote the activity of endogenous AAAD, allowing the accumulation of 5-HTP to serve as a reliable index of tryptophan hydroxylation *in vitro*. Two frequently used inhibitors of AAAD were added to the assay mixture to determine if they altered TPH activity. The results in Fig. 1 indicate that benserazide was indeed a relatively potent inhibitor of TPH. Benserazide was a competitive inhibitor with regard to BH_4 (panel A) with a K_i of 80 ± 10 μM. Benserazide displayed inhibition of the uncompetitive type with regard to tryptophan (panel B) with a K_i of 176 ± 13 μM. NSD 1015 did not alter the activity of TPH at concentrations up to 1.0 mM (data not shown).

Benserazide is a catechol, and since earlier studies demonstrated that catecholamines can inhibit TPH *in vitro* [8, 9], the abilities of several other catechol-containing compounds to interact with TPH were also tested. These results are presented in Fig. 2. It was assumed that these compounds, like benserazide, would be competitive for BH_4 so these tests were carried out with BH_4 at a concentration of 100 μM. DOPA was inhibitory with an IC_{50} of approximately 95 μM. DA and the DA receptor agonists apomorphine and SKF 38393 were also quite potent

inhibitors of TPH. DA exhibited an IC_{50} of 19 μM, while apomorphine was the most potent inhibitor presently tested with an IC_{50} of 2.3 μM. SKF 38393 was intermediate in potency to apomorphine and DA with an IC_{50} of 4.4 μM.

DISCUSSION

The present results indicate that *in vitro* assays of TPH do not require the use of AAAD inhibitors when the enzyme source is diluted or desalted since the levels of the AAAD cofactor, pyridoxal phosphate, are probably too low to support AAAD activity. AAAD inhibitors with catechol structures, such as benserazide, should be avoided in *in vitro* assays since they can inhibit TPH at concentrations below those necessary to fully inhibit AAAD. The mechanism by which benserazide inhibits TPH is identical to the manner in which this AAAD inhibitor interacts with TH [1].

Benserazide is used *in vivo* to cause the accumulation of 5-HTP as a measure of tryptophan hydroxylation, and could certainly enter 5-HT neurons and inhibit TPH through a direct interaction with the enzyme (presumably by competing with BH_4). Reinhard and Shearin [1] estimated that the brain concentrations of benserazide required to inhibit AAAD reach 200 μM, clearly sufficient to cause substantial inhibition of TPH *in vivo*. Thus, the use of benserazide as an AAAD inhibitor in studies of *in vivo* TPH should be discouraged for the same reasons discussed for TH by Reinhard and Shearin [1].

The ability of catechol-containing compounds to inhibit TH has been well characterized [1, 10, 11], but the interaction of such agents with TPH has not been explored as extensively. The structural similarities shared by TPH and TH [12] would lead one to predict that catechol compounds would also interact with the BH_4 binding site in each monooxygenase. Indeed, the DA agonists apomorphine and SKF 38393 are potent inhibitors of TPH *in vitro* and they could certainly interact with TPH *in vivo* after systemic or direct intracerebral injection (e.g. *in vivo* dialysis).

Although catecholamines are potent inhibitors of TPH *in vitro* [8, 9], one must question whether endogenous catecholamine levels could be increased sufficiently within 5-HT neurons to inhibit TPH *in vivo*. Nissbrandt *et al.* [13] demonstrated that the levels of DOPA can reach as high as 16 μM in the striatum, depending on the AAAD inhibitor used. Considering that DOPA undergoes substantial efflux from neurons [13], it is conceivable that it could enter 5-HT neurons and inhibit TPH, albeit weakly (see Fig. 2), after total AAAD inhibition. Pharmacological treatment of animals with L-DOPA or the use of L-DOPA in the therapy of Parkinson's disease could result in the production of large amounts of DA in 5-HT neurons, via the decarboxylation of exogenous DOPA by AAAD. Under these conditions, DOPA and especially DA could exert strong inhibitory effects on TPH. Finally, the use of Madopar (levodopa plus benserazide) in the treatment of Parkinson's disease might compromise the functioning of the 5-HT neuronal system due to the presence of benserazide in this preparation.

It is known that DA which has been released by drugs can be taken up into 5-HT nerve endings [14, 15]. Gibb and colleagues [16, 17] have demonstrated that the psychoactive drugs methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA), which are DA-releasing agents, cause significant inhibition of TPH *in vivo*. Prior depletion of brain DA prevents the inhibitory effects of these drugs on TPH [16, 17]. Thus, the concentration of DA within 5-HT nerve endings can reach high and possibly toxic levels *in vivo* via synthesis from exogenous DOPA or through the uptake of released (drug-induced) DA.

Estimates of *in vivo* tryptophan hydroxylation through measures of 5-HTP accumulation after AAAD inhibition could be compromised through the direct (i.e. benserazide) or indirect (e.g. NSD 1015) interaction of the AAAD inhibitors with TPH. Other DA agonists with catechol moieties are also potent inhibitors of TPH. Interpretation of results where DA agonists (e.g. apomorphine, SKF 38393) or DA releasing agents (e.g. MDMA, cocaine) alter 5-HT production in brain, especially in the model using AAAD inhibitors, should recognize that non-receptor mediated events can lead to alterations in 5-HT neurochemistry through direct inhibition of TPH. The present results also add to the growing body of evidence that DA can exert untoward effects on the 5-HT neuronal system via inhibition of TPH.

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