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Activation of brain tryptophan hydroxylase by ATP–Mg^{2+}: Dependence on calmodulin

(serotonin biosynthesis/protein phosphorylation/calcium/tryptophan 5-monooxygenase)

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ABSTRACT **Tryptophan hydroxylase [tryptophan 5-monooxygenase, L-tryptophan,tetrahydropterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4] is activated by phosphorylating conditions (ATP–Mg^{2+}) in a calcium-dependent, cyclic AMP-independent manner. Addition to the phosphorylation reaction of certain antipsychotic drugs that bind to calmodulin, the heat-stable calcium-binding protein, prevents the activation of tryptophan hydroxylase by ATP–Mg^{2+} in a concentration-dependent fashion. External addition of purified calmodulin protects the enzyme from the drug-induced effects. Calmodulin-free tryptophan hydroxylase prepared by affinity chromatography on fluphenazine-Sepharose is not activated by ATP–Mg^{2+} whereas addition of calmodulin to calmodulin-free enzyme restores the responsiveness of the hydroxylase to ATP–Mg^{2+} only in the presence of Ca^{2+}. These results indicate that the activation of tryptophan hydroxylase by phosphorylating conditions is dependent on both calcium and calmodulin.**

Tryptophan hydroxylase [tryptophan 5-monooxygenase, L-tryptophan,tetrahydropterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4] is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin (1). Recent studies in our laboratory (2) and in the laboratories of others (3, 4) have demonstrated that this important enzyme can be activated by ATP–Mg^{2+}. This apparent phosphorylation of tryptophan hydroxylase is cyclic nucleotide independent and calcium dependent (2, 3). Implicit in these studies was a potential role for a protein kinase that was dependent on calmodulin, the heat-stable calcium-binding regulator protein (5). In fact, a recent report (6) has demonstrated a role for calmodulin in the activation of tryptophan hydroxylase. In this study we report that various neuroleptic agents that inhibit calmodulin-dependent reactions block the activation of tryptophan hydroxylase by ATP–Mg^{2+}. Furthermore, removal of calmodulin from hydroxylase-containing brain extracts by affinity chromatography renders the activation of the enzyme by phosphorylating conditions absolutely dependent on addition of exogenous calmodulin.

MATERIALS AND METHODS

Assay for Tryptophan Hydroxylase. Male Sprague–Dawley rats (150–200 g) from Taconic Farms (Germantown, NY) were killed by decapitation, and the mesencephalic tegmentum was rapidly dissected from the rest of the brain, frozen on dry ice, and stored in liquid N_2. Frozen brain tissue was weighed and homogenized in conical glass homogenizers in 5 vol of 50 mM Tris·HCl, pH 7.4/2.0 mM dithiothreitol/250 μM Ca^{2+}. Homogenates were centrifuged at 40,000 × *g* for 15 min at 4°C, and the supernatant fraction was used as the enzyme source. Tryptophan hydroxylase was assayed by a modification (7) of the method of Friedman *et al.* (8). The standard reaction mixture contained the following substituents in a total volume of 50 μl: 120–130 μg of tissue extract, 50 mM Tris·HCl (pH 7.4), 2.0 mM dithiothreitol, 100 μM Ca^{2+}, 400 international units of catalase, 0.1 mM 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin (subsaturating), and 0.4 mM L-tryptophan (saturating). Reactions were started by addition of all substituents all at once to the enzyme. Each reaction component was prepared in 50 mM Tris·HCl (pH 7.4), except for the 6-methyltetrahydropterin, which was dissolved in 10 mM HCl and catalytically reduced under hydrogen in the presence of platinum oxide as described (9). Each tube was incubated for 15 min at 37°C, and the reactions were terminated by addition of 10 μl of 6 M perchloric acid. Precipitated protein was removed by centrifugation, and a 40-μl aliquot of the supernatant fraction was added to 100 μl of 8 M HCl. The fluorescence of the solution was measured in an Aminco–Bowman spectrophotofluorometer at excitation–emission wavelengths of 295–540 nm. The amount of 5-hydroxytryptophan formed enzymatically was calculated from a standard curve of authentic 5-hydroxytryptophan carried through the entire procedure. The hydroxylation of tryptophan is linear with respect to the time parameters and protein concentration used. In experiments in which phosphorylating conditions were studied, ATP (0.5 mM) and magnesium acetate (5.0 mM) were dissolved in 50 mM Tris·HCl (pH 7.4) and added to the reaction mixture. Protein concentrations were determined by the method of Bradford (10) with bovine serum albumin as standard.

Purification of Brain Calmodulin by Affinity Chromatography. In some cases brain extracts were chromatographed by affinity chromatography on a matrix with fluphenazine covalently bound to Sepharose-4B. The fluphenazine-Sepharose matrix was prepared by the method of Charbonneau and Cormier (11). Quantitative estimates of the amount of fluphenazine bound to Sepharose were made by the method of Ragland and Kinross-Wright (12) after hydrolysis of a weighed portion of gel in 6 M HCl. These estimates indicated that approximately 0.30 μmol of fluphenazine was bound to 1 g of suction-dried Sepharose. For chromatography, the gel was equilibrated with 50 mM Tris·HCl, pH 7.4/2.0 mM dithiothreitol/250 μM Ca^{2+} by passing 10–20 bed volumes of buffer over a 0.5 × 5.0 cm packed column. The amount of brain extract chromatographed (in the same buffer) never exceeded 50% of the column bed volume to avoid overloading the gel. Under these conditions, only calmodulin binds to fluphenazine-Sepharose in a calcium-dependent manner (see gels below) and tryptophan hydroxylase emerges in the effluent. Calmodulin is subsequently eluted with excess ethylene glycol bis(β-

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). For use in the enzyme assay, calmodulin purified by affinity chromatography was dialyzed exhaustively against 0.1 mM Ca^{2+}. The biological activity of calmodulin was assessed by testing its ability to stimulate the Ca^{2+}-dependent phosphorylation of synaptic plasma membranes (13).

Gel Electrophoresis. Proteins were solubilized and electrophoresed on $NaDodSO_4$/polyacrylamide slab gels (10 × 14 × 0.15 cm). The acrylamide concentration was 6% (wt/vol) in the stacking gels and 10% (wt/vol) in the resolving gels. Both stacking and resolving gels contained $NaDodSO_4$ at a final concentration of 0.1%. The running and gel buffers were prepared by the method of O'Farrell (14). After electrophoresis the gels were fixed and stained for protein with 0.1% Coomassie brilliant blue R250 in 50% methanol/10% acetic acid (vol/vol). Gels were destained by diffusion in 30% methanol/10% acetic acid (vol/vol) before they were dried.

Drugs and Chemicals. The following drugs were obtained from the indicated sources: penfluridol, pimozide, and haloperidol (McNeil Laboratories, Fort Washington, PA); chlorpromazine and trifluoperazine (Smith, Kline & French); fluphenazine (Squibb); and *cis*-flupenthixol (H. Lundbeck and Co., Copenhagen). Penfluridol, pimozide, and haloperidol were prepared for use each day in 20% (vol/vol) ethanol (5% ethanol concentration in final reaction mixture), and even at this solvent concentration, penfluridol and pimozide formed cloudy solutions. All other drugs were prepared fresh in distilled H_2O. Other chemicals were obtained from commercial sources. The pterin cofactor 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin was prepared as described (9) and standardized spectrophotometrically by means of the molar extinction coefficient (in acid) of 1.6×10^4 at 265 nm (15). Sepharose 4B was obtained from Pharmacia Fine Chemicals.

RESULTS

Effects of Neuroleptic Drugs on Activation of Tryptophan Hydroxylase. Levin and Weiss (16, 17) have demonstrated that a variety of drugs that are used clinically as antipsychotic agents bind to calmodulin in a calcium-dependent manner. Therefore, several of these drugs were tested *in vitro* for their ability to alter the activation of tryptophan hydroxylase by ATP-Mg^{2+}. The results presented in Fig. 1 indicate that all drugs tested were effective to varying degrees in blocking the activation. The drugs used and the order of their inhibitory potencies (IC_{50}) are as follows: pimozide > fluphenazine > trifluoperazine > *cis*-flupenthixol > haloperidol > penfluridol > chlorpromazine. The results with pimozide and penfluridol must be considered somewhat tentative because of the relative insolubility of these two drugs in aqueous solvents. With the exception of pimozide and penfluridol, however, the order of potency of these drugs for inhibiting the activation of tryptophan hydroxylase is similar to the order of their potencies for binding (affinity) to calmodulin (17). Various other drugs, including theophylline, morphine, naloxone, lysergic acid diethylamide, desmethylimipramine, and propranolol, did not inhibit the ATP-Mg^{2+} effect on tryptophan hydroxylase at a concentration of 100 μM (data not shown).

Purified calmodulin was added to reaction mixtures containing fluphenazine, trifluoperazine, or *cis*-flupenthixol (50 μM for each). The results presented in Fig. 2 indicate that the inhibition of the phosphorylation effect by these drugs can be effectively prevented by increasing amounts of calmodulin. As little as 50 ng resulted in a notable effect and up to 1.0 μg of calmodulin provided almost complete protection against the drug-induced inhibition of the effect of phosphorylating conditions on tryptophan hydroxylase. These data suggest that the basis for the inhibitory action of at least fluphenazine, trifluoperazine, and *cis*-flupenthixol is related to their ability to bind to calmodulin in the brain extracts. Because calmodulin does not reverse EGTA-induced inhibition of the phosphorylation effect (data not shown), the possibility that the antipsychotic drugs are inhibiting the activation of tryptophan hydroxylase by chelating Ca^{2+} seems unlikely.

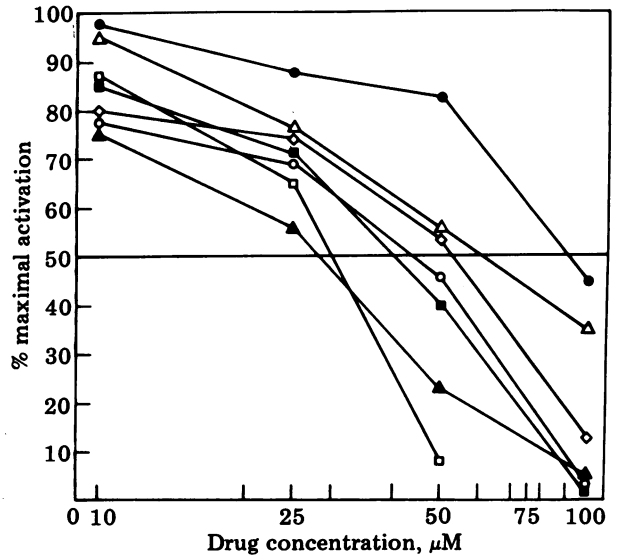

FIG. 1. Drug-induced inhibition of the effects of phosphorylating conditions on tryptophan hydroxylase. Drugs were added in the final concentrations listed on the abscissa. Drugs used were: ●, chlorpromazine; △; penfluridol; ■, fluphenazine; □, trifluoperazine; ◇, haloperidol; ○, *cis*-flupenthixol; and ▲, pimozide. Each point represents the mean of three separate experiments for each drug run in duplicate. Data are expressed as % maximal activation, calculated by dividing the activity under phosphorylating conditions in the presence of drug by the activity under phosphorylating conditions in the absence of drug.

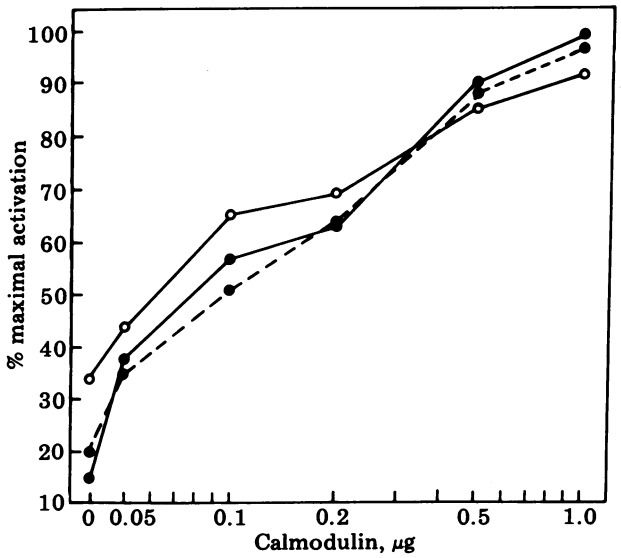

FIG. 2. Protection against drug-induced inhibition of phosphorylation of tryptophan hydroxylase by calmodulin. Phosphorylation reaction mixtures were prepared and *cis*-flupenthixol (○—○), fluphenazine (●—●), or trifluoperazine (●---●) was added in a concentration of 50 μM. Calmodulin was added to the reaction mixture and reactions were carried out as described. The amount of calmodulin added was estimated from the protein concentration, determined by the A_{280}/A_{260} ratio. Each point represents the mean of three separate experiments for each drug run in duplicate.

Affinity Chromatography. Results of experiments with affinity chromatography of the brain extracts on fluphenazine-Sepharose are presented in Table 1. If brain extracts were chromatographed on the affinity gel in the presence of Ca^{2+}, tryptophan hydroxylase emerged in the effluent. However, these enzyme preparations were no longer activated by phosphorylating conditions. Elution of the column with EGTA (2.5 mM) revealed that a single protein (see below) had bound to the matrix in a calcium-dependent manner. Addition of this eluant protein (presumably calmodulin), after dialysis against 0.1 mM Ca^{2+}, to the enzyme preparation that had been chromatographed on fluphenazine-Sepharose restored the responsiveness of the hydroxylase to ATP-Mg^{2+} (Table 1). This eluted fraction was devoid of tryptophan hydroxylase activity.

Careful inspection of the $NaDodSO_4$/polyacrylamide gels in Fig. 3 indicates that chromatography of the brain extracts on fluphenazine-Sepharose specifically removed calmodulin from the extract whereas all other proteins, including tryptophan hydroxylase, passed through in the effluent. Lane a in Fig. 3 represents the tegmental supernatant before chromatography; lane b represents the effluent proteins; lane c contains the protein fraction eluted with EGTA; and lane d is purified calmodulin prepared by the method of Klee (18). Despite some minor differences in the staining patterns, it is apparent that endogenous calmodulin was resolved from all other proteins in the brain extract (compare lanes a and b at arrow) and could be recovered from the column by elution with EGTA. The electrophoretic behavior of calmodulin purified by affinity chromatography was identical to that of calmodulin purified from pig brain acetone powder by a different method (compare lanes c and d), and the calmodulin in lane c, in the presence of Ca^{2+}, markedly enhanced the phosphorylation of specific proteins in synaptic plasma membranes (data not shown).

DISCUSSION

Our previous results clearly indicated that the activation of tryptophan hydroxylase by phosphorylating conditions (ATP-Mg^{2+}) was a calcium-dependent process (2). The present data have substantially extended these earlier findings by demonstrating that the activation of tryptophan hydroxylase is totally dependent on calmodulin. The role of calmodulin in modulating the activation of tryptophan hydroxylase is another example of a rapidly expanding group of biochemical reactions that are now known to be regulated by this important calcium-binding protein. This phenomenon has been investigated by two approaches.

Table 1. Phosphorylation effects on tryptophan hydroxylase after chromatography on fluphenazine-Sepharose

Enzyme	Addition	Assay conditions: Control	Assay conditions: Phosphorylating
Control	—	5.21 ± 0.07	8.44 ± 0.40
Chromatographed	—	5.65 ± 0.21	5.72 ± 0.18
	Calmodulin	5.32 ± 0.04	9.22 ± 0.42
	Calmodulin + EGTA	5.20 ± 0.15	4.62 ± 0.19

Control enzyme or enzyme chromatographed on fluphenazine-Sepharose was assayed under control or phosphorylating (0.5 mM ATP; 5.0 mM Mg^{2+}) conditions. Calcium was present at 100 μM. Calmodulin was purified by affinity chromatography and 1.0 μg was added where indicated. Calmodulin alone had no tryptophan hydroxylase activity. The concentration of EGTA was 200 μM. Enzyme activity is expressed as nmol of 5-hydroxytryptophan per mg per 15 min and represents the mean ± SEM of six separate experiments run in duplicate.

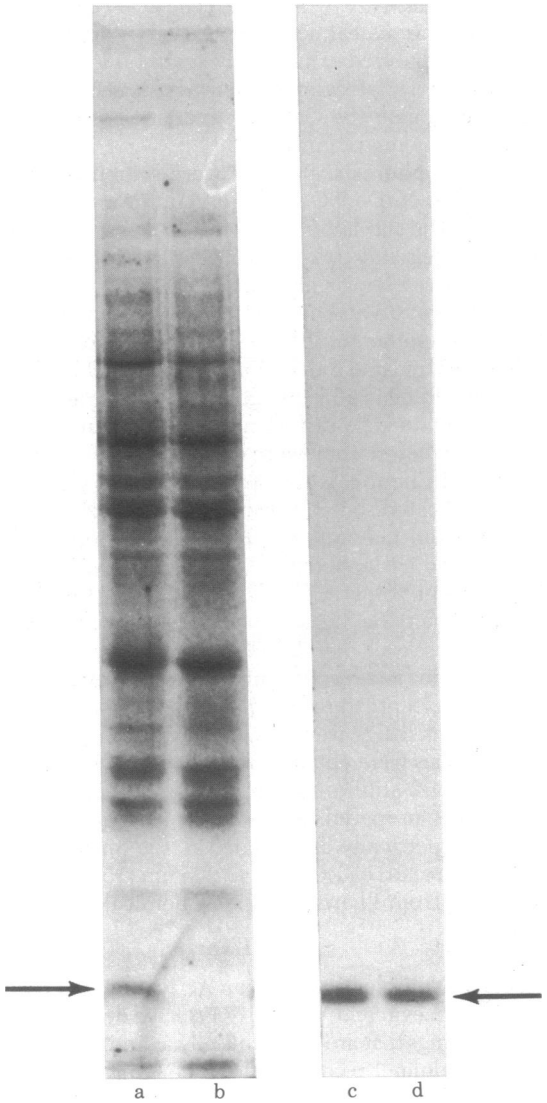

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of brain proteins. Lane a, extract (100 μg of protein) before chromatography on fluphenazine-Sepharose; lane b, effluent (100 μg of protein) from fluphenazine-Sepharose; lane c, EGTA eluant (2 μg of protein) from affinity column after dialysis against 0.1 mM Ca^{2+}; lane d, purified calmodulin (2 μg of protein) from pig brain acetone powder prepared by the method by Klee (18). Arrows indicate the position of calmodulin.

First, a number of clinically effective antipsychotic drugs from a variety of chemical classes were effective in inhibiting the activation of tryptophan hydroxylase by phosphorylating conditions. The ability of purified calmodulin to protect the enzyme from the inhibitory effect of the antipsychotic drugs suggests that these drugs block the phosphorylation effect by binding to endogenous calmodulin. Levin and Weiss (16, 17) have shown that many of the drugs presently used bind to calmodulin *in vitro* in the presence of calcium, and these investigators have concluded that the ability of antipsychotic drugs to inhibit calmodulin-activated phosphodiesterase (19, 20) and catecholamine-sensitive adenylate cyclase (21, 22) also results from the binding of the drugs to calmodulin. The ability of antipsychotic drugs to bind calmodulin may prove to be an important mechanism of action of these drugs, especially when the role of calmodulin in a variety of enzymatic and cellular processes is considered (23). Recent findings suggest that certain antipsychotic drugs may lower 5-hydroxyindoleacetic acid in

the cerebrospinal fluid of manic patients (24). Such a result would be anticipated if one of the actions of these drugs was to block the activation of tryptophan hydroxylase *in vivo*.

Second, the effects of chromatography of the brain extracts on fluphenazine-Sepharose on the responsiveness of tryptophan hydroxylase to phosphorylating conditions were studied. The fluphenazine-Sepharose affinity gel was quite effective in binding endogenous calmodulin, thereby resulting in a calmodulin-free tryptophan hydroxylase. This chromatographed enzyme preparation did not respond to phosphorylating conditions until calmodulin was added back into the reaction mixtures. Reconstitution of the phosphorylation effect on tryptophan hydroxylase was calcium dependent.

In summary, the process by which brain tryptophan hydroxylase is activated under *in vitro* conditions that favor protein phosphorylation is strictly dependent on both calcium and calmodulin. The stimulation of tryptophan hydroxylase activity by Ca^{2+}-dependent proteolysis (25) was not changed by the removal of calmodulin (unpublished observations), ruling out a proteolytic mechanism in the foregoing results. Furthermore, because it was also observed that the basal activity of tryptophan hydroxylase was not altered significantly by affinity chromatography on fluphenazine-Sepharose (Table 1), it appears that a cyclic AMP-independent, Ca^{2+}-dependent protein kinase confers calcium and calmodulin dependence on the ATP-Mg^{2+}-induced activation process.

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