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CALCIUM-DEPENDENT ACTIVATION OF TRYPTOPHAN HYDROXYLASE BY ATP AND MAGNESIUM

Donald M. Kuhn*, Robert L. Vogel and Walter Lovenberg

Section on Biochemical Pharmacology
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20014

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SUMMARY

Tryptophan hydroxylase [EC 1.14.16.4; L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating)] in rat brainstem extracts is activated 2 to 2.5-fold by ATP and Mg^{++} in the presence of subsaturating concentrations of the cofactor 6-methyltetrahydropterin ($6MPH_4$). The activation of tryptophan hydroxylase under these conditions results from a reduction in the apparent K_m for $6MPH_4$ from 0.21 mM to 0.09 mM. The activation requires Mg^{++} and ATP but is not dependent on either cAMP or cGMP. The effect of ATP and Mg^{++} on enzyme activity was enhanced by μM concentrations of Ca^{++} and totally blocked by EGTA. These data suggest that tryptophan hydroxylase can be activated by a cyclic nucleotide independent protein kinase which requires low calcium concentrations for the expression of its activity.

INTRODUCTION

Tryptophan hydroxylase [EC 1.14.16.4; L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating)] catalyzes the initial and rate limiting step in the biosynthesis of the neurotransmitter serotonin (1). Studies in several laboratories have demonstrated that Ca^{++} can activate tryptophan hydroxylase in crude brainstem extracts (2,3,4) via an apparent Ca^{++}-dependent proteolytic reaction (3). Incubation of brain extracts with ATP, Mg^{++}, and cAMP has also been found to activate tryptophan hydroxylase by a process which possibly involves protein phosphorylation (3). In the current experiments we have investigated the potential interaction of these two enzyme activation systems. It appears that the activation of tryptophan hydroxylase by Ca^{++}-dependent proteolysis and by phosphorylating conditions represent distinct phenomena: the former requires high Ca^{++} concentrations and results in a reduction of the apparent K_m for both substrate and cofactor (4) whereas the latter is stimulated by very low Ca^{++} concentrations and affects only cofactor

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kinetics. While this manuscript was in preparation, two additional, independent reports appeared (5,6) describing results which are largely in agreement with the data presented below.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Zivic Miller) weighing 150-200 g were sacrificed by decapitation. The mesencephalic tegmentum and tectum, hypothalamus, septal nuclei, and pineal gland were rapidly dissected from the brain, frozen on dry ice, and stored in liquid nitrogen until used for enzyme assays.

6-Methyltetrahydropterin, dimethyltetrahydropterin($DMPH_4$) and tetrahydrobiopterin(BH_4) were purchased from Regis Chemical Co. Stock solutions (approximately 0.01 M) of these compounds were prepared (7) and standardized spectrophotometrically by means of the molar extinction coefficient of 1.6×10^4 at 264 nm.

Frozen brain regions were weighed and homogenized in conical glass homogenizers in 4 volumes of 0.05 M Tris-HCl (pH 7.4) containing 0.002 M dithiothreitol. Pineals (in pairs) were homogenized in 130 µl of the same buffer. Homogenates were centrifuged at 40,000 x g for 20 min. and the supernatant fraction was used as the enzyme source. Tryptophan hydroxylase activity was measured by a modification (8) of the method of Friedman, Kappelman and Kaufmann (9). The standard reaction mixture contained the following in a total volume of 50 µl: 145-155 µg of tissue extract, 2.5 µmol of Tris-HCl (pH 7.4), 0.01 µmol of dithiothreitol, 400 units of catalase (bovine liver) and 0.02 µmol of ℓ-tryptophan. Reactions were initiated with the addition of the cofactor (usually 5 nmol) and incubations were carried out for 30 min. at 37°C. Reactions were terminated by the addition of 10 µl of 6 N 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 at excitation/emission wavelengths of 295/540 nm. The amount of 5-hydroxytryptophan (5-HTP) formed was calculated from a standard curve of 5-HTP carried through the entire procedure. Reaction tubes incubated without cofactor served as blanks.

In experiments where phosphorylating conditions were studied, the following components were added to the reaction mixtures: ATP, 25 nmol; cAMP, 10 nmol: and magnesium acetate, 0.5 µmol.

In some experiments, Ca^{++}-dependent neutral protease activity was determined by the method of Guroff (10). Protein was estimated by the method of Lowry et al. (11).

RESULTS

The exposure of rat midbrain tryptophan hydroxylase to phosphorylating components results in a 2.0 - 2.5-fold stimulation of catalytic activity. ATP, cAMP and Mg^{++} reduce the apparent K_m for $6MPH_4$ from 0.21 mM to 0.09 mM while having no effect on V_{max} (13.8 nmol/mg/30 min). Similar kinetic studies with tryptophan (25×10^{-6}M to 4×10^{-4}M) at a constant cofactor concentration (8×10^{-4}M) indicated that phosphorylation does not change the K_m for tryptophan (control K_m = .080 mM; phosphorylation K_m = .084 mM).

Table 1. Requirements for the stimulation of tryptophan hydroxylase

CONDITION	Tryptophan hydroxylase activity nmol 5-HTP/mg/30 min
Control	5.99 ± .38 (5)
Phosphorylating conditions	9.68 ± .76 (5)
-Mg^{++}	6.58 ± .47 (5)
-ATP	5.94 ± .57 (5)
-cAMP	10.10 ± 1.0 (5)
-cAMP + db-cAMP (0.2 mM)	7.75 ± .42 (4)
-cAMP + cGMP (0.2 mM)	9.82 ± .72 (4)
-ATP + AMP-P(NH)P (0.5 mM)	5.21 ± .86 (2)

Activity was measured under standard assay conditions using 0.1 mM $6MPH_4$ as cofactor. Results are expressed as the mean ± S.E.M. The number of experiments (assayed in duplicate) is given in parenthesis. PO_4 = phosphorylating conditions.

The requirements for the phosphorylation-induced increase in tryptophan hydroxylase are presented in Table 1. It is apparent that the stimulation is largely dependent on both ATP and Mg^{++}. The omission of cAMP did not reduce the magnitude of the stimulation. Neither db-cAMP nor cGMP had any further effect on the ATP-Mg^{++} activation. The addition of either ATP, cAMP, or Mg^{++}, alone, did not increase enzyme activity. Moreover, the ATP analog AMP-P(NH)P could not replace ATP in the activation reaction.

The addition of purified cAMP-dependent protein kinase (bovine heart) was also without further stimulation. The cAMP-dependent protein kinase inhibitors adenosine, adenine, ADP and the heat stable protein kinase modulator (bovine heart) were tested for antagonism of the ATP-Mg^{++} effect. Only adenosine produced a slight (13%) but nonsignificant reversal. All other inhibitors were without effects.

The ATP-Mg^{++} effect on tryptophan hydroxylase was not confined to the

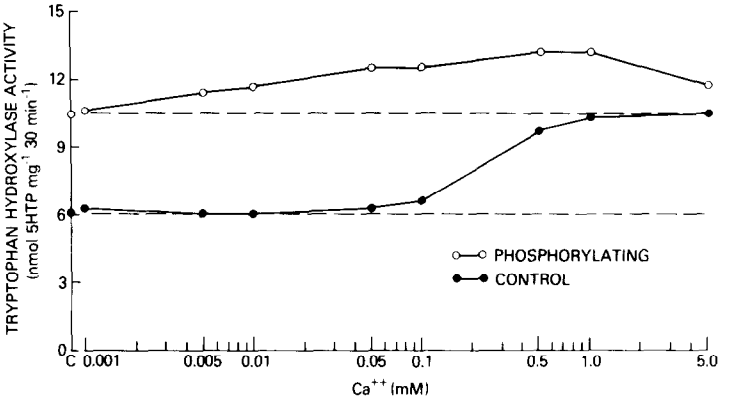

Figure 1. The effects of Ca^{++} on the phosphorylation induced activation of tryptophan hydroxylase. Ca^{++} was added in the indicated concentrations and tryptophan hydroxylase was assayed as described in Methods under control or phosphorylating conditions. The dashed lines represent phosphorylating (top) and control (bottom) conditions. The $6MPH_4$ concentration was 0.1 mM.

mesencephalic tegmentum. The stimulation was also observed in other brain areas including the mesencephalic tectum, hypothalamus, and septum, as well as in the pineal when subsaturating concentrations of $6MPH_4$ were used. Furthermore, the ATP-Mg^{++} effect was apparent in the tegmentum when subsaturating concentrations of either BH_4 or $DMPH_4$ were used in place of $6MPH_4$ (data not shown).

The role of Ca^{++} in the ATP-Mg^{++} activation was also assessed. Figure 1 demonstrates that Ca^{++} in concentrations as low as 5-10 μM can stimulate the ATP-Mg^{++} effect. It is known that much larger concentrations of Ca^{++} are required to activate the protease which is virtually inactive at Ca^{++} concentrations below 0.5 mM (10). Homogenization of brain tissue in the presence of EGTA (10 mM) prevented the activation of tryptophan hydroxylase by ATP-mg^{++} which, in turn, could be reinstated by the addition of Ca^{++} (Table 2). The addition of EGTA (0.1 - 2.0 mM) to reaction mixtures would also block the activation. Since Ca^{++} can activate both the hydroxylase and protease, it was important to determine if ATP and Mg^{++} affected tryptophan hydroxylase by

Table 2. Effects of EGTA of the ATP-Mg^{++} activation of tryptophan hydroxylase

CONDITION	Tryptophan hydroxylase activity nmol 5-HTP/mg/30 min
EGTA Homogenate	2.4
+ ATP-Mg^{++}	2.6
+ Ca^{++} (5.0 mM)	3.7
+ ATP-Mg^{++} + Ca^{++} (5.0 mM)	5.4

Tegmental tissue was homogenized in the normal buffer containing 10 mM EGTA and tryptophan hydroxylase was assayed in the 40,000 x g supernatant as described in Methods. The $6MPH_4$ concentration was 0.1 mM. The final concentration of EGTA in the reaction mixture was 2.0 mM. The results presented are the means of duplicate determinations. Similar results have been observed in three separate experiments.

stimulating the Ca^{++}-dependent protease. Table 3 demonstrates that Ca^{++} (5.0 mM) increased both hydroxylase and protease activity whereas phosphorylating conditions only increased the activity of the hydroxylase.

DISCUSSION

The activation of tryptophan hydroxylase by ATP and Mg^{++} suggests that the activity of this enzyme may be regulated by a protein phosphorylation reaction. The kinetic changes observed with tryptophan hydroxylase are similar to those previously reported for tyrosine hydroxylase incubated in the presence of a protein phosphorylation system (7,12-17). The major difference between these phenomena is that tryptophan hydroxylase activation is not dependent on cAMP whereas the activation of tyrosine hydroxylase is. The possibility that a cAMP dependency was being masked by endogenous cAMP was examined by chromatographing tegmental extracts on a Sephadex G-25 column (0.9 x 25 cm). This technique, which removes all free cAMP, had no effect on the ability of ATP and Mg^{++} to activate the enzyme.

Calcium and ATP-Mg^{++} apparently activate tryptophan hydroxylase by differ-

Table 3. The effects of Ca^{2+} and phosphorylating conditions on the activity of tryptophan hydroxylase and Ca^{2+}-dependent neutral proteinase from rat midbrain

CONDITION	Tryptophan hydroxylase Activity	Proteinase Activity
	nmol 5-HTP/mg/30 min	nmol TYR/mg/30 min
Control	4.90 ± .27	2.42 ± .20
Ca^{2+} (5.0 mM)	8.44 ± .61	5.27 ± .12
Phosphorylating	9.8 ± .83	2.46 ± .15

Assays were carried out as described in Methods. Results are expressed as the mean ± S.E.M. of three separate experiments assayed in duplicate in the presence of 0.1 mM $6MPH_4$.

ent mechanisms. In high concentrations, Ca^{++} stimulates a tryptic-like proteolytic cleavage of tryptophan hydroxylase (3) which results in a reduction of the K_m for both substrate and cofactor (4). The ATP-Mg^{++} effect requires and can be enhanced by low Ca^{++} concentrations and changes only cofactor kinetics. These kinetic data are supported by the results of Lysz and Sze (6) whereas Hamon et al. (5) found that phosphorylating conditions apparently change the K_m for tryptophan. The lack of dependence on cAMP and the Ca^{++} requirement in the ATP-Mg^{++} effect on tryptophan hydroxylase suggests that this cation is either directly stimulating a protein kinase or generating an active kinase from a prokinase. The latter contention recently received experimental support. Inoue et al., (18) described a cyclic nucleotide independent protein kinase in rat brain which is produced from its proenzyme by the action of a Ca^{++}-dependent protease. This protein kinase, like the ATP-Mg^{++} effect (Table 1), is not sensitive to cAMP-dependent protein kinase inhibitors.

Examination of protein kinase activity using ATP-γ-^{32}P as the ^{32}P donor, with either endogenous proteins or histone as the ^{32}P acceptors, indicated that our tryptophan hydroxylase extracts also contain significant amounts of cAMP independent protein kinase. In agreement with Inoue et al., (18), the cAMP

independent protein kinase activity is greatly enhanced by a short preincubation with Ca^{++} (unpublished observations).

Several lines of evidence suggest that the Ca^{++}-dependent activation of tryptophan hydroxylase by ATP-Mg^{++} may serve as a mode of regulation of this important enzyme in vivo. For example, the ATP-Mg^{++} effect is observed with enzyme from several different brain areas, it occurs with the presumed natural pterin cofactor (BH_4) as well as with synthetic analogs ($DMPH_4$ and $6MPH_4$), and it is enhanced by very low Ca^{++} concentrations. Although Ca^{++} can apparently stimulate tryptophan hydroxylase via proteolysis (3), this is not a likely mode of regulation due to the large Ca^{++} concentrations (mM) required and the irreversible nature of a proteolytic effect.

Thus, it is possible to speculate that tryptophan hydroxylase may be activated by a cAMP-independent protein kinase which, in turn, is either dependent upon Ca^{++} or is activated, as described (18), by a Ca^{++}-dependent protease. Since our experiments were carried out using a crude enzyme source, additional studies using purified tryptophan hydroxylase, the cAMP independent prokinase (18), and Ca^{++}-dependent protease are necessary so that a more complete understanding of the role of Mg^{++} and ATP in regulating tryptophan hydroxylase will be possible.

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