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ACTIVATION OF TRYPTOPHAN HYDROXYLASE FROM CENTRAL SEROTONERGIC NEURONS BY CALCIUM AND DEPOLARIZATION

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Abstract—The activity of tryptophan hydroxylase in a low speed supernatant preparation from rat thalamus, midbrain and medulla pons, assayed in the presence of subsaturating concentrations of 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine ($6MPH_4$), was enhanced 100 per cent by addition of 10 mM $CaCl_2$ to the reaction medium. A similar increase in activity was also observed if $CaCl_2$ (10 or 100 mM final concn) was added directly to the enzyme and the unbound $CaCl_2$ then removed by gel filtration on Sephadex G-25. This increase in activity could not be reversed by the addition of ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA). It was associated with a modest increase in V_{max} (16 per cent) and an approximate doubling of the affinity of the enzyme for substrate and cofactor (apparent K_m for tryptophan decreased from 79 to 46 μM; apparent K_m for $6MPH_4$ decreased from 148 to 101 μM). An increase in activity was also observed when tryptophan hydroxylase was prepared from slices of the brain preparation which had been (1) depolarized with a medium containing 66 mM KCl; (2) incubated overnight in an Na^+-free medium or (3) treated with ouabain (0.05 or 0.1 mM), procedures believed to increase free intraneuronal levels of Ca^{2+}. The increase in activity was, however, not observed when the slices were incubated in a Ca^{2+}-free, K-rich medium to which 100 μM EGTA had been added. A kinetic analysis of the enzyme prepared from slices depolarized in K^+ revealed an increase in V_{max} and a decrease in the apparent K_m for substrate (74–45 μM) and artificial cofactor (177–118 μM). The activation observed after depolarization could not be reversed with EGTA.

Tryptophan hydroxylase [EC 1.14.16.4 tryptophan-5-monooxygenase; L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating)] is the rate-limiting enzyme in the formation of 5-hydroxytryptamine (5-HT) and as such determines the overall rate of 5-HT synthesis in the serotonergic neurones of the mammalian central nervous system [1, 2]. Studies on the properties of this enzyme and of the factors which determine its activity *in vivo* are thus of great importance for understanding ways in which the supply of 5-HT may be regulated in serotonergic neurones and matched to ongoing physiological requirements. Investigations on the regulation of 5-HT synthesis have shown that tryptophan hydroxylase is not saturated by its substrate tryptophan under physiological conditions since tryptophan loading *in vivo* increases brain levels of both 5-HT and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) [1, 2]. In fact the K_m of the partially purified enzyme in the presence of the presumed natural cofactor, tetrahydrobiopterin, is 50 μM [3] while the concentration of tryptophan in brain, estimated by assuming a uniform distribution throughout this tissue, is approximately 30 μM [4, 5]. Thus, any process which alters the concentration of tryptophan in serotonergic neurones would be predicted to change the ongoing rate of 5-HT synthesis. So far, three major factors have been identified which have a significant role in determining tryptophan levels within the central nervous system. These are the free plasma tryptophan concentration [5–8] which is in turn determined by the dietary intake of tryptophan [8], the concentration of tryptophan relative to other neutral amino acids which compete for the same uptake site at the neuronal membrane [8, 9], and the uptake process itself [10, 11] which may be altered in the presence of drugs [12, 13]. Although tryptophan levels clearly have an important role in determining ongoing rates of 5-HT synthesis, there is other evidence suggesting that neuronal activity is a more important factor in producing rapid adjustments in synthesis rate *in vivo*.

In 1968 Sheard and Aghajanian [14] found that electrical stimulation of the serotonergic perikarya in the midbrain raphe nuclei of the rat produces a rapid increase in the forebrain levels of the 5-HT metabolite 5-HIAA. An initial drop of 20 per cent in endogenous 5-HT occurred during the first 15 min of stimulation, but no further decrease was observed thereafter, even though 5-HIAA levels continued to rise. The authors therefore inferred that synthesis of 5-HT had increased to compensate for the enhanced release and metabolism of 5-HT. Subsequently, Shields and Eccleston [15] demonstrated that the formation of 5-HT from radiolabeled tryptophan was increased by electrical stimulation of the raphe neurons in a frequency-dependent manner. In these experiments, electrical stimulation altered neither the concentration nor specific acti-

vity of tryptophan in the brain. Thus, changes in tryptophan hydroxylase activity rather than substrate availability appeared to be responsible for the enhanced synthesis of 5-HT seen during nerve stimulation. This interpretation was consistent with an earlier finding that nerve stimulation increased both 5-HT and 5-HIAA levels even when tryptophan levels were not rate limiting[16]. Estimates *in vivo* of brain tryptophan hydroxylase activity can be made more directly by measuring the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) in the presence of an aromatic amino acid decarboxylase inhibitor[17]. Under these conditions, electrical stimulation of the midbrain raphe causes a marked increase in the accumulation of 5-HTP in the forebrain, again without any change in the levels of endogenous tryptophan[18]. Thus, all the evidence suggests that the activity of tryptophan hydroxylase, like that of the closely related enzyme tyrosine hydroxylase, which is rate limiting in the synthesis of catecholamines[19–21], is increased during neuronal depolarization.

Preliminary studies have shown that tryptophan hydroxylase can be activated by addition of Ca^{2+} to low speed supernatant preparations[22, 23]. Since the increase in 5-HT synthesis from tryptophan which occurs in response to neuronal depolarization is a Ca^{2+}-dependent phenomenon[24], this Ca^{2+}-dependent activation of tryptophan hydroxylase has been studied in detail and the results are reported here. In addition the present studies show that tryptophan hydroxylase activity is enhanced when the enzyme is prepared from depolarized slices of brain tissue containing serotonergic neurones and that this activation, like the enhanced synthesis of 5-HT, is a Ca^{2+}-dependent process.

MATERIALS AND METHODS

Materials. Purified bovine liver catalase in water with 0.5% thymol (activity 1.57×10^6 i.u./ml at 30°), dithiothreitol (DTT, A grade), NADPH (A grade), synthetic cofactor, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine ($6MPH_4$), and ouabain (Strophanthin G).$8H_2O$ were obtained from CalBiochem, San Diego, CA. D- and L-Tryptophan, ammonium sulfate (enzyme grade) and Tris base (ultrapure) were products of Schwarz Mann, Orangeburg, NY. Ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) and 5-HTP were purchased from the Sigma Chemical Co., St Louis, MO; brocresine (3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate) was a gift from Lederle Laboratories, Pearl River, NY. All other reagents were of maximal commercially available purity.

Dissection of brain tissue. Male Sprague–Dawley rats (Flow Laboratories, Rockville, MD) weighing 150–200 g were killed by decapitation. The brains were removed and immediately placed on ice. For the source of enzyme a preparation consisting of thalamus, midbrain and medulla pons was used. To obtain this, the cerebellum was cut away and discarded, the cerebral hemispheres were pushed back and a cut was made just behind the level of the anterior commissure. In some experiments, preparations dissected from single brains were sliced and incubated prior to isolation of the enzyme supernatant; in other experiments the dissected tissue from three to ten rat brains was pooled and used immediately to prepare the enzyme.

Preparation and incubation of brain tissue slices. The freshly dissected, weighed, ice-cold tissue from individual rats was placed on moistened filter paper mounted on the stage of a Dupont Sorvall MT-2 tissue sectioner and chopped into slices 250 μm thick. The slices were scooped into an 18-ml polycarbonate 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, 1 mM; glucose, 10 mM; and Tris acetate, pH 7.4, 10 mM. To depolarize slices, 40 per cent of the NaCl was replaced by equimolar KCl. In some experiments $CaCl_2$ was omitted and EGTA added to the medium to remove remaining traces of Ca^{2+}. In other experiments the NaCl was replaced entirely by sucrose (300 mM), or ouabain was added to the medium. If the slices were to be preincubated in one type of medium, they were separated from this medium at the end of the preincubation period by filtration using Whatman number 1 filter paper and gently lifted into fresh medium. The centrifuge tubes containing the slices were then placed in a water bath maintained at 26° and shaken at 140 strokes/min. In preliminary experiments, slices were incubated at 37° for 15 and 30 min, but this was found to cause almost total loss of enzyme activity. At 26° loss of tryptophan hydroxylase activity was about 30 per cent after 15 min of incubation and increased to 50 per cent after 30 min. Initially the incubation medium was bubbled with 100% oxygen, with shaking. However, there was no difference between the activity of tryptophan hydroxylase isolated from slices incubated in control or K-enriched media which had simply been shaken in room air, and the activity of enzyme from slices which had been shaken and gassed with 100% oxygen. Therefore, the incubations were carried out in air with shaking. At the end of the 10-min incubation period the slices were spun down at 10,000 rpm (12,000 *g*) for 5 min in a Dupont Sorvall RC-5 refrigerated centrifuge. The supernatant was decanted and the pellet used to prepare the crude tryptophan hydroxylase extract.

Preparation of tryptophan hydroxylase extract. Freshly dissected, weighed brain tissue pooled from up to ten animals, or individual pelleted slice preparations were homogenized with 0.05 M Tris acetate buffer, pH 7.4 (1:1.5, w/v) using a Kontes Duall all-glass homogenizer (clearance 0.01 cm) and the homogenate was centrifuged at 18,000 rpm (39,000 *g*) for 30 min in a Dupont Sorvall RC-5 centrifuge at 4°. DTT, final concentration 2 mM, was added to the resulting low speed supernatant which was then passed over a column of Sephadex G-25 (coarse type, Pharmacia, Piscataway, NJ) equilibrated with 0.05 M Tris acetate, pH 7.4, containing 2 mM DTT at 7° (cold room temperature). The column size was 25×2.5 cm for large batches of enzyme and 22×1.3 cm for enzyme prepared from individual sliced brain preparations. This gel filtration step was necessary to reduce the fluorescence of the blank samples in the tryptophan hydroxylase assay. The protein fraction (pink) which emerged in

the void volume was collected and used immediately or frozen at − 70° and stored at − 20° for up to 3 days.

Preparation of dihydropteridine reductase. Dihydropteridine reductase (EC 1.6.99.7) was prepared from sheep liver through the first ammonium sulfate fraction according to the method of Kaufman [25]. The sheep livers were obtained from Pel-Freez Biologicals, Rogers, AR and maintained at − 20° until the time of preparation. The enzyme preparation was dissolved in a volume of 0.025 M Tris–HCl, pH 7.4, equivalent to one-seventh the original volume and dialyzed overnight against 0.01 Tris–HCl, pH 7.4. The dialyzed preparation was quick-frozen in 3.0-ml batches and stored at − 20° until use.

Tryptophan hydroxylase assay. Tryptophan hydroxylase was assayed by the formation of 5-HTP according to the procedure of Friedman *et al.* [3] with some minor modifications: glucose 6-phosphate and glucose 6-phosphate dehydrogenase were omitted and brocresine was used instead of NSD 1034 [*N'*-(3-hydroxybenzyl)-*N'*-methylhydrazine] as the aromatic amino acid decarboxylase inhibitor. Sheep liver pteridine reductase was added in the initial studies on Ca^{2+} activation. However, the presence of the liver enzyme plus NADPH did not enhance tryptophan hydroxylase activity more than with NADPH alone, presumably because adequate amounts of the dihydropteridine reductase were already present in the low speed supernatant preparation from the brain tissue. Therefore, in the experiments on enzyme prepared from incubated brain slices, the sheep liver enzyme was omitted; however, NADPH was always added to the assay medium. The reaction was carried out in a volume of 300 μl and contained in μmoles: Tris acetate, pH 7.4, 12; DTT, 0.12; brocresine, 0.04; NADPH, 0.06; $6MPH_4$, 0.015 or 0.03 (in experiments to test for enzyme activation); sheep liver pteridine reductase, 20 μl (800 μg protein); catalase 1000 units; and enzyme supernatant 15–80 μl. In initial studies, divalent cations (e.g. Ca^{2+}, Ba^{2+} and Mg^{2+} as their chloride salts) were added directly to the reaction medium. Later $CaCl_2$ (10 or 100 mM) was added to the enzyme preparation at 4° after it had been passed over Sephadex G-25. In this case, both control enzyme and enzyme to which $CaCl_2$ had been added were then passed over Sephadex a second time, prior to assay, in order to remove the unbound Ca^{2+} from the treated enzyme (see Results). After a 10-min preincubation period at 37° with shaking, the reaction was initiated by the addition of substrate, L-tryptophan (0.03 or 0.06 μmole in the enzyme activation studies); D-tryptophan was added to the blanks. The reaction was stopped with 30 μl of 70% perchloric acid and the precipitated protein removed by centrifugation in a Beckman microfuge, model B, using 1.5-ml disposable plastic centrifuge tubes. The native fluorescence of 5-HTP was measured by adding 50 μl of concentrated HCl to 200 μl of the clear supernatant using a Farrand model 801 spectrophotofluorometer, excitation 305 nm, emission 535 nm. Internal standards were run in a complete blank reaction mixture.

Kinetics were determined on the linear portion of the time course and protein concentration curves. Estimations of the K_m of $6MPH_4$ and tryptophan were made using the method of Lineweaver and Burk [26]. The reaction was linear for up to 25 min and from 80 to 500 μg protein. Protein was determined by the method of Lowry *et al.* [27] using bovine serum albumin as standard.

Determination of Ca^{2+} in tissue and enzyme preparations. Reagents and samples were, wherever possible, handled in plasticware which had been rinsed with EGTA (0.1%) and de-ionized water. Any glassware used was first soaked in concentrated (70%) perchloric acid for 1 hr and rinsed with de-ionized water. Protein was precipitated from tissue homogenates or supernatant by adding an equal volume of ice-cold trichloroacetic acid, chilling on ice and centrifuging for 10 min at 10,000 rpm (12,000 *g*). To 200 μl of clear supernatant were added 1.6 ml of de-ionized, distilled water and 200 μl of 10% lanthanum chloride. The standard Ca^{2+} solution was prepared by dissolving a known amount of $CaCO_3$ in concentrated HCl and adjusting the volume with de-ionized, distilled water. Standards and water blanks also contained 1% lanthanum chloride and 1% trichloroacetic acid. Samples were read at 423 nm using an atomic absorption spectrophotometer (model 353, Instrumentation Laboratory Inc., Lexington, MA).

RESULTS

Effect of Ca^{2+} and other divalent cations on the activity of tryptophan hydroxylase from rat thalamus, midbrain and medulla pons. Figure 1 summarizes the effect of increasing concentrations of $CaCl_2$ on the activity of tryptophan hydroxylase prepared from a homogenate of thalamus, midbrain and medulla pons. The low speed supernatant enzyme preparation was incubated with different Ca^{2+} concentrations for 5 min prior to starting the enzyme reaction by addition of the substrate L-tryptophan. In this experiment the concentration of tryptophan was 200 μM and that of $6MPH_4$ was 50 μM. An increase in enzyme activity of similar magnitude was also obtained in the presence of $CaCl_2$ using 100 μM $6MPH_4$ and 100 μM tryptophan in the assay. A maximal increase in tryptophan hydroxylase activity of 220 per cent of control was obtained with 10 mM $CaCl_2$ under both assay conditions.

Another divalent cation, Ba^{2+}, enhanced tryptophan hydroxylase activity by about 50 per cent at a concentration of 10 mM. Mg^{2+}, on the other hand, was found to be without any significant effect.

Addition of the chelating agent, EGTA, did not reverse the increase in enzyme activity seen with Ca^{2+}. In a concentration of 1 or 10 mM, EGTA reduced control enzyme activity by 40 per cent. However, the same concentration of EGTA added to the enzyme in the presence of Ca^{2+} produced an even more pronounced inhibition. For example, enzyme activity was increased 70 per cent by 1 mM $CaCl_2$ but was reduced to less than 20 per cent of the control value when 1 mM EGTA was added together with 1 mM $CaCl_2$.

Sephadex G-25 chromatography of enzyme activated by addition of $CaCl_2$. In these experiments $CaCl_2$ (100 mM final concn) was added directly to

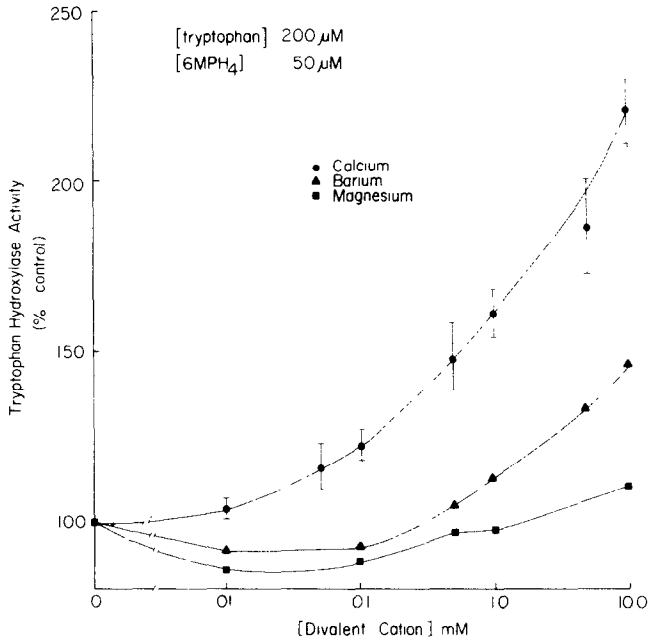

Fig. 1. Effect of increasing concentrations of Ca^{2+}, Ba^{2+} and Mg^{2+} on the activity of tryptophan hydroxylase. The divalent cations were added directly to the assay medium and the reaction was carried out in the presence of 200 μM tryptophan and 50 μM $6MPH_4$. The results are expressed as per cent of control activity (9.6 ± 0.6 ng 5-HTP/mg of protein/min). The values obtained in the presence of Ba^{2+} and Mg^{2+} are the mean of duplicate determinations: those for Ca^{2+} are the mean ± the standard error of the mean of a minimum of four determinations.

the enzyme preparation at 4°. A sample of this enzyme was saved and the rest was passed over a column of Sephadex G-25 at 7° to remove unbound Ca^{2+}. Untreated enzyme was also run over the Sephadex column to serve as control. From Table 1 it can be seen that the activity of enzyme from which unbound Ca^{2+} was separated was as great as that of enzyme from which the $CaCl_2$ (100 mM) had not been removed or of control enzyme to which 10 mM $CaCl_2$ was added in the assay medium. Furthermore, no increase in activity was observed after addition of 10 mM $CaCl_2$ to the assay medium of the Ca^{2+}-treated chromatographed enzyme. The total Ca^{2+} concentrations of the control and Ca^{2+}-treated enzyme preparations were 4 and 65 μM, respectively, after Sephadex chromatography.

Two attempts were made to reverse the increase in enzyme activity observed after addition of 100 mM $CaCl_2$ followed by Sephadex chromatography. The first involved the addition of increasing concentrations of EGTA to the enzyme assay medium.

Table 1. Activation of tryptophan hydroxylase by addition of calcium—effect of Sephadex G-25 chromatography*

Enzyme pretreatment	Tryptophan hydroxylase activity additions to assay	
	None	$CaCl_2$† (10 mM)
None‡	15.2 ± 0.4	29.9 ± 2.3
$CaCl_2$§ (100 mM)	31.1 ± 2.1	32.5 ± 1.0
Sephadex G-25 chromatography‖	12.3 ± 0.4	21.1 ± 0.7
$CaCl_2$¶ (100 mM) followed by Sephadex G-25 chromatography	34.7 ± 0.7	30.9 ± 2.2

* Tryptophan hydroxylase activity was determined in the presence of 100 μM tryptophan and 100 μM $6MPH_4$ and is expressed as ng 5-HTP/mg of protein/min. Each value is the mean ± the standard error of the mean of eight determinations.

† $CaCl_2$ was added to the assay medium to determine the extent of activation produced by the pretreatment with 100 mM $CaCl_2$.

‡ Enzyme was prepared as described in Materials and Methods.

§ $CaCl_2$ (final concn 100 mM) was added to enzyme preparation maintained at 4° until assayed.

‖ Enzyme was passed over Sephadex G-25 for the second time. The final concentration of Ca^{2+} was 4 μM.

¶ $CaCl_2$ was added to the enzyme preparation at 4° and enzyme was then passed over Sephadex G-25 (second time). The final concentration of Ca^{2+} was 65 μM.

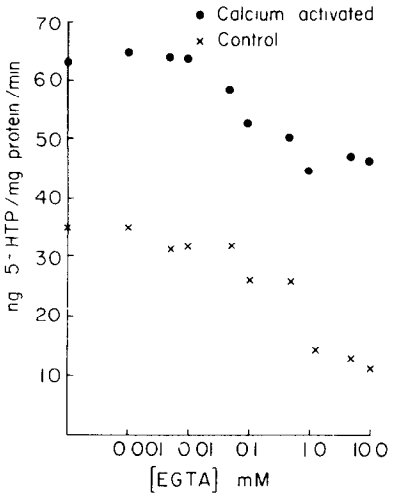

Fig. 2. Effect of increasing concentrations of EGTA on the activity of control and calcium-activated tryptophan hydroxylase. $CaCl_2$ (10 mM) was added directly to the low speed supernatant preparation which was then chromatographed over Sephadex G-25. EGTA was added to the reaction medium. The Ca^{2+} concentrations of the control and activated enzyme preparations after gel filtration were 2 and 65 μM respectively.

In this experiment both the control and the Ca^{2+}-activated enzymes were inhibited in parallel (Fig. 2). However, the EGTA failed to reverse the Ca^{2+}-induced activation of the enzyme. The second approach was used to try to avoid the inhibition seen when EGTA was included in the assay medium. In this experiment, EGTA was added directly to the control and Ca^{2+}-activated enzymes immediately after Sephadex chromatography, and was then promptly separated from these enzyme preparations together with any chelated Ca^{2+} by a further gel filtration step. The concentration of EGTA chosen (100 μM) was twice the total Ca^{2+} concentration of the Ca^{2+}-activated enzyme after gel filtration. Even under these conditions, 20 per cent inhibition of both control and activated enzymes was observed. More importantly, however, the activation produced by Ca^{2+} was not reversed.

The activity of the enzyme which had been treated with 100 mM $CaCl_2$ followed by chromatography on Sephadex G-25 was stable to freezing and thawing and could be kept frozen for about 3 days without serious loss of activity. However, the enzyme was completely inactivated if it was frozen without first removing the unbound Ca^{2+}. An increase in tryptophan hydroxylase activity of similar magnitude was obtained after the addition of 10 mM instead of 100 mM $CaCl_2$ to the enzyme followed by Sephadex chromatography.

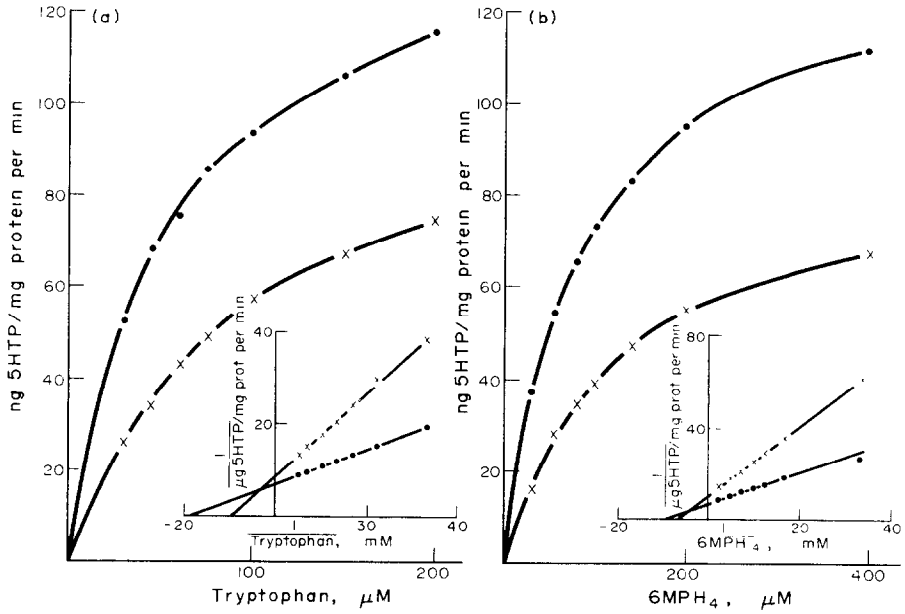

Fig. 3. Effect of calcium on the kinetic properties of tryptophan hydroxylase. $CaCl_2$ (10 mM) was added directly to the low speed supernatant enzyme preparation which was then chromatographed over Sephadex G-25. Control enzyme was chromatographed only. Velocity is expressed as ng 5-HTP formed/mg of protein/min and is the mean of duplicate determinations. (A) Effect of L-tryptophan concentration on the rate of formation of 5-HTP by control (×) and calcium-activated (●) tryptophan hydroxylase in the presence of 1 mM $6MPH_4$. A double reciprocal plot of the data is shown inset on the right. The values of the apparent K_m for tryptophan in this experiment are: control enzyme, 89 μM; activated enzyme, 50 μM. The values of V_{max} are 64 and 90 ng 5-HTP/mg of protein/min, respectively, for control and activated enzymes. (B) Effect of $6MPH_4$ concentration on the rate of formation of 5-HTP by control (×) and calcium-activated (●) tryptophan hydroxylase in the presence of 500 μM tryptophan. A double reciprocal plot of the data is shown inset on the right. The values of the apparent K_m for $6MPH_4$ in this experiment are: control enzyme, 149 μM; activated enzyme, 85 μM. The values of V_{max} are 56 and 75 ng 5-HTP/mg of protein/min, respectively, for control and activated enzymes.

Table 2. Effects of calcium addition on the kinetic properties of tryptophan hydroxylase*

Treatment	I Tryptophan			II $6MPH_4$		
	K_m (μM)	V_{max}	ΔV_{max}	K_m (μM)	V_{max}	ΔV_{max}
None	79 ± 5	47 ± 6		148 ± 7	63 ± 8	
			13.4 ± 3.0			16.8 ± 2.2
$CaCl_2$ (10 mM)	46 ± 3	60 ± 7		101 ± 6	79 ± 9	
	P < 0.001	NS	P < 0.01	P < 0.001	NS	P < 0.001

* Determinations of apparent K_m and V_{max} of tryptophan hydroxylase with seven tryptophan concentrations ranging from 12.5 to 200 μM were made in the presence of 1 mM $6MPH_4$ (I). At this concentration the reduced pterin cofactor inhibits the enzyme, and therefore values of V_{max} obtained under these conditions are lower than those obtained when the concentration of $6MPH_4$ was varied from 25 to 400 μM in the presence of 500 μM tryptophan (II). Each value of K_m and V_{max} is the mean ± the standard error of the mean of intercepts generated from six separate plots (duplicate determinations on three different enzyme preparations). V_{max} results are expressed as ng 5-HTP/mg of protein/min. Mean values of V_{max} did not differ significantly between control and activated enzyme. However, the mean increase in V_{max} of activated over control enzyme (ΔV_{max}) was highly significant.

† Enzyme was activated by addition of 10 mM $CaCl_2$ followed by Sephadex chromatography. Control enzyme was chromatographed without addition of $CaCl_2$.

Effect of Ca^{2+} on the kinetic properties of tryptophan hydroxylase. For the kinetic studies, the activity of tryptophan hydroxylase was maximally increased by the addition of 10 mM $CaCl_2$ to the enzyme followed by Sephadex chromatography. Control enzyme was chromatographed without the addition of $CaCl_2$. In these experiments the final concentration of Ca^{2+} in the chromatographed enzyme preparations was found to be 4.4 ± 0.7 μM for control and 41.6 ± 3.7 μM for the Ca^{2+}-activated enzyme.

The effect of Ca^{2+} on the kinetic properties of tryptophan hydroxylase is shown in Fig. 3 for one experiment. The values of the apparent K_m for both tryptophan and $6MPH_4$ were decreased after treatment of the enzyme with Ca^{2+}, while there was a slight increase in V_{max}. Values obtained for duplicate determinations on three different enzyme preparations have been summarized in Table 2. The apparent K_m for tryptophan decreased from 79 to 46 μM, that for $6MPH_4$ from 148 to 101 μM. In the presence of 1 mM $6MPH_4$, V_{max} increased by a mean value of 13.4 ng 5-HTP/mg of protein/min (paired analysis of values of V_{max} before and after Ca^{2+} activation for different enzyme preparations). A similar increase in V_{max} of the Ca^{2+}-activated enzyme was also observed when the kinetic analysis was made in the presence of 500 μM tryptophan (16.8 ng 5-HTP/mg of protein/min). However, the absolute values for V_{max} determined from experiments carried out in the presence of saturating concentrations of reduced pterin cofactor were lower than those obtained from experiments carried out in the presence of saturating concentrations of tryptophan, a finding which indicates that high concentrations of cofactor inhibit the enzyme. Therefore,

Table 3. Role of calcium in the increase of tryptophan hydroxylase activity produced by depolarization of slices of a thalamus–midbrain–medulla pons preparation*

Expt.	Modifications to the incubation medium	Tryptophan hydroxylase activity		Calcium concentration (μM)	
		Control	K-enriched	Control	K-enriched
I	None	21.1 ± 1.8	43.4 ± 4.4	24	20
	Ca^{2+}-free	24.7 ± 1.8	53.4 ± 4.8	15	11
II	None	28.0 ± 1.1	42.0 ± 1.3	55	48
	Ca^{2+}-free + EGTA (50μM)	26.2 ± 0.9	30.2 ± 0.9	40	52
	Ca^{2+}-free + EGTA (100 μM)	21.3 ± 0.8	19.7 ± 1.0	45	55
	Ca^{2+}-free + EGTA (500 μM)	21.5 ± 1.0	18.1 ± 1.0	50	55
III	None	25.8 ± 1.3	37.0 ± 0.4		
	$MgCl_2$ (20 mM)	28.5 ± 0.8	36.9 ± 1.3		

* Tryptophan hydroxylase was assayed in the presence of 200 μM tryptophan and 50 μM $6MPH_4$ and is expressed as ng 5-HTP/mg of protein/min. Each value is the mean ± the standard error of the mean of four determinations. Calcium determinations were made on total calcium (bound and free) in the low speed supernatant enzyme preparation after gel filtration.

Tissue slices were preincubated for 15 min on ice in control medium or Ca^{2+}-free control medium with or without added EGTA. The slices were separated from the preincubation medium by filtration and then incubated in control or K-enriched media with or without Ca^{2+} and EGTA. Tissue slices were incubated for 10 min in air at 26° as outlined in Materials and Methods.

Table 4. Effect of ouabain and sodium-free incubation medium on the activity of tryptophan hydroxylase from slices of a thalamus–midbrain–medulla pons preparation*

Expt.	Modifications to the incubation medium	Tryptophan hydroxylase activity	Increase in activity (% control)
I	None	59.9 ± 1.0	
	NaCl replaced with sucrose	62.9 ± 1.4	105
II	None; overnight at 7°	47.9 ± 0.9	
	NaCl replaced by sucrose; overnight at 7°	79.5 ± 1.1	166
III	None	51.1 ± 0.5	
	Ouabain (0.01 mM)	60.9 ± 0.8	119
	Ouabain (0.05 mM)	72.2 ± 1.2	141
IV	None	59.9 ± 1.0	
	Ouabain (0.1 mM)	95.4 ± 1.2	159
	Ouabain (1.0 mM)	65.9 ± 1.4	110

* Tryptophan hydroxylase was assayed in the presence of 200 μM tryptophan and 50 μM $6MPH_4$ and is expressed in ng 5-HTP formed/mg of protein/min. Each value is the mean ± the standard error of the mean of six determinations.

these kinetic measurements were repeated using a lower cofactor concentration (300 μM). Under these conditions the values of the apparent K_m of the control and Ca^{2+}-activated enzyme for tryptophan were unchanged (85 and 46 μM respectively). V_{max} increased from 62 to 85 ng 5-HTP/mg of protein/min after Ca^{2+} treatment.

Effect of depolarization on tryptophan hydroxylase activity. The activity of tryptophan hydroxylase prepared from slices of a brain preparation which included thalamus, midbrain and medulla pons was found to be enhanced after depolarization of the slices in an incubation medium in which 40 per cent of the NaCl had been replaced by KCl. In these studies, tryptophan hydroxylase was assayed in the presence of 200 μM L-tryptophan and 50 μM $6MPH_4$. The activity of enzyme from slices incubated in control medium was found to be 40.3 ± 4.2 ng 5-HTP/mg of protein/min in nine separate experiments. This increased to 63.8 ± 6.0 ng 5-HTP/mg of protein/min for enzyme from depolarized brain tissue. The increase in activity produced by depolarization could not be further enhanced by the addition of 10 mM $CaCl_2$ to the assay medium.

The possible role of Ca^{2+} in producing this increase in enzyme activity was investigated by altering the composition of the medium in which the slices were incubated. Omitting $CaCl_2$ from both control and K-enriched incubation media did not abolish the activation resulting from depolarization (Table 3). However, since Ca^{2+} could have leaked out from the tissue damaged during the slicing procedure (the Ca^{2+} concentration of the Ca^{2+}-free medium at the end of the incubation was found to be 40 μM), the experiment was repeated in the presence of EGTA. It was found that the increase in enzyme activity produced by depolarization was completely blocked in the Ca^{2+}-free medium to which 100 μM EGTA had been added (Table 3), and under these conditions the Ca^{2+} concentration of the

Table 5. Effect of depolarization of slices of rat brain with a K-enriched incubation medium on the kinetic properties of tryptophan hydroxylase*

	I Tryptophan			II $6MPH_4$	
Incubation medium	K_m (μM)	V_{max}	ΔV_{max}	K_m (μM)	V_{max}
Control	74 ± 7	55 ± 6		177 ± 14	65 ± 4
			12.8 ± 2.7		
K-enriched	45 ± 4	67 ± 8		118 ± 13	90 ± 3
	P < 0.01	NS	P < 0.01	P < 0.02	P < 0.001

* Slices of a brain preparation consisting of thalamus, midbrain and medulla pons were incubated for 10 min at 26° in control or K-enriched media, centrifuged, homogenized and assayed for tryptophan hydroxylase activity as described in Materials and Methods. Values of apparent K_m and V_{max} obtained by varying the concentration of tryptophan in the presence of 1 mM $6MPH_4$ (I) are the mean of five separate determinations; those obtained by varying the concentration of $6MPH_4$ in the presence of 500 μM tryptophan (II) are the mean of six separate determinations. V_{max} results are expressed as ng 5-HTP/mg of protein/min. Mean values of V_{max} obtained in the presence of 1 mM $6MPH_4$ did not differ significantly between control and activated enzyme. However, the mean of the increases in V_{max} of activated over control enzyme obtained from individual plots was significant ($P < 0.01$, paired analysis). Values for V_{max} determined in the presence of 1 mM $6MPH_4$ are lower than those obtained in the presence of 500 μM tryptophan since saturating concentrations of $6MPH_4$ inhibit tryptophan hydroxylase.

medium was below the limits of detection (1 μM).

In another experiment Mg^{2+} was tested to see whether it would block the increase in activity of enzyme from depolarized tissue. However, 20 mM $MgCl_2$ added to control and K-enriched media containing normal concentrations of $CaCl_2$ had no effect on control or enhanced tryptophan hydroxylase activity.

Finally, the effect on tryptophan hydroxylase activity of incubating the slices in a control medium in which all the NaCl had been replaced by sucrose or to which ouabain had been added was examined. A 10-min incubation in the Na^+-free medium at 26° left tryptophan hydroxylase activity unchanged. However, when the slices were left in the Na^+-free medium overnight (15 hr) at 7° and were then incubated at 26° for 10 min, a marked increase in enzyme activity was observed (Table 4).

Incubation of slices in the presence of ouabain also resulted in an increase in tryptophan hydroxylase activity. This effect was concentration dependent. An enhancement of enzyme activity was observed with enzyme prepared from slices which had been incubated with 0.05 and 0.1 mM ouabain, but not with 0.01 or 1.0 mM. The increase in enzyme activity was not due to a direct effect of the drug on the enzyme since no change in enzyme activity was observed when these same concentrations of ouabain were incubated directly with the enzyme in the assay.

An attempt was made to reverse the activation produced by depolarization by directly adding increasing concentrations of EGTA to the enzyme reaction medium (1 μM–10 mM). The results were similar to those presented in Fig. 2. There was a parallel inhibition of activity of enzyme from both control and depolarized slices, but the activation was not reversed.

Effect of depolarization on the kinetic properties of tryptophan hydroxylase. Depolarization caused a decrease in the apparent K_m for tryptophan from 74 to 45 μM (Table 5). There was a slight increase in V_{max} for the enzyme prepared from depolarized tissues compared with that for control which amounted to 12.8 ng 5-HTP/mg of protein/min. It should, however, be pointed out that values of V_{max} obtained under these conditions (1 mM $6MPH_4$) are low because the reduced pterin cofactor inhibits the enzyme at high concentrations. In the presence of 300 μM $6MPH_4$, the apparent K_m for tryptophan decreased from 74 to 57 μM on depolarization and the V_{max} increased from 66 to 84 ng 5-HTP/mg of protein/min. Depolarization also caused a small decrease in the apparent K_m of the enzyme for $6MPH_4$ from 177 to 118 μM. Values of V_{max} determined in

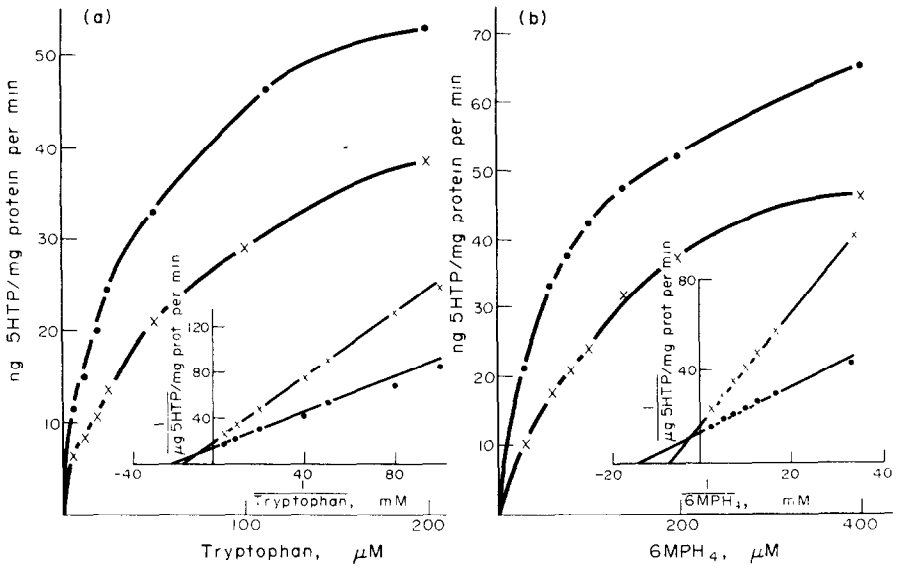

Fig. 4. Effect of depolarization on the kinetic properties of tryptophan hydroxylase. Enzyme was prepared from slices of a preparation of thalamus–midbrain–medulla pons incubated for 10 min at 26° in control or K-enriched medium, as described in Materials and Methods. Velocity is expressed as ng 5-HTP formed/mg of protein/min and is the mean of duplicate determinations. (A) Effect of L-tryptophan concentration on the rate of formation of 5-HTP by tryptophan hydroxylase from control (×) and depolarized (●) slices in the presence of 1 mM $6MPH_4$. A double reciprocal plot of the data is shown on the right. The values of the apparent K_m for tryptophan in this experiment are: control enzyme, 77 μM; enzyme from depolarized tissue slices, 46 μM. The values of V_{max} are 67 and 85 ng/5-HTP/mg of protein/min, respectively, for the control enzyme and the enzyme from depolarized slices. (B) Effect of $6MPH_4$ concentration on the rate of formation of 5-HTP by tryptophan hydroxylase from control (×) and depolarized slices (●) in the presence of 500 μM L-tryptophan. The values of the apparent K_m for $6MPH_4$ in this experiment are: control enzyme, 154 μM; enzyme from depolarized tissue slices, 83 μM. The values of V_{max} are 58 and 77 ng 5-HTP/mg of protein/min, respectively, for the control enzyme and the enzyme from depolarized slices.

the presence of 500 μM tryptophan were 65 and 90 ng 5-HTP/mg of protein/min for control and depolarized tissue respectively. Results from an individual experiment are shown in Fig. 4.

DISCUSSION

Indirect evidence outlined in the introductory paragraphs strongly suggests that tryptophan hydroxylase activity is enhanced by depolarization of serotonin-containing neurones in the brains of intact anaesthetized animals. In the present paper the activity of tryptophan hydroxylase prepared from slices of rat brain containing serotonergic perikarya, axons and some terminals [28] increases when the slices are depolarized in a K-enriched medium. This procedure has also been found to enhance the conversion of tryptophan to 5-HT in slices of rat hippocampus [24], an area which contains the terminals of 5-HT neurones which project from the midbrain raphe [28]. The increase in tryptophan hydroxylase activity after depolarization, reported here, can be accounted for by a small increase in the V_{max} and an approximate doubling of the affinity of the enzyme for the substrate tryptophan and the artificial cofactor, $6MPH_4$. Changes in the kinetic properties of the closely related enzyme, tyrosine hydroxylase, have also been observed after depolarization of sympathetic [19], central noradrenergic [20] or central dopaminergic neurones [21]. Calcium ions which are essential for the enhanced synthesis of 5-HT [24], as well as norepinephrine in tissues depolarized with potassium [29, 30], appear to play a role in the activation of tryptophan hydroxylase produced by depolarization. This divalent cation is known to be required for the activation of tyrosine hydroxylase seen when sympathetic neurones are depolarized [19]. Although in the present experiments incubation of slices in a Ca^{2+}-free medium did not abolish the increase in enzyme activity seen with depolarization, addition of EGTA to the Ca^{2+}-free, K-enriched medium did prevent the activation from occurring. It seems reasonable to suppose that the EGTA is required to remove Ca^{2+} ions present in the extracellular space which leak from the tissue slices. There is, in addition to this observation, other evidence suggesting a role for Ca^{2+} ions in the activation produced by depolarization. This comes from experiments with ouabain and with the Na^+-free incubation medium. Experiments on squid axon have shown that removal of Na^+ from the bathing medium increases the concentration of free Ca^{2+} in the axon [31, 32]. Furthermore, ouabain or an Na^+-free incubation medium has been found to enhance the spontaneous and evoked release of transmitter at the neuromuscular junction, processes which are known to depend on the concentration of ionized Ca^{2+} within the nerve terminal [32]. Thus, the results with ouabain and the Na^+-free medium showing an increase in tryptophan hydroxylase activity are consistent with the hypothesis that raised intraneuronal concentrations of Ca^{2+} are required for the enzyme activation to occur. In this connection it is of interest that the synthesis of norepinephrine from tyrosine in sympathetically innervated tissues or slices of rat cortex is dramatically enhanced by ouabain or an Na^+-free medium [33, 34].

One question which arises with the depolarization-induced activation of tryptophan hydroxylase concerns the fact that the preparation used in these studies consists of cell bodies and axons as well as some terminals, whereas the studies on 5-HT synthesis have been carried out on tissues containing predominantly terminals. Clearly, enhanced synthesis of 5-HT must have an important role at the nerve terminals replenishing transmitter released in response to nerve stimulation. The enzyme activation, however, appears to occur throughout the neurone. The reason for this may be that the intraneuronal Ca^{2+} concentrations are raised in response to nerve activity not simply at the nerve terminal where transmitter release occurs, but along the entire length of the neurone [32].

The question whether the activation produced by depolarization is related to the activation resulting from addition of Ca^{2+} directly to the enzyme preparation is unclear. In both cases, similar changes in the kinetic properties of the enzyme are obtained. Moreover, the addition of $CaCl_2$ to the enzyme which has already been activated by depolarization does not activate the enzyme further. Neither activation can be reversed with EGTA, which suggests that the direct binding of Ca^{2+} ions to the enzyme itself is unlikely to be involved in either activation process. However, in spite of these common features, the fact remains that the amounts of Ca^{2+} which must be added directly to the enzyme preparation to produce an activation are enormous and highly unphysiological. In contrast, the activation produced by K-depolarization occurs in the presence of physiological concentrations of Ca^{2+}. In a recent paper by Hamon *et al.* [35] the mechanism of the activation of tryptophan hydroxylase produced by the addition of mM concentrations of Ca^{2+} to low speed supernatant preparations of rat hind brain has been examined in some detail. They found that the Ca^{2+} activates a neutral proteinase as well as increasing the activity of tryptophan hydroxylase. When the neutral proteinase was separated from tryptophan hydroxylase by gel filtration on Sephadex G-200, addition of Ca^{2+} no longer increased tryptophan hydroxylase activity. Hamon *et al.* concluded that the activation of tryptophan hydroxylase by Ca^{2+} addition resulted from partial proteolysis of the enzyme by this Ca^{2+}-dependent neutral proteinase. Their conclusion was further supported by the fact that tryptophan hydroxylase activity was also enhanced after treatment with trypsin [35, 36]. Once established, this activation was not reversed with EGTA. In the present experiments, tryptophan hydroxylase was found to be activated when the concentration of the added Ca^{2+} had been reduced from 100 mM to 50 μM by gel filtration. Since the temperature of the preparation was maintained between 4 and 7° throughout these procedures, it is rather unlikely that an activated neutral proteinase could have acted on the tryptophan hydroxylase prior to removal of the Ca^{2+}. Thus, if the action of such a proteinase is indeed responsible for the activation of tryptophan hydroxylase observed under these conditions, then the

amounts of Ca^{2+} involved in the activation process must be rather small. The fact that a high concentration of Ca^{2+} must be added to the enzyme preparation initially may simply reflect the presence of large numbers of low affinity binding sites in the preparation rather than the Ca^{2+} requirements of the proteinase. Of course the possibility that some other undefined Ca^{2+}-dependent process may be responsible for the activation of tryptophan hydroxylase under the conditions of these particular experiments cannot be ruled out at the present time.

There has been a report that low concentrations of Ca^{2+} (μM) will enhance the activation of tryptophan hydroxylase which occurs *in vitro* in the presence of phosphorylating conditions [37]. However, it seems unlikely that this is the explanation for the activation of the enzyme reported here after removal of most of the added Ca^{2+} by gel filtration, since this procedure would also be expected to remove Mg^{2+} and ATP. (These substances are in any case probably completely removed by the gel filtration step carried out *prior* to the addition of Ca^{2+}.) The effect of Ca^{2+} on the activation of tryptophan hydroxylase in the presence of ATP and Mg^{2+} reported by Hamon *et al.* [37] may, however, shed some light on the role Ca^{2+} ions play in the activation of the enzyme that occurs when slices of brain tissue rich in serotonergic neurones are incubated under conditions known to increase the intraneuronal concentration of free ionized Ca^{2+}. It is conceivable that under these circumstances the raised intraneuronal concentration of Ca^{2+} activates a Ca^{2+}-dependent protein kinase, which then phosphorylates the tryptophan hydroxylase molecule directly or some other activator molecule [37]. Such a hypothesis is consistent both with the observed activation of tryptophan hydroxylase *in vitro* by ATP and Mg^{2+} reported by Hamon *et al.* [37] and the recent characterization of Ca^{2+}-dependent protein kinases in brain [38].

In conclusion, tryptophan hydroxylase activity is enhanced during depolarization of brain slices by a mechanism which requires the presence of Ca^{2+} ions, and results in an increased affinity of the enzyme for its substrate and cofactor as well as a modest increase in V_{max}. The interest of these observations lies in their possible relevance to the regulation of 5-HT synthesis in the intact neurone. Studies are currently in progress to determine whether tryptophan hydroxylase becomes activated as a result of nerve stimulation.

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