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ACTIVATION OF TRYPTOPHAN HYDROXYLASE FROM SLICES OF RAT BRAIN STEM INCUBATED WITH $N^6,O^{2'}$-DIBUTYRYL ADENOSINE-3':5'-CYCLIC MONOPHOSPHATE

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Recent in vitro studies on tryptophan hydroxylase, the rate-limiting enzyme in 5-hydroxytryptamine (5-HT) synthesis, have revealed that enzyme activity is increased under phosphorylating conditions (1-3) and that this increase in enzyme activity is further enhanced when calcium, but not adenosine 3':5'-cyclic monophosphate (cAMP) is included in the reaction medium together with the ATP and magnesium (1,2). These observations suggest a molecular mechanism for the calcium-dependent increase in tryptophan hydroxylase activity observed when enzyme is prepared from slices of brain stem depolarized in a high potassium medium (4,5) and for the enhanced synthesis of 5-HT which occurs in intact nervous tissue in response to electrical stimulation (6-8) and which is also calcium dependent (9). According to this scheme, calcium which enters the nerve during depolarization triggers a calcium-dependent phosphorylation of tryptophan hydroxylase, or an activator protein, and this, in turn, produces an activation and/or stimulation of the enzyme which results in the enhanced conversion of tryptophan to 5-hydroxytryptophan (5-HTP). These experiments on crude preparations of enzyme appear to exclude a role for cyclic nucleotides in the regulation of tryptophan hydroxylase activity, in marked contrast to the closely related enzyme, tyrosine hydroxylase (10). It was therefore surprising to discover that tryptophan hydroxylase was activated when prepared from brain stem slices which had been incubated with $N^6,O^{2'}$-dibutyryl adenosine-3':5'-cyclic monophosphate (dibutyryl cAMP). This finding is the subject of the present report.

The procedures used in this study for the preparation and incubation of the slices of brain stem (diencephalon, midbrain, medulla pons), for the isolation of the enzyme from the slice preparation in a low speed supernatant fraction, and for the tryptophan hydroxylase assay have all been described in detail elsewhere (4,5). In the present experiments the slices of each brain stem were incubated at 25° in 5.0 ml of oxygenated medium to which different concentrations of dibutyryl cAMP (Sigma Chemical Co.) were added. At the end of a ten minute incubation period each sliced brain stem was separated from the incubation medium by centrifugation and used as the source of the low speed supernatant preparation of tryptophan hydroxylase. Enzyme was assayed in the presence of 200 μM L-tryptophan and a suboptimal concentration (50 μM) of reduced artificial pterin cofactor, 6-methyl-5,6,7,8-tetrahydropterin (6-MPH_4, Calbiochem Behring Corp.) using a modification of the method of Friedman et al. (11) as described elsewhere (4,5). The activity of each brain stem enzyme preparation was assayed in sextuplet. Results are expressed in pmoles 5-HTP formed per mg protein per minute and are the means ± S.E.M. of values from at least three separate brain stem preparations.

From Table 1 it can be seen that dibutyryl cAMP produced a concentration-dependent increase in tryptophan hydroxylase activity which was maximal at 2.5 mM (70 percent increase

Table 1. Increase in the activity of tryptophan hydroxylase prepared from slices of rat brain stem incubated with dibutyryl cyclic AMP*

Concentration of dibutyryl cAMP in incubation medium (mM)	Tryptophan hydroxylase activity (pmoles 5HTP/mg protein/min)		% Control
	231 ± 7	(7)	
0.1	236 ± 12 (NS)	(3)	102
1.0	327 ± 7†	(5)	142
2.5	393 ± 25†	(5)	170
5.0	290 ± 7‡	(3)	125

*Numbers in parentheses indicate the number of brain stem slice preparations tested. Significance of the differences between the activity of enzyme from treated and control brain stem slices was determined by Student's *t*-test. NS = not significant.
†$P < 0.001$.
‡$P < 0.005$.

Table 2. Effect of dibutyryl cAMP on the kinetic properties of tryptophan hydroxylase from slices of rat brain stem*

	Tryptophan†		6-MPH_4‡	
	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Control	91 ± 8	244 ± 6	140 ± 8	251 ± 14
2.5 mM Dibutyryl cAMP	62 ± 4	316 ± 11	104 ± 4	308 ± 7
	P<0.05	P<0.005	P<0.02	P<0.025

* Values of K_m and V_{max} are the means ± the S.E.M. of results from three separate enzyme preparations each of which was obtained by pooling slices from three brain stems (see text for details). V_{max} is expressed in pmoles 5-HTP per mg protein per minute.

† Tryptophan concentration was varied in the presence of 300 μM 6-MPH_4. This concentration of 6-MPH_4 is subsaturating, but higher concentrations are inhibitory (12).

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

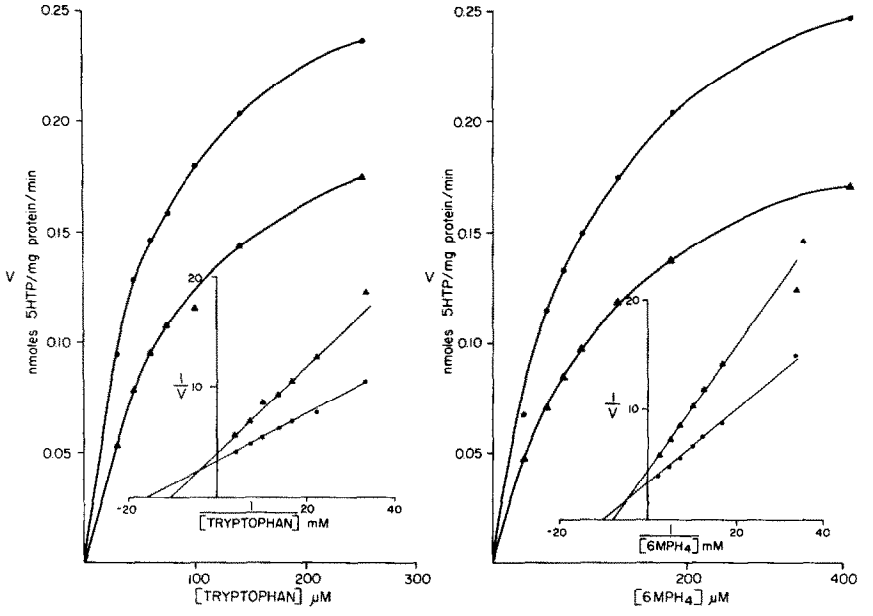

Fig. 1. Effect of dibutyryl cAMP on the kinetic properties of tryptophan hydroxylase. Effect of L-tryptophan (A) and 6-MPH_4 (B) concentration on the rate of formation of 5-HTP by tryptophan hydroxylase from control (▲) and dibutyryl cAMP-treated slices (●) in the presence of 300 μM 6-MPH_4 (A) and 500 μM L-tryptophan (B). A double reciprocal plot of the data is inset at right. (A) K_m for tryptophan: control 87 μM; dibutyryl cAMP, 59 μM. V_{max}: control 238; dibutyryl cAMP, 296 pmoles 5-HTP/mg protein/min. (B) K_m for 6-MPH_4: control, 145 μM; dibutyryl cAMP, 109 μM. V_{max}: control, 238; dibutyryl cAMP, 310 pmoles 5-HTP/mg protein/min.

over control enzyme activity) but then declined to a 25 percent increase at 5.0 mM. Dibutyryl cAMP had no effect on tryptophan hydroxylase activity when added directly to the control enzyme in the assay medium.

Kinetic properties of the control and dibutyryl cAMP-treated enzyme preparations were determined in three separate experiments carried out in duplicate or triplicate. Each control and experimental enzyme preparation was made from three brain stem slice preparations. Values of K_m and V_{max} were determined from the reciprocals of the intercepts of Lineweaver-Burk plots obtained by linear regression and the mean ± S.E.M. calculated from the separate values for the three experiments.

Table 2 summarizes the kinetic properties of the enzyme after incubation of the slice preparation with 2.5 mM dibutyryl cAMP. Results from one enzyme preparation are illustrated in Fig. 1. There was a significant fall in the value of the apparent K_m for both tryptophan and 6-MPH_4 and a modest increase in V_{max}. The alterations in the kinetic properties of the enzyme are very similar to those observed after depolarization of brain stem slices with a potassium-enriched incubation medium (4).

There are several interesting questions which are raised by these data. The first is why enzyme activity is enhanced following incubation of the brain stem slice preparation with dibutyryl cAMP when no increase in the activity of crude supernatant preparations of enzyme is observed after addition of cAMP directly to the reaction medium alone or together

with optimal concentrations of ATP and magnesium, with or without exogenous cAMP-dependent protein kinase (1). One explanation for this discrepancy may lie with the concentration of ATP in the 5-HT neurones of the slice. It turns out that cAMP will enhance the activity of crude supernatant preparations of enzyme in the presence of ATP, magnesium and exogenous cAMP-dependent protein kinase if the ATP concentration is suboptimal (1). Thus, a low ATP concentration in the slice may be the critical factor which permits dibutyryl cAMP to activate the enzyme. However, such an effect must presumably be mediated over an endogenous cAMP-dependent protein kinase which leaves the requirement for exogenous cAMP-dependent protein kinase in the supernatant preparation unexplained. A second question relates to the similarity between the kinetic changes in the enzyme obtained with dibutyryl cAMP and with potassium depolarization and whether a common mechanism may be involved. It is worth noting that one essential component of the depolarization-induced activation of tryptophan hydroxylase is the influx of extracellular calcium into the neurone. In the absence of extracellular calcium no increase in enzyme activity occurs in response to depolarization. In fact, treatments which raise the free intracellular concentration of calcium even in the absence of depolarization will activate the enzyme (4,5). There is, however, a problem in the interpretation of these data since depolarization as well as many of the other manipulations which increase the concentration of free intracellular calcium (e.g. metabolic poisons, ouabain) also increase levels of cAMP in neural tissue (13).

To study the possible role of cAMP or other nucleotides in the regulation of tryptophan hydroxylase, a comparative investigation of the effect on enzyme activity of a wide variety of phosphodiesterase inhibitors is currently being undertaken. In this way, it is hoped to distinguish between increases in enzyme activity due to the accumulation of cyclic nucleotide (which should be common to all inhibitors if the enzyme is truly sensitive to cyclic nucleotide) and other effects, such as calcium release, which are exhibited by some phosphodiesterase inhibitors. In addition, it is planned to determine whether the increase in tryptophan hydroxylase activity induced by any of these substances is paralleled by the enhanced formation of 5-HT from tryptophan in brain stem slices.

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