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DECREASE IN THE ACTIVITY OF TRYPTOPHAN HYDROXYLASE FROM SLICES OF RAT BRAIN STEM INCUBATED IN A LOW CALCIUM OR A CALCIUM-FREE MANGANESE-SUBSTITUTED MEDIUM

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Synthesis of 5-hydroxytryptamine (5-HT) from tryptophan in the 5-HT-containing neurons of the mammalian central nervous system is enhanced in a frequency dependent manner by electrical stimulation of the 5-HT perikarya which lie within the midbrain raphe nuclei and is depressed when firing of these neurons is blocked by surgical or pharmacological manipulation (1-3). Recent studies on brain slices indicate further that extracellular calcium ions are essential for the depolarization-induced increase in 5-HT synthesis to occur (4). The regulatory step in 5-HT synthesis is at the level of tryptophan hydroxylase (5), the rate-limiting enzyme in 5-HT formation which converts tryptophan to 5-hydroxytryptophan (5-HTP). In vitro studies employing slices of brain stem, which contain 5-HT perikarya as well as sectioned axons and terminal projections, suggest that the increase in 5-HT synthesis which results from nerve impulse activity may be brought about by a calcium-dependent activation of tryptophan hydroxylase which is triggered by neuronal depolarization (6,7). Preparations of tryptophan hydroxylase isolated from depolarized brain stem slices show an increased affinity for substrate and artificial cofactor, 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$), and a slight increase in V_{max} compared with enzyme from slices incubated in control medium (6,7). These observations taken together with the fact that the 5-HT perikarya of the raphe nuclei fire tonically in vivo (2,8) suggest that tryptophan hydroxylase may normally exist in a partially activated state in the intact neuron and further, that its activity may be reduced if the enzyme is prepared from neuronal tissue in which nerve impulses have been suppressed. The observation of Mosko and Jacobs (9) that the 5-HT perikarya of the midbrain raphe continue to fire, in vitro, in slices of midbrain made it possible to test this hypothesis with the brain stem slice preparation employed in earlier investigations (6,7). In the present experiments enzyme was prepared from slices of rat brain stem which had been incubated in the presence of tetrodotoxin (Calbiochem-Behring Corp., La Jolla, CA; TTX) or in a sodium-free medium, treatments which block the inward sodium current of the action potential (10,11). In addition, since there is growing evidence that calcium, as well as sodium, ions contribute to the action potential generated in the cell bodies of vertebrate neurons (12-14), in some experiments calcium ions were omitted from the control or experimental incubation media or were replaced with the calcium antagonist, manganese. The activity of enzyme from slices incubated in modified media was then compared with that of enzyme from slices incubated in control medium.

The procedures used in this study for the preparation and incubation of the slices of brain stem (diencephalon, midbrain and 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 been described in detail elsewhere (6,7). In the present experiments the slices of each individual brain stem were incubated at 25° in 5.0 ml of oxygenated medium which had

the following standard composition: NaCl, 150 mM; KCl, 6 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; glucose, 10 mM; Tris acetate buffer, pH 7.4, 10 mM. Modifications to this medium included replacement of all the NaCl with sucrose (300 mM), omission of the $CaCl_2$ or replacement of it with $MnCl_2$ (2 mM) and addition of TTX (300 nM) to control medium or to medium containing $MnCl_2$ instead of $CaCl_2$. 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 at 37° in the presence of 200 μM L-tryptophan and 50 μM $6MPH_4$ (Calbiochem-Behring Corp.) by a modification of the method of Friedman et al. (15) as previously described (6,7). Results were calculated in pmoles 5-HTP formed per mg protein per min and are expressed as per cent of control enzyme activity from the same experiment. They are the mean ± the S.E.M. of values from at least three separate brain stem preparations each of which was assayed in sextuplet.

Table 1 summarizes the effects of the different modifications to the incubation medium on the activity of tryptophan hydroxylase. Neither the absence of sodium ions nor the presence of TTX had any effect on tryptophan hydroxylase activity (column I). Enzyme activity was also unaffected by simple removal of calcium ions from control, sodium-free or TTX-containing media (column II). On the other hand if calcium ions were replaced with 2 mM manganese a significant fall in enzyme activity of about 25 per cent occurred regardless of whether or not sodium was present in the medium or whether the voltage-dependent sodium channels had been blocked by TTX (column III). These results suggest that external calcium

TABLE 1

Effect of TTX and ionic modifications to the incubation medium on the activity of tryptophan hydroxylase from slices of rat brain stem

Incubation medium[b]	Tryptophan Hydroxylase Activity[a]		
	I	II	III
	Concentration of $CaCl_2$ and $MnCl_2$ in the medium		
	2 mM $CaCl_2$ 0 mM $MnCl_2$	0 mM $CaCl_2$ 0 mM $MnCl_2$	0 mM $CaCl_2$ 2 mM $MnCl_2$
Control	100	98 ± 1 (3)	77 ± 3 (3)
Sodium-free	97 ± 2 (5)	96 ± 3 (3)	74 ± 4 (4)
TTX	98 ± 2 (3)	---	74 ± 1 (3)

[a]Enzyme activity is expressed as per cent of that of enzyme from brain stem slices incubated in unmodified control medium (100) and is the mean ± S.E.M. of three or more brain stem enzyme preparations (number in parentheses). Average control enzyme activity was 203 ± 6 pmoles 5-HTP/ mg protein/ min (n = 10).

[b]Calcium and manganese concentrations indicated under column headings.

ions in some way enhance the level of tryptophan hydroxylase in control slices and furthermore that the effects of calcium on tryptophan hydroxylase activity are mediated via manganese-sensitive calcium channels and not the TTX-sensitive sodium channels in the nerve membrane (11,12-14). To explore the role of calcium ions further, brain stem slice preparations were incubated in control medium containing different calcium and manganese concentrations (Table 2). A reduction in the concentration of calcium to 0.2 or 0.5 mM depressed enzyme activity, whereas an increase in the concentration of calcium to 10 mM enhanced tryptophan hydroxylase activity slightly. The per cent increase or decrease in enzyme activity over control which resulted from incubation of the slices in high or low calcium was not statistically significant. However the difference between the activities of the enzyme preparations seen after low (0.2 or 0.5 mM) and high (10 mM) calcium treatments was

significant ($P < 0.01$, Student t-test). Addition of 2 or 10 mM manganese to the standard 2 mM calcium medium did not alter enzyme activity (columns II and III, Table 2).

TABLE 2

Effect of different concentrations of calcium on the activity of tryptophan hydroxylase from rat brain stem slices

Concentration of $CaCl_2$ in medium (mM)	Tryptophan Hydroxylase Activity[a]		
	I	II	III
	Concentration of $MnCl_2$ in the medium		
	0 mM	2 mM	10 mM
0	98 ± 1	77 ± 3	--
0.1	102 ± 1		
0.2	86 ± 3		
0.5	78 ± 6.5		
2.0	100	104 ± 0.5	94 ± 4
5.0	97 ± 1.5		
10.0	119 ± 5.3		

[a]Enzyme activity is expressed as per cent of that from the brain stem slice preparation incubated in standard control medium in the same experiment, and is the mean ± S.E.M. of results from three brain stem enzyme preparations. Average control enzyme activity was 195 ± 5 pmoles 5-HTP/ mg protein/ min (n = 12).

TABLE 3

Kinetic properties of tryptophan hydroxylase from slices of brain stem incubated in calcium free, manganese-substituted medium containing TTX

Brain stem slice incubation medium	I Tryptophan[a]		II $6MPH_4$[b]	
	K_m (μM)	V_{max}[c]	K_m (μM)	V_{max}[c]
Control	76 ± 3	212 ± 4	128 ± 6	286 ± 5
TTX + 2 mM $MnCl_2$ 0 mM $CaCl_2$	76 ± 5	163 ± 4	138 ± 13	236 ± 11
	NS	$P < 0.001$	NS	$P < 0.02$

[a]Tryptophan concentration was varied in the presence of 300 μM $6MPH_4$. This concentration of $6MPH_4$ is subsaturating, but higher concentrations are inhibitory (6,7).

[b]$6MPH_4$ concentration was varied in the presence of 500 μM tryptophan.

[c]V_{max} is expressed in pmoles 5-HTP/ mg protein/ min.

The enzyme preparations were each made from tissue pooled from three sliced brain stems. The K_m and V_{max} for each enzyme preparation were determined from the reciprocals of the intercepts of three Lineweaver-Burk plots obtained by linear regression. Statistical significance of differences in values of K_m and V_{max} for control and treated enzyme were determined by the Student t-test. NS, not significant.

The kinetic properties of the enzyme prepared from slices incubated in a calcium-free medium to which 2 mM $MnCl_2$ and 300 nM TTX had been added are compared with those of enzyme from control brain stem slices in Table 3. No alteration was observed in the K_m for either substrate or cofactor, but there was a slight decrease in the V_{max} of the treated enzyme which was significant.

From these data it appears that calcium ions in the extracellular medium play a role in regulating tryptophan hydroxylase activity. The recent evidence that calcium ions contribute to the action potential of neuronal cell bodies via selective calcium channels (12-14) and the observation that the 5-HT perikarya of the midbrain raphe fire spontaneously *in vitro*

make it likely that calcium as well as sodium enters active 5-HT perikarya in slice preparations and that this calcium in some way enhances tryptophan hydroxylase activity. When calcium ions are prevented from entering the active neurons by substitution with manganese, then enzyme activity is lowered. The fact that removal of calcium alone does not have this effect may have to do with changes in membrane properties which occur in the absence of a suitable divalent substituent. The decrease in enzyme activity is observed with calcium removal provided it is incomplete. Conversely a fivefold increase in calcium concentration results in a slight increase in enzyme activity.

Further studies are clearly warranted to determine whether the activity of tryptophan hydroxylase from intact animals is decreased when neuronal firing is blocked in vivo, and whether activity is increased when neuronal firing rate is raised.

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