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ACTIVATION AND INACTIVATION OF STRIATAL TYROSINE HYDROXYLASE: THE EFFECTS OF pH, ATP AND CYCLIC AMP, *S*-ADENOSYLMETHIONINE AND *S*-ADENOSYLHOMOCYSTEINE

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Abstract—The activity of tyrosine hydroxylase (TH) in the corpus striatum of haloperidol treated and control rats has been examined. The activation of TH by haloperidol caused a decrease in the K_m for tetrahydrobiopterin but no change in the V_{max}. This effect was totally abolished when homogenates were prepared at high values of pH. A similar activation could be produced *in vitro* by preincubating with *S*-adenosylmethionine; conversely, enzyme activity was reduced by preincubating with *S*-adenosylhomocysteine. ATP and cyclic AMP activated the enzyme when incubated together with TH *in vitro* but the activity was reduced when the enzyme was preincubated with these substances. A possible role for carboxymethylation in controlling tyrosine hydroxylase activity is discussed.

The activities of enzymes responsible for the synthesis of catecholamine neurotransmitters can alter in response to changes in the environment of an animal and this may relate to behavioural phenomena. Tyrosine hydroxylase (L-tyrosine, tetrahydropteridine, oxygen oxido-reductase (3 hydroxylating), EC 1.14.16.2., TH) the rate limiting step in catecholamines synthesis, is made in the cell body of catecholamine-containing neurons and transported to the nerve endings. In the case of the dopamine-containing neurones of the corpus striatum the cell bodies are situated some distance away from the striatum in the substantia nigra. As the rate of axonal transport of proteins is relatively slow, changes of enzyme activity that occur within hours are unlikely to be due to *de novo* synthesis of the enzyme. The only way that the enzyme activity can be changed, therefore, is by post translocational modification. This can be brought about by changing the number of active enzyme units (i.e. activating previously inactive molecules) and would be seen experimentally as a change in V_{max}. Alternatively the enzyme may be activated by an alteration in the rate at which substrates react with the enzyme, i.e. by a change in the respective Michaelis constant (K_m).

It has already been shown that TH can be 'activated' *in vivo* by the administration of neuroleptic drugs such as haloperidol [1]. The response to such drugs is a reduction in the K_m for the pterin substrate. Similar changes in the enzyme kinetics have also been reported to occur *in vitro*, when adding adenosyl triphosphate (ATP) and cyclic adenosyl monophosphate (cAMP) to the incubation medium [2] (phosphorylating conditions). However, some authors have also shown that prolonged preincubation with these compounds causes a decrease in the overall enzyme activity, although the K_m for the pterin substrate is still lowered [3].

Some proteins in the brain may be modified by carboxymethylation [4] rather than by phosphorylation. The enzyme responsible for this reaction uses *S*-adenosylmethionine (SAM) as a substrate, is strongly inhibited by *S*-adenosylhomocysteine (SAH), and the resulting carboxymethyl groups will spontaneously hydrolyse at high values of pH. Since, in a previous report [5], we have shown that the preparation of homogenates at high pH causes a change in the activity of TH we have examined the effects of adding not only ATP and cAMP but also SAM and SAH to the incubation medium.

MATERIALS AND METHODS

Animals

All rats used were from the Porton Wistar strain bred at Babraham. They were usually males weighing between 200 and 400 g.

Materials

Radioactive L-[side chain 2,3-^{3}H]-tyrosine (10–30 Ci/mmol) was purchased from the Radio Chemical Centre, Amersham, Bucks. Bovine plasma albumin was obtained from Armour Pharmaceuticals, Eastbourne, England, Woelm alumina was purchased from ICN Pharmaceuticals, and SAM and SAH from Sigma. We are indebted to Dr K. J. M Andrews and Roche Products for the gift of tetrahydrobiopterin (BPH_4) without which this work would not have been possible. All other compounds were of the highest purity obtainable.

Preparation of homogenates

The animals were killed by decapitation and the

striatum was rapidly dissected out and weighed. Homogenates were then prepared using a close fitting all glass, hand homogenizer. The tissue was homogenized in either 0.1 or 0.05 M dimethylglutarate (DMG) NaOH buffer, pH 6.0, at a concentration of 100 mg (fresh tissue weight)/ml. All homogenates were used within 30 min of preparation. For some experiments the pH of the 'buffer' was raised to pH 8.5 or slightly above; at this value the DMG has little buffering capacity. In all cases where control samples were used, the animals were matched (usually litter mates) and tissue samples were always incubated under the same experimental conditions.

Incubation of homogenates

The assay procedure used for tyrosine hydroxylase has been reported previously [6, 7]. Samples of homogenates, prepared as above, were incubated within 30 min of preparation at 37° for 30 min with radioactive tyrosine having a specific activity of approximately 218 Ci/mole. The final volume of incubation mixture was 100 μl and consisted of 40 mM DMG/NaOH buffer (pH 6.0), 0.2 mM $FeSO_4$, 4 mg/ml bovine plasma albumin (BPA), 2.5 mM dithiothreitol (DTT), 100 mM NaCl, 2500 units catalase, 1% by volume Triton X-100 and varying concentrations of BPH_4, as indicated. The final concentration of Na^+ ions was 150 mM (measured in a flame photometer). Incubation was started by the addition of tyrosine and stopped by the addition of 0.15 M perchloric acid. Separation of dihydroxyphenylalanine (L-dopa) was carried out on alumina columns as previously described [6, 7]. Activity was always expressed as nmol L-dopa synthesised per mg tissue wet weight per hour (nmol/mg h). Kinetic constants were calculated using the method described by Bliss [8]. In some experiments, homogenates prepared in weak (20–50 mM) solutions of DMG at pH 8.5 were used and added to incubation media buffered at pH 6.0. This addition of homogenates prepared at high pH did cause a small change in the incubation pH but this was less than 0.15 of a pH unit. Under optimal conditions a change in pH of this magnitude caused no detectable change in the velocity of L-dopa synthesised by tyrosine hydroxylase.

In some experiments homogenates prepared at pH 6.0 were preincubated in the presence of SAM or ATP and cyclic AMP, or SAH. These were added to tubes containing ice-cold incubation mixture from which BPA, BPH_4 and [^{3}H]-tyrosine had been omitted. The mixtures were then preincubated for 30 min at 37° after which they were cooled and BPH_4 added. The incubation, at 37°, for estimating TH activity was then started by the addition of [^{3}H] tyrosine as previously described.

Statistical analysis

Statistical analysis of enzyme activity was carried out using Student's t-test. To obtain a statistical significance of differences in values for K_m and V_{max}, a model fitting technique was used. Each set of data was fitted to a two parameter hyperbolic model and the residual sum of squares noted. One parameter (e.g. V_{max}) was restricted to a single value and new regression lines calculated, the remaining parameters being allowed to vary and the increase in residual sum of squares was noted. Finally the model was fitted to the combined data from the two experiments to be compared and the change in the residual sum of squares noted. An analysis of variance was then constructed apportioning the variation to the restrictions in the various parameters. The significance of the variations in the parameters was tested by comparing the corresponding variance with the residual variance obtained by fitting a separate model for each set of data. This gave values for K_m and V_{max} which were identical with those obtained using the Bliss method, but enabled a reliable statistical comparison.

RESULTS

Activation of TH by haloperidol in vivo

Rats were injected i.p. with haloperidol (1.5–3.0 mg/kg) and killed 30 min later. It was found that the V_{max} of the striatal TH from these treated animals was not different from that prepared from matched controls when the homogenates were incubated in the presence of 240 μM tyrosine (saturating concentration) and different concentrations of BPH_4. There was a reduction in the K_m for BPH_4 from 303 ± 123 μM to 122 ± 41 μM ($P < 0.02$). When the tyrosine concentration was lowered to 40 μM (subsaturating), the K_s for BPH_4 was again reduced, this time for the enzyme from haloperidol-treated animals from 782 ± 161 μM to 331 ± 64 μM ($P < 0.05$) and the V_{max} was unchanged (see Fig. 1). K_m and K_s

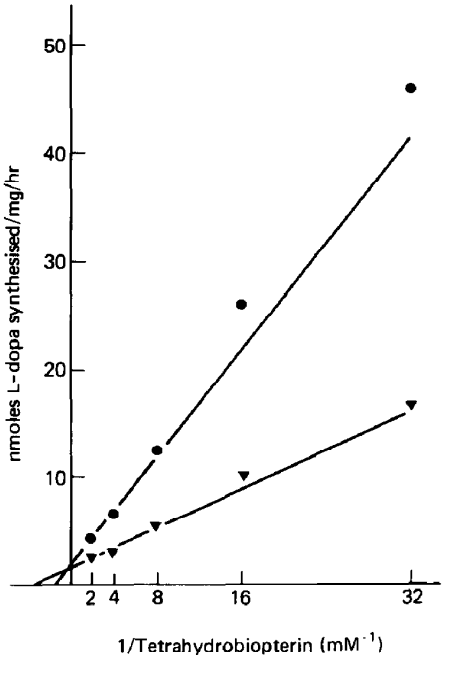

Fig. 1. The effect of treatment with haloperidol on the synthesis of L-dopa by homogenates of rat striatal tissue at various concentrations of tetrahydrobiopterin. ▼, Haloperidol-treated animals, K_s 331 ± 64 μM; ●, Control, K_s 782 ± 161 μM. The values for K_s are significantly different ($P < 0.05$).

Table 1. Activation and inactivation of tyrosine hydroxylase

A.	Percentage change in initial velocity			
	Method of activation or inactivation	%	(Tyrosine)	(BPH_4)
1.	Haloperidol *in vivo*	113–188	40 μM	254 μM
2.	Preparation of homogenates at pH 8.5 (incubation at pH 6.0)	74	40	254
3.	Incubation with (1 mM) ATP cyclic (0.5 mM) AMP	126	40	254
4.	Preincubation with (1 mM) cyclic AMP (1 mM ATP)	60	40	254
5.	Preincubation with (0.5 mM) SAM	112	40	64
6.	Preincubation with (1.0 mM) SAH	40	40	64
	Preincubation with (1.0 mM) SAH	31	40	254
7.	Preincubation (1.0 mM) SAH (1.0 mM) ATP (0.5 mM) cyclic AMP	48	40	254

B.	Changes in kinetic constants		
	Method effecting change	(Tyrosine)	% Change K_s BPH_4
1.	Haloperidol *in vivo*	40 μM	42–79
	Haloperidol *in vivo*	Saturating (240 μM)	40 (K_m)
2.	Haloperidol *in vivo* preparation at pH 8.5	40 μM	364
3.	Preincubation with SAM	40 μM	57
4.	Preincubation with SAH	40 μM	140

Activation and inactivation is expressed as a percentage with controls being 100%. Therefore an activation of initial velocities is greater than 100% and inactivation less than 100%. Conversely, with K_s values, an activation is shown by a reduction in K_s and vice versa.

represent Michaelis constants calculated from enzyme activity in the presence of saturating and subsaturating concentrations of tyrosine respectively. In Fig. 1, the upper line does not, at first sight, appear to be the best fit. This is because of the statistical weighting of the points corresponding with high concentrations of substrate. These results indicate that as a result of treatment with haloperidol, there is an increase in the initial velocity of the striatal TH but not in the amount of enzyme present. It was also noted that when the difference between treated and control animals was small, this was due to a high rate of L-dopa synthesis in the control rather than a low value for the homogenate from the treated animals. This suggests a variable level of 'activation' in the controls which is increased to a maximum value by treatment with haloperidol.

When homogenates, prepared at pH 8.5 and incubated at pH 6.0 were compared with homogenates that were both prepared and incubated at pH 6.0, it was found that the activity of the enzyme from both haloperidol-treated and untreated rats was reduced. In one example there was a reduction from 0.471 ± 0.016 to 0.301 ± 0.018 nmole/mg hr for haloperidol-treated rats and 0.418 ± 0.029 to 0.309 ± 0.018 for control rats.

To aid the comparison between the different methods of activation and inactivation and to eliminate inter control variations, the changes are expressed as percentages of the matched control values, which is the rate constant, K_s or K_m of the enzyme from an untreated animal prepared and measured at the same time and incubated at pH 6.0. Thus haloperidol can be seen to activate the enzyme rate to 113% of control and preparation of the tissue at pH 8.5 reduces the rate to 74% of control. Table 1 lists the percentage changes that occur under the different experimental conditions.

The initial rate of L-dopa synthesis at subsaturating

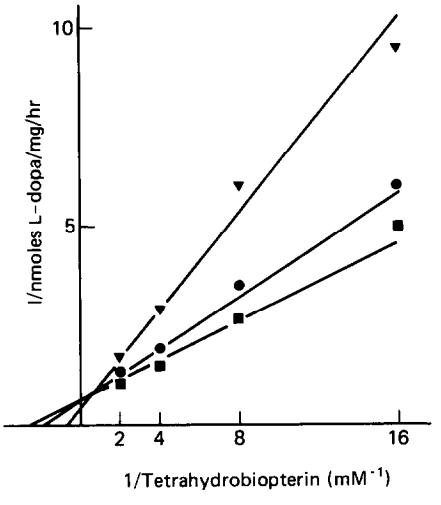

Fig. 2. The effect of homogenization at pH 8.5 on L-dopa synthesis at varying concentrations of tetrahydrobiopterin by homogenates of rat striatal tissue. ▼, Striatal tissue from haloperidol-treated animal prepared at pH 8.5 (K_s 1580 ± 543 μM); ■, Striatal tissue from haloperidol-treated animal prepared at pH 6.0 (K_s 414 ± 90 μM); ●, Striatal tissue from a control animal prepared at pH 6.0 (K_s 521 ± 111 μM). The K_s of 1580 μM is significantly different from that of the control prepared at pH 6.0 ($P < 0.05$).

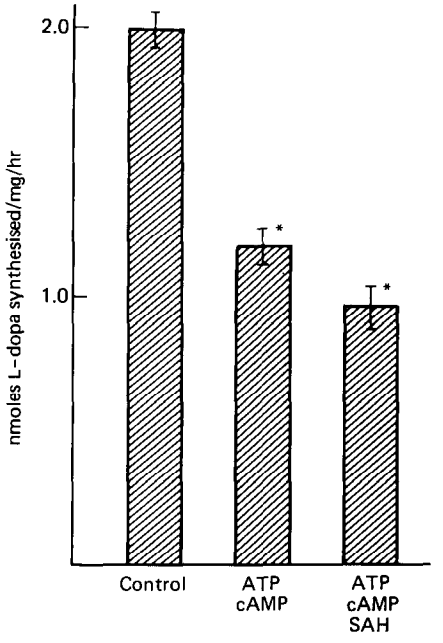

Fig. 3. Inactivation of TH by preincubation with 1.0 mM ATP and 0.5 mM cyclic AMP, and also with *S*-adenosylhomocysteine (SAH) 1.0 mM. *These values are significantly different from the control value ($P < 0.01$) using Student's t test. In all cases $n = 10$.

concentrations of BPH_4 by striatal homogenates from both haloperidol-treated and control rats was similar if the homogenates were prepared at pH 8.5 (Fig. 2). The similarity in the enzyme activity when the homogenates were prepared at pH 8.5 was also reflected in the K_s values which were increased from 414 ± 90 μM for homogenates (pH 6.0) from haloperidol-treated animals and 521 ± 111 μM for controls to near 1500 μM in both cases (results not illustrated). Thus the homogenization of striatal tissue from treated and control animals at pH 8.5 results in enzyme preparations showing similar initial activities, similar K_s values and moreover the activating effect of treatment with haloperidol is more than reversed. It would seem that the natural enzyme is present in an already partially activated form.

Activation and inactivation in vitro

In vitro: Activation of TH, similar to that obtained *in vivo* with haloperidol, has previously been achieved by incubating the enzyme homogenate under conditions conducive to protein phosphorylation [2]. We have compared such incubating conditions with two others using adenosyl derivatives which might affect the enzyme. It was found that incubating the homogenates in the presence of 1 mM ATP and 0.5 mM cyclic AMP increased the initial enzyme activity to 126% of controls ($P < 0.01$). However, 30 min preincubation with these two compounds reduced initial enzyme activity to 60% of controls (Fig. 3), in agreement with the observations of Vrana *et al.*, [3]. Incubations of homogenates with either SAH or SAM produced no effect but 30 min preincubation with 0.5 mM SAM produced a small but significant increase in the activity to 112% of control values ($P < 0.01$) whereas preincubation with 1.0 mM SAH reduced the activity to 31% of control values ($P < 0.01$). This activation by SAH proved to be additive to that produced by the phosphorylating pre-incubation conditions (Fig. 4 and Table 1A). The magnitudes of these changes were variable especially in the case of the preincubation with SAM, as was the case with the *in vivo* haloperidol activation. It was again noted that SAM produced the smallest activation when the controls were high, indicating a variable level of activation of the enzyme in the control situation. The activation by SAM was a result of a lowering of the K_s for BPH_4 from 572 ± 28 μM to 325 ± 42 μM ($P < 0.001$) a decrease to 57% of the control (Table 1B and Fig. 5) with no change in the V_{max}. Similarly the inactivation by SAH

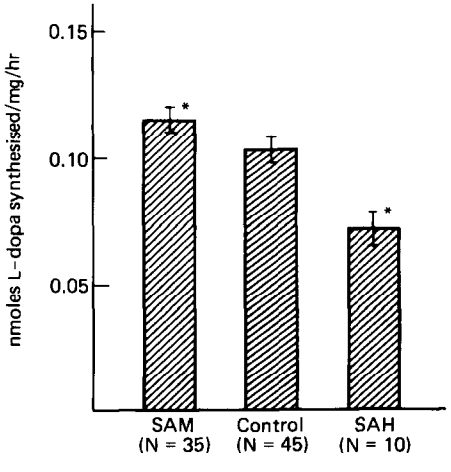

Fig. 4. The effect of preincubation with *S*-adenosylmethionine and *S*-adenosylhomocysteine on TH activity in striatal homogenates. Mean activity of striatal homogenates preincubated with 5 mM SAM or 10 mM SAH. *These values are significantly different from the control value ($P < 0.01$) using Student's t test.

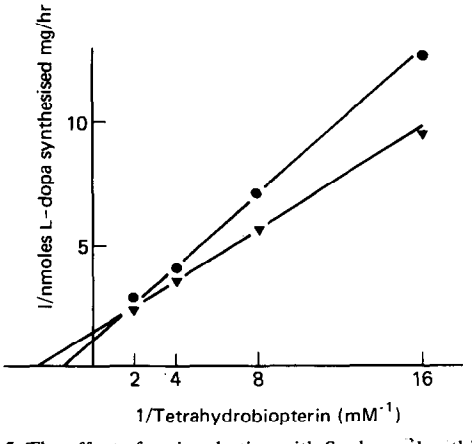

Fig. 5. The effect of preincubation with *S*-adenosylmethionine on the synthesis of L-dopa in striatal homogenates at various concentrations of tetrahydrobiopterin. ▼, Preincubated 30 min with 0.5 mM *S*-adenosylmethionine, K_s 325 ± 42; ●, Preincubated 30 min in absence of SAM, K_s 572 ± 28.

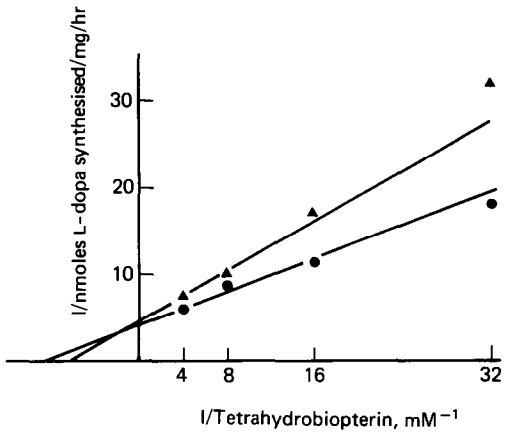

Fig. 6. Inactivation of striatal tyrosine hydroxylase by *S*-adenosylhomocysteine. ▲, Preincubation with 1.0 mM SAH, K_s 163 μM; ●, Control; K_s 113 μM.

was a consequence of an increase in the K_s to 140% of the control value with no change in the V_{max} (Fig. 6). This contrasts with the effects of preincubation with ATP which does cause a decrease in V_{max} [3]. Figure 6 appears to be a curve typical for a reversible competitive inhibitor, but since there was no effect unless there was a 30 min preincubation it cannot be SAH itself which is the inhibitor. Table 1 summarises the changes in TH activity that occur with the procedures used in the present experiments. The *in vitro* changes are all of a similar order of magnitude to that produced by haloperidol *in vivo*.

DISCUSSION

It is now established that TH can be activated by nerve stimulation, calcium, and phosphorylating conditions [9, 10]. We have confirmed that *in vivo* activation of TH is brought about by injection of haloperidol. This activation is a consequence of a change in the properties of the enzyme as reflected by the K_m for BPH_4, and not the quantity of enzyme present as indicated by the constancy of the measured values of V_{max}. Homogenization of the tissue at pH 8.5 instead of pH 6.0 reduced the activity of the enzyme in striatal homogenates from both haloperidol-treated, and control rats, again by changing the K_m for BPH_4, and not by inducing a change in the amount of enzyme. Similar findings have also been seen in the caudate nuclei of piglets where the early-weaning causes an activation of TH which can be reversed by homogenization at pH 8.5 and is a result of a change in the K_m for BPH_4 and not a change in V_{max} [15]. We have found evidence that the preparation of the homogenates at pH 8.5 produces preparations of TH that are indistinguishable whether they are prepared from haloperidol-treated or control animals, and appear to have minimal activity. We have also observed more consistency in the activity of enzyme preparations from haloperidol-treated rats than in enzyme preparations from controls, indicating the activity of TH was increased to a maximum value by haloperidol treatment, and that the controls were in various intermediate stages of activation.

All of the *in vitro* modifications used here produced effects of a similar magnitude to that produced *in vivo* by haloperidol. With the exception of preincubation under phosphorylating conditions, activation was due to changes in the K_m for BPH_4 and not to changes in the amount of enzyme. It is important to consider whether these changes have any biological significance. It is thought that the BPH_4 concentration at the site of synthesis is 10 μM [11]. From our results it can be seen that a change in the K_m for BPH_4 of 303 μM to 122 μM would increase the rate of L-dopa synthesis by a factor of 2.5, a change which is large enough to have biological significance.

We have shown that incubation with ATP and cyclic AMP produces activation of TH [12] and that 30 min preincubation under these conditions reduces the activity of the enzyme. This result contrasts with that of Lazar *et al.* [13], who found, using a soluble supernatant TH from the striatum of rats, that both with haloperidol and control samples 'phosphorylation' at pH 7.0 increased the activity of the enzyme at pH 6.0 and that both K_m and V_{max} in the presence of different concentrations of BPH_4 were increased. This result might be explained by the fact that the assay method used was different although Vrana *et al.* [3] used a similar method ($^{14}CO_2$ release) to that of Lazar *et al.*, and obtained results similar to ours. Our experiments seem to confirm the fact that phosphorylation plays a role in the modulation of TH activity but our results with SAM and SAH indicate that this is not the only mechanism that might be involved.

Thirty minutes preincubation with, but not incubation with, SAM causes an activation of TH, whereas preincubation but not incubation with SAH, causes an inactivation of the enzyme, both effects being caused by a change in the K_m for BPH_4 and not by a change in the V_{max}. The inhibition due to SAH was also found to be additive to that of preincubation with ATP and cyclic AMP. Diliberto and Axelrod [4] have shown that the enzyme protein methylase II is present in considerable quantities in the striatum, that it has an optimum pH of 6.0 [14], uses SAM as the methyl donor, that SAH is a potent product inhibitor, and that the protein carboxymethyl groups produced are rapidly hydrolysed at pH values in excess of 7.0. The activation of TH by SAM, its inactivation by SAH and by preparation of homogenates at pH 8.4, strongly suggest that protein methylation, as well as phosphorylation, could be involved in the control of TH activity and thus in the control of L-dopa synthesis in the striatum of the rat.

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