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ABSENCE OF INCREASED TYROSINE HYDROXYLATION AFTER INDUCTION OF BRAIN TYROSINE HYDROXYLASE FOLLOWING RESERPINE ADMINISTRATION

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Abstract—Reserpine was administered to rats by i.p. injection, and seven days later, with a control group, crude synaptosomal suspensions were prepared from hippocampus and cerebellum for the estimation of rates of synaptosomal tyrosine hydroxylation; additionally, samples of hippocampus, cerebellum and hypothalamus were homogenized in a hypotonic buffer containing Triton for the *in vitro* estimation of tyrosine hydroxylase. In the samples from reserpine pretreated animals, *in vitro* tyrosine hydroxylase activities were considerably elevated compared to controls (234 per cent of controls in the cerebellum, 154 per cent in hippocampus and 181 per cent in hypothalamus). However, the synaptosomal tyrosine hydroxylation rates were not elevated in the drug-treated group. The rate of product formation with time was linear in preparations from both control and reserpine pretreated animals. The results suggests that enzyme induction leads to an increased potential for tyrosine hydroxylation which may not be expressed due to interaction with regulatory mechanisms operating on the enzyme.

Tyrosine hydroxylase (E.C. 1.14.16.2; tyrosine 3-monooxygenase) controls a rate limiting step in the synthesis of catecholamines; in addition to mechanisms which control the level of specific enzyme protein (e.g. transneuronal induction [1] and nerve growth factor [2]) there is a wide range of factors which modify the activity of this enzyme in both peripheral and central neurons. Enzyme induction is initiated by increased presynaptic activation. However there is a delay of several days before increased amounts of enzyme reach the nerve terminals by axoplasmic transport. The *in vivo* consequence of enzyme induction may be an elevated rate of tyrosine hydroxylation at a time when the increased presynaptic stimulation has ceased. Alternatively the activity of induced enzyme may be suppressed and represent an increased potential for tyrosine hydroxylation should this be required. As a first step towards evaluating these possibilities we have compared the effect of reserpine administration on the *in vitro* activity of this enzyme with the effect on the rate of tyrosine hydroxylation in a synaptosomal preparation from rat brain. Our results indicate that the induced enzyme activity observed in the *in vitro* assay is suppressed by regulatory mechanisms retained in the synaptosomal preparation.

Reserpine administration leads to a 2–3 fold increase in the amount of tyrosine hydroxylase in the nucleus locus coeruleus, which contains the cell bodies of noradrenergic neurons. The increase in tyrosine hydroxylase reaches a peak at 48–72 hr after reserpine administration and persists for three weeks [3]. Areas of the brain which receive terminals from this nucleus show a rise in enzyme level with a delay which is related to their distance from the nucleus; this delay is about six days for the cerebellum and eleven days for the frontal cortex [4].

MATERIALS AND METHODS

L-[3,5-^{3}H]Tyrosine was purchased from the Radiochemical Centre, Amersham, Bucks., and was purified prior to use by alumina and Dowex-50 column chromatography essentially as described by Coyle [5] with a final specific radioactivity of 0.74 Ci/m-mol (unlabelled tyrosine was purchased from Sigma Chemical Co., London). We are grateful to Dr. K. J. M. Andrews, Roche Products Ltd., Welwyn Garden City, for the gift of 5,6,7,8-tetrahydrobiopterin. Reserpine was supplied in 2% benzyl alcohol.

Estimation of synaptosomal tyrosine hydroxylation. Tissues were immediately homogenised in 0.32 M sucrose. A crude synaptosomal fraction (P2) was prepared and assayed for synaptosomal tyrosine hydroxylation by an adaptation of the method of Karobath [7] which is fully described in another communication [6]. The sucrose homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was then centrifuged at 16,000 *g* for 20 min to sediment the P_2 pellet, which was suspended in incubation medium and immediately used as a source of synaptosomes (protein concentration, 13–18 mg/ml). The incubation medium had a final composition of NaCl (125 mM); $CaCl_2$ (1 mM); $MgCl_2$ (1 mM); glucose (10 mM); ascorbic acid (1 mM) buffered to a pH of 6.2 with sodium phosphate buffer at a final concentration of 15 mM. The medium was oxygenated immediately prior to use. Incubation was started by the addition of 5 μl of L-[3,5-^{3}H]tyrosine (0.74 mCi/μmol) to 45 μl of the P_2 suspension to give a final tyrosine concentration of 20 μM. After 20 min at 37° in a metabolic shaker the incubation was stopped by cooling and addition of 100 μl of 5% trichloroacetic acid.

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Following mixing 100 μl of the supernatant was put onto a small double column of Dowex 50 H^+ (approx. vol. 150 μl) on top of Dowex 1 OH^- (approx. vol. 100 μl). The tritiated water formed was eluted into a scintillation vial with 0.5 ml of H_2O, and counted with 0.4% butyl-PBD in toluene/ethanol at an efficiency of 30 per cent. Activity is expressed as fmol H_2O formed/min of incubation/mg protein in the synaptosomal suspension.

Assay for in vitro *tyrosine hydroxylase activity*. The measurement of *in vitro* tyrosine hydroxylase activity was an adaptation of the procedure of Buda *et al.* [8]. Samples were prepared by homogenisation in 5 vol. 5 mM sodium phosphate buffer (pH 6.2 with 0.2% Triton X-100) and assayed by incubation of 25 μl of sample with 5 μl of catalase (1000 U; Boehringer), 10 μl of 5 mM tetrahydrobiopterin in 140 mM mercaptoethanol, 5 μl of 1 M sodium acetate buffer pH 5.8 and 5 μl of L-[3,5-^{3}H]tyrosine (0.74 mCi/μmol) to a final concentration of 55 μM. Incubation was at 37° for 10 min, and was stopped by cooling and addition of 100 μl of 5% trichloroacetic acid. The tritiated water formed was separated and counted as described for the synaptosomal assay. Activity is expressed as fmol H_2O formed/min of incubation/mg protein.

RESULTS AND DISCUSSION

Male Sprague–Dawley rats weighing about 250 g were given 10 mg/kg reserpine by i.p. injection and killed seven days later, together with an untreated control group. The cerebellum, hippocampus and hypothalamus were removed; one half of the cerebellum, one hippocampus and the entire hypothalamus were stored over liquid nitrogen for later analysis of *in vitro* tyrosine hydroxylase activity. The remaining hippocampus and hypothalamus were immediately utilised for preparation of a crude synaptosomal suspension and the estimation of synaptosomal tyrosine hydroxylation.

The results of the *in vitro* tyrosine hydroxylase assay are shown in Table 1. In common with Black [4] we find a very substantial increase in cerebellar tyrosine hydroxylase following reserpine administration. This finding is extended by the results in Table 1 to a further projection area of the locus coeruleus, the hippocampus, and to the hypothalamus. In the latter region the locus coeruleus probably contributes no more than 40 per cent of the noradrenergic innervation. Despite this the elevation in amount of tyrosine hydroxylase is comparable to that observed in the other two regions studied, suggesting that terminals originating from several groups of cell bodies are affected.

In Table 2 are presented the results of the estimation of tyrosine hydroxylation rates in the synaptosomal preparations; the control figures in Tables 1 and 2 reflect the difference between the preparations as well as the tyrosine concentrations used in the two assays. Synaptosomal tyrosine hydroxylation retains the sensitivity to end-product inhibition and to cyclic AMP activation [6] shown by an *in vitro* preparation of the enzyme [12, 13]. Table 2 shows that reserpine pretreatment failed to alter the rate of the reaction in either hippocampus or cerebellum. Despite an increase in the amount of enzyme in the samples from reserpine pretreated animals, there is no increase in synaptosomal tyrosine hydroxylation, suggesting that the activity of the enzyme becomes suppressed by factors retained in the synaptosomal preparation, bringing the total activity to control levels.

Table 2. Synaptosomal tyrosine hydroxylation in reserpinised and control rats

	Control	Reserpine
Cerebellum	264 ± 31	258 ± 30
Hippocampus	284 ± 23	295 ± 41

Figures are fmol/min/mg protein in synaptosomal suspension, ± S.E.M. Each determination was in duplicate, with $n = 5$ in each case.

We have considered the possibility that the tyrosine hydroxylation rates are artificially lowered in the synaptosomal preparation by the action of reserpine, which limits the availability of functional vesicles, leading to the accumulation in the soluble compartment of dopamine or its metabolites during the incubation. This may result in feedback inhibition of tyrosine hydroxylase. An expected consequence of this would be a non-linear time course for product accumulation, with feedback inhibition building up during the incubation. We investigated this possibility by injecting three animals with 10 mg/kg i.p. reserpine. The animals were killed, with controls, after 7 days and the rate of synaptosomal tyrosine hydroxylation by cerebellar preparations at various incubation times from 5 to 25 min was determined. The result was two identical and linear plots of product accumulation with time (data not shown). This observation is incompatible with the possibility that the rate of hydroxylation in the synaptosomes is a consequence of reduced uptake of dopamine into vesicles.

We therefore conclude that elevated levels of tyrosine hydroxylase, in response to an inducing stimulus such as reserpine, lead to an increased potential for tyrosine hydroxylation in terminals which may not be expressed due to interaction with regulatory mechanisms. These mechanisms may be a consequence of one or more of the potential regulatory factors which have been shown to modify tyrosine hydroxylase activity, such as availability of substrates and co-factors [9–11],

Table 1. *In vitro* tyrosine hydroxylase activity in reserpinised and control rats

	Control	Reserpine	% of control	Significance
Cerebellum	587 ± 50	1381 ± 175	235	$P < 0.01$
Hippocampus	931 ± 65	1441 ± 68	154	$P < 0.001$
Hypothalamus	25225 ± 1691	45700 ± 8463	181	$P < 0.05$

Figures are fmol/min/mg protein ± S.E.M.
Each determination was in duplicate with $n = 5$ in each case.

product feedback inhibition [12], levels of Ca^{2+} and cyclic adenosine monophosphate [11, 13], interaction with lipid [14], and conformational transitions [15].

It has been suggested that transynaptic enzyme induction represents a long term adaptation to increased transmitter utilisation. The results of our experiments with intact synaptosomes indicate that an increase in enzyme protein does not necessarily result in a raised level of transmitter synthesis.

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