Specific immunolysis of serotonergic nerve terminals using an antiserum against tryptophan hydroxylase

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An antiserum to tryptophan hydroxylase purified from whole rat brain when incubated with rat striatal synaptosomes in the presence of complement caused release of 18% of LDH, 20% loss of potassium and 60% loss of tryptophan hydroxylase. Uptake of 5-HT was reduced by 60%. Anti-tryptophan hydroxylase alone, or complement alone were without action. The antiserum plus complement had no effect on DA uptake and did not release TH or GAD. These results suggest selective lysis of serotonergic nerve terminals had occurred. The antiserum plus complement reduced choline uptake by 45%. However, this did not seem due to lysis of cholinergic terminals, as ChAT was not released.

Synaptosome *Serotonergic nerve terminal* *Lysis* *Antiserum* *Tryptophan hydroxylase*

1. INTRODUCTION

It is possible to investigate the presence of cell surface antigens on synaptosomes by monitoring the immunolysis which occurs after exposure of these preparations to a specific antibody and a source of complement [1–3].

We recently reported that antisera to choline acetyltransferase (ChAT), glutamate decarboxylase (GAD) and tyrosine hydroxylase (TH) evoked complement-mediated lysis of specific subpopulations of synaptosomes prepared from both the cerebral cortex and corpus striatum of rat [4]. These observations suggest that the antisera used were capable of recognizing specific antigens in the outer face of the synaptosomal membrane.

We now describe a similar effect produced by an antiserum to tryptophan hydroxylase, purified from whole rat brain. This antiserum, in the presence of complement, appears to lyse serotonergic synaptosomes (prepared from the corpus striatum of rat) but appears not to act on dopaminergic or GABAergic nerve terminals. Choline uptake into the treated striatal synaptosomes is reduced. However, this action is apparently not associated with the immunolysis of cholinergic nerve terminals.

2. MATERIALS AND METHODS

Tryptophan hydroxylase was purified from whole rat brain (full details to be published elsewhere). Briefly, brains were homogenized in an activating medium containing 50 mM Tris-HCl (pH 8), 20 mM dithiothreitol, 2 mM Ca^{2+}, 50 μM Fe^{2+} and 0.1 mM phenylmethylsulphonylfluoride, and after centrifugation, the resulting supernatant was applied to a 6,7-dimethyl-5,6,7,8-tetrahydropterine affinity column. After rinsing, the enzyme was eluted with 10 mM $NaHCO_3$ buffer (pH 10.8) containing 0.1 mg/ml catalase. The enzyme was then subjected to phenyl-Sepharose and finally hydroxyapatite chromatography. The hydroxyapatite fractions from 5 purifications (which had a specific activity of about 370 nmol/min per mg protein and were pure according to the criterion of SDS electrophoresis) were concentrated to about 500 μl and injected at 8-day intervals subcutaneously into the rabbit. The first 3 injections contained Freund's complete adjuvant. Eight days

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after the final injection, the rabbit was killed, the blood collected and the serum prepared. The presence in the serum of antibodies to tryptophan hydroxylase was tested by immunoprecipitation and immunoblotting.

Lyophilized guinea pig complement, obtained from Gibco, was prepared for use as in [4].

The sodium-dependent uptake of 5-[1,2(n)-^{3}H]hydroxytryptamine creatinine sulphate (23.4 Ci/mmol, New England Nuclear) into rat corpus striatal synaptosomes was measured at 0.05–2 μM 5-HT as in [4] in the presence of ascorbic acid (0.1 mg/ml) and nialamide (10 μM). Uptake of 5-HT was also assayed in synaptosomes that had been lysed by the addition of 0.25% (v/v) Triton X-100. Eadie-Hofstee plots of V vs V/S were constructed to determine V_{max} and K_m values for the uptake process. Straight lines were fitted to the data by linear regression.

The sodium-dependent uptake of [^{3}H]choline (15 Ci/mmol, Amersham) and [2,5,6-^{3}H]dopamine hydrochloride (9.6 Ci/mmol, Amersham) at 0.125 μM was also measured [4].

Tryptophan hydroxylase activity was measured essentially as described in [5] by the formation of 5-hydroxy[4-^{3}H]tryptophan from [5-^{3}H]tryptophan (27.6 Ci/mmol, New England Nuclear), and the subsequent acid-dependent quantitative release of ^{3}H as ^3H_2O; unreacted substrate being removed by activated charcoal. Catalase (0.5 mg/ml) was included in the incubation mixture.

ChAT activity was measured using 0.4 mM [1-^{14}C]acetyl coenzyme A (4 mCi/mmol, Amersham) as in [6] using incubation volumes of 35 μl.

GAD activity was assayed by the production of $^{14}CO_2$ from L-[1-^{14}C]glutamic acid (59 mCi/mmol, Amersham), at a final concentration of 2.5 mM as described [2].

Tyrosine hydroxylase activity was assayed by the production of [^{3}H]dopa from L-[2,6-^{3}H]tyrosine (40 Ci/mmol, Amersham) using columns of activated aluminium oxide to trap reaction products. Incubations of 75 μl volume were performed in Eppendorf tubes, and contained 10 μM tyrosine, 100 μM sodium acetate (pH 5.8), 6-methyl-5,6,7,8-tetrahydropterine in 28 mM mercaptoethanol and catalase (0.5 mg/ml). After 20 min incubation at 37°C the reaction was stopped by the addition of 1 ml 0.4 N $HClO_4$. The tubes were then centrifuged at 10000 × g for 5 min at 4°C. To the supernatant was added 6.5 ml of 1.55% disodium EDTA and 80 mM KH_2PO_4. This solution was adjusted to pH 8.6 and passed over an aluminium oxide column (approx. 400 mg). The column was washed with 20 ml water. The reaction products were eluted with 4 ml of 0.5 N acetic acid. This was mixed with 11 ml ACS II scintillant (Amersham International) for subsequent radioactivity counting. Recovery from the column was routinely 50%. Lactate dehydrogenase (LDH) activity [7], potassium levels [4] and protein content [8] were measured as described.

The actions of the antiserum were tested essentially as in [2,4] by incubating striatal synaptosomes (500–1000 μg/ml) with (a) antisera (20–250 μl) plus complement (1/20 of final volume), (b) antisera alone (20–250 μl), (c) complement alone (1/20 of final volume), (d) non-immune serum (20–250 μl) plus complement (1/20 of final volume) or (e) phosphate buffer alone in a total volume of 0.5–1.0 ml for 30 min at 37°C. After incubation the synaptosomes were deposited by centrifugation at 10000 × g for 30 s. The supernatant was retained for the measurement of tryptophan hydroxylase, ChAT, GAD, tyrosine hydroxylase and LDH activity. After surface washing, the pellet was resuspended in Krebs-phosphate medium. An aliquot of the synaptosomal suspension (500–1000 μg/ml) was used to measure 5HT, choline and dopamine uptake, to assay for tryptophan hydroxylase, ChAT, GAD, tyrosine hydroxylase and LDH activities, and to measure potassium content.

Release of enzymes from synaptosomes due to the combined action of antiserum plus complement could thus be calculated, and was expressed as a percentage of the total after subtraction of the amount present in the incubation medium at the start of the incubation and that due to non-specific release.

3. RESULTS AND DISCUSSION

When the antiserum (50 μl) was incubated with the phenyl-Sepharose eluted fraction (250 μl) at 4°C for 15 h and then centrifuged, no tryptophan hydroxylase activity remained in the supernatant. Normal rabbit serum was incapable of such immunoprecipitation.

Immunoblotting tests revealed only one major

band which corresponded to the major stained band obtained on a gradient polyacrylamide slab gel, run under non-denaturing conditions.

When striatal synaptosomes were incubated with different dilutions of anti-tryptophan hydroxylase serum in the presence of complement, lysis occurred as reflected by the release of LDH into the supernatant to a maximum of 18% of the total present (fig.1). The response was proportional to the amount of antibody used, with optimal concentrations lying between 0.8 and 1.0 μl antiserum/μg synaptosomal protein. With optimal amounts of antibody, the potassium content of the treated synaptosomes was reduced to 80% of untreated controls (control values 201 ± 15 nequiv./mg protein, mean ± SD, n = 4) 5-HT uptake into these treated synaptosomes was reduced to 40% of control values (fig.1). Tryptophan hydroxylase activity was not detected in the supernatant obtained after centrifuging treated synaptosomes. However, measurement of the enzyme activity in the pelleted synaptosomes revealed that after exposure to a mixture of antisera and complement, up to 60% of the enzyme activity had been lost (fig.2). Such loss did not occur after exposure of synaptosomes to antisera alone.

This suggests that after exposure to both antisera and complement, striatal serotonergic synaptosomes are lysed with both a loss of 5HT uptake and a release of tryptophan hydroxylase. A loss of 5HT uptake also occurs after lysis of synaptosomes using Triton X-100. The V_{max} for the process is then reduced to 20 pmol/2 min per mg and the K_m increased to 8.5 μM, the correlation coefficient for the plot of V vs V/S being −0.54 (3 separate synaptosome preparations, 5 substrate concentrations used in each). Tryptophan hydroxylase released during immunolysis cannot be detected in the medium. This could be because antibodies to the enzyme are inhibiting its activity, as it is known to inhibit the purified enzyme (not shown). However, since there was no loss of activity during incubation with antiserum alone, it is unlikely that antibodies were able to penetrate synaptosomes and inactivate the soluble tryptophan hydroxylase within. Also, any enzyme bound to the outer surface of the synaptosome membrane must be a relatively small proportion of the total tissue activity approximating to the level of error in the assay method. Alternatively, it could reflect the inherent instability of the free enzyme, when unprotected in incubation media.

That such immunolysis was selective for serotonergic terminals in the striatum was demonstrated by the absence of TH and GAD release (total control values 120 ± 13 and 580 ± 60 pmol/min per mg, respectively, mean ± SD, n

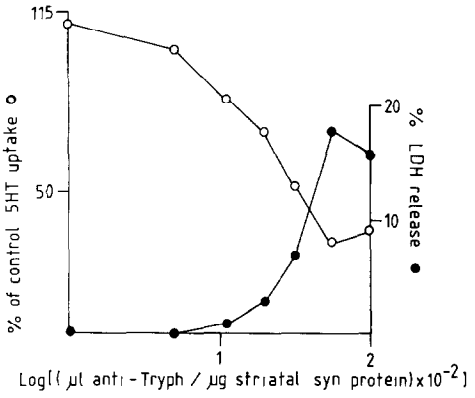

Fig.1. Uptake of 5-HT into (○) and release of LDH from (●) striatal synaptosomes treated with anti-tryptophan hydroxylase serum plus complement. Separate synaptosome preparations were used for each dose of antiserum tested. Uptake of 5-HT into control synaptosomes, using 0.05 μM 5-HT was 12 ± 2.3 pmol/2 min per mg (mean ± SD, n = 5). Total LDH activity in control synaptosomes was 1.3 ± 0.15 μmol/min per mg (mean ± SD, n = 4).

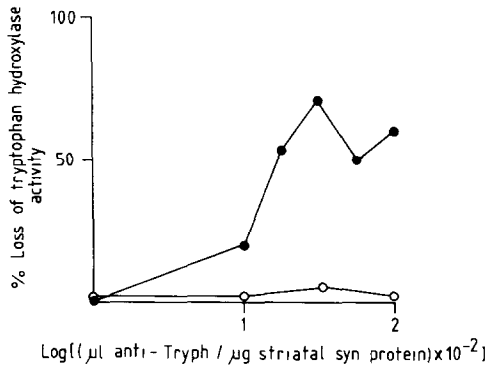

Fig.2. Loss of tryptophan hydroxylase activity from striatal synaptosomes treated with anti-tryptophan hydroxylase serum plus complement (●) and anti-tryptophan hydroxylase serum alone (○). Total tryptophan hydroxylase activity in untreated synaptosomes was 8.3 ± 0.6 pmol/min per mg (mean ± SD, n = 7).

= 8 and total values after exposure to antibody and complement 135 ± 25 and 620 ± 70 pmol/min per mg, respectively, mean ± SD, n = 5) and the lack of action on dopamine uptake (control uptake 62 ± 9 pmol/2 min per mg, mean ± SD, n = 9 and uptake after exposure to antibody and complement 70 ± 12 pmol/2 min per mg, mean ± SD, n = 4). However, choline uptake was reduced by this treatment. This effect did not seem to be caused by the lysis of cholinergic terminals as ChAT was not released (fig.3), and is therefore likely to reflect a direct action on the choline transport system.

The results reported here suggest that an antiserum against tryptophan hydroxylase, in the presence of complement, will cause specific lysis of serotonergic synaptosomes. Tryptophan hydroxylase has been considered to exist in both a soluble and particulate form [9]. However, it has been suggested that this merely reflects its presence in cell bodies and nerve terminal regions respectively. Immunological studies indicate that the same antibody reacts with the 'soluble' form in 5HT cell bodies and the 'particulate' form in 5-HT nerve terminals in the septum [10]. However, Joh et al. [10] observed that the tryptophan hydroxylase was associated with endoplasmic reticulum in the cell bodies and with neurotubules in terminal regions.

If the tryptophan hydroxylase present in striatal synaptosomes is associated with neurotubules, it is possible that the enzyme is exposed at the outer surface of the synaptosome during vesicular exocytosis, thus allowing antiserum and complement to interact.

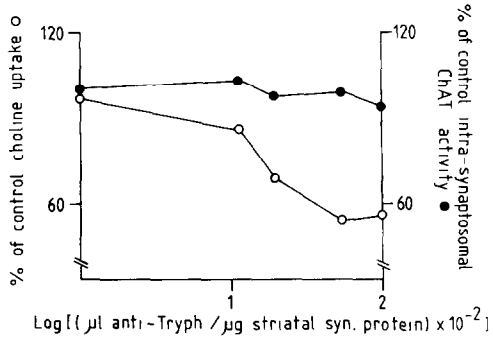

Fig.3. Uptake of choline into (○) and release of ChAT from (●) striatal synaptosomes treated with anti-tryptophan hydroxylase serum plus complement. Uptake of choline into control synaptosomes, using 0.125 μM choline, was 40 ± 5 pmol/2 min per mg (mean ± SD, n = 4). Total ChAT activity in control synaptosomes was 736 ± 90 pmol/min per mg (mean ± SD, n = 4).

However, our results might also be explained by the existence of a peptide chain common to both tryptophan hydroxylase and a synaptosomal membrane component which serves as a phenotypic marker for serotonergic nerve terminals. Results obtained previously using antisera against ChAT, GAD and TH [1,2,4] have suggested that this phenomenon might apply to many neurotransmitter systems. These cell surface antigens may be very important in phenotypic classification in the mammalian CNS.

NOTE ADDED IN PROOF

Concerning the details of the purification of tryptophan hydroxylase referred to in section 2 as to be published elsewhere, this work has now been accepted for publication: Cash, C.D., Vayer, P., Mandel, P. and Maitre, M. (1985) Eur. J. Biochem., in press.

ACKNOWLEDGEMENT

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