Specific lysis of GABAergic synaptosomes by an antiserum to glutamate decarboxylase

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An antiserum to pure glutamate decarboxylase (CGAD) when incubated with rat cortical synaptosomes in the presence of complement caused release of 33–53% of lactate dehydrogenase (LDH) and 22–41% of total GAD. In addition most of the γ-aminobutyrate (GABA) present was released. Anti-GAD antiserum alone, or complement alone, were without action. The antiserum plus complement had no effect on noradrenaline or choline uptake, and did not release cholineacetylase (ChAT). Anti-ChAT serum plus complement released 30–37% of ChAT and 10–13% of LDH. It prevented choline uptake. This serum did not produce GAD release or prevent GABA, choline or noradrenaline uptake. When cortical synaptosomes were exposed to both antisera plus complement, their actions were strictly additive. The data indicate specific lysis of GABAergic and cholinergic synaptosomal sub-populations.

Synaptosome *GABAergic* *Lysis* *Antiserum* *GAD* *LDH*

1. INTRODUCTION

A major limitation in the use of synaptosomes from mammalian brain in the study of synaptic biochemistry is the heterogeneity of the preparations. Neurones which employ different neurotransmitters each contribute their nerve-terminals to the preparations obtained by density-gradient centrifugation [1]. Antisera directed against specific components of these neurotransmitter systems could theoretically be used to achieve a separation of these sub-populations of synaptosomes, if the relevant specific antigens were located on the outer face of the synaptosomal membrane. Such techniques have been applied to the separation of lymphocytes [2].

We recently reported that an antiserum to a choline acetyltransferase preparation allowed complement-mediated lysis of the cholinergic subpopulation of cortical synaptosomes [3]. We now describe a similar effect produced by an antiserum to glutamate decarboxylase, purified from mouse brain. This preparation, in the presence of complement, appears to lyse GABAergic synaptosomes, whilst having no action on either the cholinergic or the noradrenergic sub-populations.

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Abbreviations: BSA, bovine serum albumin; GAD, glutamate decarboxylase (EC 4.1.1.15); ChAT, choline acetyltransferase (EC 2.3.1.6); LDH, lactate dehydrogenase (EC 1.1.1.27); GABA, γ-aminobutyric acid; NA, noradrenaline; DABA, diaminobutyric acid; Butyl-PBD, 2-(4'-tert-Butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole

2. METHODS

Antibodies against purified GAD from mouse brain were produced in rabbits by bi-weekly injections of a total of 50–60 μg of protein over a period of 8 weeks. Antisera obtained were characterized by immunodiffusion, enzyme inhibition,

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immunoelectrophoresis and microcomplement fixation test [4–6].

Antibodies against ChAT were prepared as in [3]. GAD activity in synaptosome preparations was assayed by the production of $^{14}CO_2$ from L-[U-^{14}C] glutamic acid (280 mCi/mmol, Amersham). Incubations of 1-ml volumes were performed in glass scintillation vials sealed with rubber suba-seals (no.33), and contained 25 mM glutamate, 1 mM dithiothreitol, 0.2 mM pyridoxal phosphate, 1 mg/ml BSA in 50 mM sodium phosphate buffer (pH 6.8). An Eppendorf tube, with cap removed, was placed in each scintillation vial and contained 20 μl 20% NaOH absorbed in a small strip of filter paper. The reaction was started by placing the sealed vials into a shaking water bath at 37°C. After 30 min, 50 μl of ION H_2SO_4 was injected through the suba-seal and into the incubation mixture to stop the reaction. The vials were kept at room temperature overnight to allow all the $^{14}CO_2$ released during the reaction to be trapped by the alkaline-soaked filter paper. The filter paper was then removed and placed in 10 ml scintillant (toluene with 0.33% w/v butyl PBD plus 44% v/v 2-methoxyethanol). Radioactivity was measured in a Packard Tri-Carb 300C liquid scintillation counter. Blank values (1–5%) obtained by incubating ^{14}C-glutamate in the absence of synaptosome preparations were subtracted from sample values.

The sodium-dependent uptake of [methyl-^{3}H] choline chloride (15 Ci/mmol, Amersham) at 0.0625–2 μM choline, and of 4-amino-n-[2,3-^{3}H] butyric acid (64 Ci/mmol, Amersham) at 0.125–4 μM, into rat cerebrocortical synaptosomes [7] was measured as in [3]. The sodium-dependent uptake of DL-[7-^{3}H] noradrenaline hydrochloride (13 Ci/mmol, New England Nuclear) at 0.0625–2 μM was measured in a similar manner, in the presence of ascorbic acid (0.1 mg/ml) and nialamide (10 μM). Uptake of DL-[7-^{3}H] noradrenaline and [methyl-^{3}H] choline chloride was also assayed in synaptosomes that had been hypotonically lysed in 8 mM Tris–HCl (pH 8.1) at a protein concentration of 1 mg/10 ml for 30 min at 0–4°C. Synaptosomes were pelleted at 10000 × g and resuspended in 0.32 M sucrose at a protein concentration of 2 mg/ml. In some experiments synaptosomes were pre-incubated with Krebs incubation medium containing 1 μM dopamine prior to the assay of NA uptake.

Choline acetyltransferase activity was measured using 0.4 mM [1-^{14}C] acetyl coenzyme A (4 mCi/mmol, Amersham) as in [8] using incubation volumes of 35 μl. Lactate dehydrogenase activity was measured as in [9]. Samples were prepared for amino acid analysis as in [10].

The actions of the antisera were tested by incubating cortical synaptosomes (400 μg protein/ml) in 500 μl of Krebs-Ringer phosphate which contained either GAD antiserum (50 or 100 μl) or ChAT antiserum (100 μl) plus 50 μl of guinea pig complement (Gibco Labs), or the antisera alone for 30 min at 37°C (maximal effects were measured after 30 min incubation). In some experiments synaptosomes were incubated with both anti-GAD (50 μl) and anti-ChAT (100 μl) sera in the presence of complement (50 μl). After incubation, the synaptosomes were deposited by centrifugation at 10000 × g. The supernatant was retained for the measurement of GAD, ChAT and LDH activity and GABA. The pellets were first washed with sodium free Krebs–Ringer medium before being resuspended in 0.32 M sucrose (2 mg synaptosomal protein/ml) for the assay of choline, GABA and NA uptake, where 25 μl of this suspension was added to 250 μl final incubation volume. ChAT and LDH activity in the pellets was measured after resuspension in 1% Triton X-100 (v/v). The activity of GAD in the pellets was assayed after lysis produced by resuspension in 8 mM Tris–HCl (pH 8.1) and freezing and thawing, twice.

3. RESULTS AND DISCUSSION

The results of table 1 show that the anti-GAD serum released 58–68% of synaptosomal GAD at a dilution of 1:10 in the incubation medium, provided complement was added. Increasing the concentration of this antiserum 2-fold did not increase the extent of the GAD release. Under these conditions (1:10 dilution) GABA was also found to be released into the supernatant though no GABA was detectable in the absence of complement. The enzyme ChAT was not released by the anti-GAD serum (± complement).

Measurement of LDH showed that this soluble enzyme was released into the incubation medium to the extent of 43–58% by this antiserum at the

Table 1

Effects of antisera on release from cerebrocortical synaptosomes prepared from rat

Treatment	GAD activity (% released)	GABA release	ChAT activity (% released)	LDH activity (% released)
Krebs medium	n.d.	n.d.	n.d.	5– 7
Control serum (1:5) + complement	n.d.	n.m.	5– 6	6– 8
Anti-GAD serum (1:10) + complement	58–68	158 ± 20	n.d.	43–58
Anti-GAD serum (1:5) + complement	49–66	n.m.	n.d.	40–60
Anti-GAD serum (1:10)	n.d.	n.d.	n.d.	4– 7
Anti-GAD serum (1:5)	n.d.	n.m.	n.d.	3– 6
Complement	14–27	n.d.	n.d.	4– 6
Anti-ChAT serum (1:5) + complement	n.d.	n.m.	36–43	17–20
Anti-ChAT serum (1:5) + Anti-GAD serum (1:10) + complement	45–60	n.m.	29–39	62–79

Values given are means ± S.D. from at least 4 separate synaptosome preparations and are calculated after subtraction of that free in the medium at the start of each incubation. Values for GABA release are in units of pmol/20 μg synaptosomal protein/30 min. Total activities/mg synaptosomal protein were: GAD, 200 ± 60 pmol $CO_2 . min^{-1}$; ChAT, 360 ± 27 pmol ACh $. min^{-1}$ and LDH, 1.8 ± 0.16 μmol $. min^{-1}$

n.d., not detectable; n.m., not measured

Table 2

Effect of antiserum to purified GAD on uptake into cerebrocortical synaptosomes prepared from rat

Treatment	Sodium-dependent choline uptake		Sodium-dependent GABA uptake		Sodium-dependent uptake of 0.0625 μM NA
	V_{max}	K_m	V_{max}	K_m	
Krebs medium	50 ± 6	0.52 ± 0.05	760 ± 69	4.8 ± 0.4	14.2
Anti-GAD serum (1:10) + complement	53 ± 6	0.51 ± 0.06	782 ± 80	4.4 ± 0.5	13.8
Anti-ChAT serum (1:5) + complement	n.d.	n.d.	680 ± 80	5.4 ± 0.7	15.2

Values given for choline and GABA uptake are means ± S.D. from at least 4 separate synaptosome preparations. NA uptake values are from 2 preparations and are in units of pmol $.$ mg synaptosomal protein$^{-1} . 2$ min^{-1}. V_{max} values for choline and GABA uptake are given in the same units. K_m is given in units of μM. n.d., not detectable

1:10 dilution, and by 40–60% at the 1:5 dilution, provided complement was present. Neither complement alone, nor antiserum alone caused any release of LDH, ChAT or of GABA above control levels. Complement itself contained GAD activity (43 ± 9 pmol $CO_2 . min^{-1} . 50\ \mu l^{-1}$, $n = 6$) and this fully accounts for the apparent releasing action of complement shown in table 1. The GAD antiserum was without action on either choline or noradrenaline uptake (table 2).

These results indicate that this antiserum contains antibodies capable of lysing about half of the cortical synaptosomes, whilst releasing both GAD and GABA. The lack of action on choline or on noradrenaline uptake (table 2), and the absence of any ChAT release (table 1) suggests that there is a considerable degree of specificity with regard to the sub-population(s) which is vulnerable to lysis by the GAD antiserum plus complement, and strongly indicates that it may well be confined to the GABAergic terminals.

Dopamine (1 μM) blocks noradrenergic uptake by cerebrocortical synaptosomes (fig.1), and hypotonic lysis greatly reduced both noradrenaline and choline uptake (fig.2), indicating that the usual form of sodium-dependent high affinity uptake was being measured. Uptake measured under these conditions (fig.2) no longer conformed to Michaelis-Menten kinetics (choline uptake; $r = -0.66$, $y = 10 - 1.68x$), noradrenaline uptake; $r = -0.66$, $y = 20 - 2.5x$).

When both anti-GAD and anti-ChAT sera were added to cerebrocortical synaptosome preparations together with complement, the total LDH release observed (62–79%) was equivalent to the sum of the actions of each antiserum when added alone (table 1). ChAT antiserum, whilst releasing ChAT, was without effect on either GAD or GABA, and did not influence NA uptake.

If the anti-GAD serum is acting specifically on GABAergic terminals, the lack of effect on GABA uptake (table 2) has to be satisfactorily explained, especially as the antiserum appears to release GABA itself (table 1). The GABA uptake measured in our system has been shown to be reduced by hypotonic lysis of synaptosomes, and is blocked by DABA [3], indicating that it is the standard form of sodium-dependent high-affinity GABA uptake. The most likely explanation is that GABA is taken up by structures other than GABAergic terminals, such as free mitochondria which can employ GABA as an effective metabolic substrate [11].

The results reported here and previously [3] on the extent of LDH release by either GAD antiserum or by ChAT antiserum indicate that the contribution of GABAergic terminals to rat cortical synaptosome preparations is about 40%, whilst cholinergic terminals contribute about 10%. This is confirmed by the strictly additive effect of exposing synaptosomes to both antisera simultaneously. The additive nature of these actions, and the change in specific properties (e.g., choline uptake loss, GAD release) effected by each antiserum makes it most unlikely that the location of GAD and of ChAT in the outer synaptosomal membrane is an artefact due to surface adsorption during synaptosome preparation. The surface

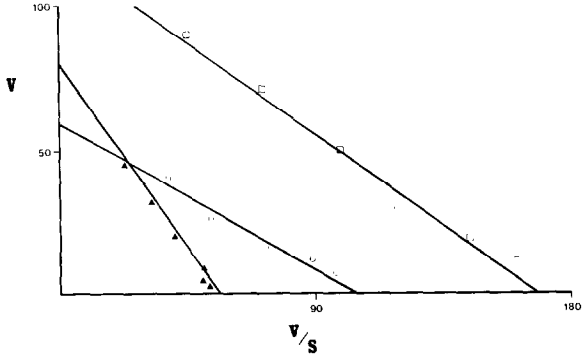

Fig.1. Typical Eadie-Hofstee plots of sodium-dependent choline (○—○) and noradrenaline (□—□) uptake into control synaptosomes. Noradrenaline uptake into synaptosomes in the presence of 1 μM dopamine is also shown (▲—▲). Data are the means of triplicate determinations at each substrate concentration. Lines through the points were determined using linear regression analysis.

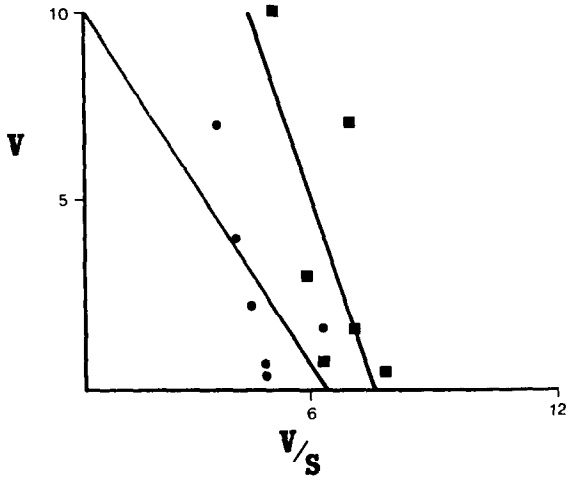

Fig.2. Typical Eadie-Hofstee plots of sodium-dependent choline (●—●) and noradrenaline (■—■) uptake into lysed synaptosomes. Data calculated as in fig.1.

localization of specific markers of this kind for each neuronal type would be of great value in directing growing nerve-fibres to their target sites during brain development, even though the bulk of the enzyme is located elsewhere (e.g., in the cytoplasm).

It is possible that neurotransmitter synthesising enzymes may be expressed on the surface of neurones employing other neurotransmitters, and we are currently investigating whether or not this is a general rule.

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