Amino acid neurotransmitters in the CNS

Characteristics of the acidic amino acid exchange

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D-Aspartate exchange, defined as amino acid-stimulated D-[^{3}H]aspartate efflux, was investigated in a preparation of rat brain synaptosomes. The efflux of radiolabelled D-aspartate was found to be enhanced by micromolar concentrations of externally added D- and L-aspartate, L-glutamate, L-cysteate and L-cysteinesulphinate. The stimulation of release by external amino acids followed Michaelis-Menten kinetics; the apparent K_m values (in μM) were: 14.65 ± 0.98 for D-aspartate; 8.00 ± 1.5 for L-aspartate; 22.31 ± 1.62 for L-glutamate; 6.76 ± 0.3 for L-cysteate and 7.89 ± 1.23 for L-cysteinesulphinate. The V_{max} values for efflux were 2.16–4.06 nmol/min per mg protein. The exchange process was found to require external NaCl but was very little affected by increase in the external [K^+]. The demonstration of exchange as a part of the transport process provides support for the suggestion that in synaptosomal preparations a substantial portion of influx and efflux of amino acid neurotransmitters occurs via a reversible membrane carrier.

Amino acid neurotransmitter *Amino acid exchange* *Neurotransmitter transport* *Aspartate*

1. INTRODUCTION

Studies of the early 1970's [1] have suggested that in mammalian central nervous system, acidic amino acid transmitters, L-glutamate and L-aspartate, and the D-stereoisomer of the latter, D-aspartate, share the same high-affinity transport system. This postulate was based on the observation that uptake of any one of the above amino acids was inhibited in a competitive manner by any other member of the group with a K_i which was not very different from the respective K_m for transport [1,2]. It has also been shown that L-cysteate and L-cysteinesulphinate, which are transported with high affinities [3–5], are potent competitive inhibitors of both L-glutamate [1] and D- and L-aspartate [2] influx while the latter 3 amino acids affect in the same manner uptake of L-cysteate [3] and L-cysteinesulphinate [4,5]. It seems therefore that either all 5 amino acids are transported on the same carrier protein or that transporters which are responsible for uptake of individual amino acids have overlapping specificities.

It is well known that many transport systems can mediate exchange in addition to net uptake. An exchange process of external for internal amino acid has been described for the central nervous system neurotransmitter γ-aminobutyric acid (GABA) in preparations of rat brain synaptosomes [6–9] and shown to involve a high-affinity transporter. However, much less information is available about other amino acid neurotransmitters although preliminary information from two laboratories [10,11] has indicated that the glutamate carrier may mediate homoexchange in addition to net accumulation.

In our earlier studies [12] we have evaluated in some detail the properties of synaptosomal high-

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affinity aspartate transport and suggested that they may be shared by other acidic amino acid neurotransmitters. The goal of this work was two-fold: (i) to determine whether the transporter for acidic amino acids mediates, like that for GABA, an exchange reaction; and (ii) to obtain information on the characteristics and degree of this process. Characterization of such a reaction could help us to throw additional light on the operation of acidic amino acid carrier protein(s). As in [12], aspartate was used as the representative neurotransmitter.

2. MATERIALS AND METHODS

Synaptosomes were isolated as described in [13] and protein measured as in [14].

2.1. *Measurements of aspartate homo- and heteroexchange*

Synaptosomes (about 10 mg protein/ml) were preloaded with 2 μM radioactive D-aspartate by 15 min incubation at 30°C (i.e. until a steady state was attained). Before further dilution, an aliquot was withdrawn and centrifuged through silicone oil to determine the level of radioactivity in the synaptosomal pellet and in the medium at zero time. Synaptosomes were then diluted into appropriate buffers with KCl and NaCl concentrations altered as required and containing either no or various amounts of cold amino acids (L- and D-aspartate, L- and D-glutamate, L-cysteate and L-cysteinesulphinate). Samples were taken at 30, 60, 90 and 120 s, rapidly centrifuged through silicone oil and the radioactivity in the pellets and supernatants measured as described [12]. Homoexchange was calculated by deducting values of the rates of efflux in the absence of cold amino acids from corresponding values for efflux measured in the presence of external (added) amino acids. All rates were determined by linear regression analysis of the increase of radioactivity in the supernatants. Total concentrations of aspartate in the pellets and in the supernatants were measured by HPLC as in [12].

3. RESULTS

3.1. *Kinetic properties of D-aspartate homo- and heteroexchange*

Here, we have defined 'exchange' as stimulation of release of an internal amino acid by addition to the external medium of either the same ('homoexchange') or a different ('heteroexchange') amino acid. To study this reaction we have labelled the internal aspartate pool by incubation with the radioactive D-analogue until a steady state was attained. The advantage of using D-aspartate was that while it shares all the properties of L-glutamate and L-aspartate transport, it is metabolized very slowly (less than 3% during 60 min incubation at 37°C [2]) and hence the radiolabel does not distribute into other products during the loading interval.

The efflux of D-aspartate from preloaded synaptosomes was measured as a function of time in the presence (stimulated) and absence ('control') of external unlabelled amino acids (L- and D-aspartate, L- and D-glutamate, L-cysteate and L-cysteinesulphinate). The basal efflux of labelled D-aspartate in Krebs-Henseleit-Hepes medium containing 5 mM potassium and 140 mM sodium was very slow whereas the addition of either amino acid markedly stimulated the release of radiolabel. The time course of stimulated efflux was almost

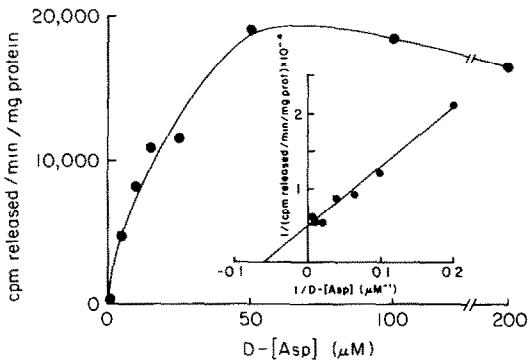

Fig.1. The dependence of the rates of D-[^{3}H]aspartate homoexchange on the external aspartate concentration. Synaptosomes were preincubated in Krebs-Henseleit-Hepes buffer for 15 min at 30°C with 2 μM D-[^{3}H]aspartate and diluted 10-fold into medium with and without various concentrations of unlabelled D-aspartate. The rates of radiolabelled aspartate efflux were determined as described in section 2. Aspartate homoexchange was calculated by deducting values of control efflux from corresponding values for efflux measured in the presence of added amino acid. $K_{m\ app}$ for homoexchange calculated from the experiment presented here is 16.4 μM.

identical to that of D-[^{3}H]aspartate uptake into synaptosomes when measured under the same conditions (i.e. medium composition, temperature and protein content; not shown). At a protein concentration of 0.8–1 mg/ml the rates were linear for up to 2 min and therefore the initial 3–4 time points were used for measurements of exchange velocities (see section 2).

Stimulation of D-[^{3}H]aspartate efflux by added amino acids followed Michaelis-Menten kinetics with respect to their external concentrations and was saturated at 20–50 μM D- or L-aspartate, 50–100 μM L-glutamate and 20–40 μM L-cysteate or L-cysteinesulphinate (see fig.1 for results with D-aspartate). By contrast, much higher concentrations of D-glutamate were required to stimulate D-aspartate efflux: the reaction was not saturated even at 2 mM amino acid. The external concentrations (in μM) of the amino acids required for half-maximal stimulation of D-[^{3}H]aspartate efflux (K_{app} for homoexchange) were: 14.65 ± 0.98 for D-aspartate, 8.00 ± 1.5 for L-aspartate, 22.31 ± 1.62 for L-glutamate, 6.76 ± 0.3 for L-cysteate and 7.89 ± 1.23 for L-cysteinesulphinate (table 1). The V_{max} values (table 1) as determined by double-reciprocal plots similar to that shown in fig.1 were 2.16–4.06 nmol/min per mg protein, i.e. not substantially different from the maximal velocity of D-aspartate uptake [12]. It was assumed in these calculations that labelled D-aspartate was evenly distributed within the endogenous pool(s) of aspartate.

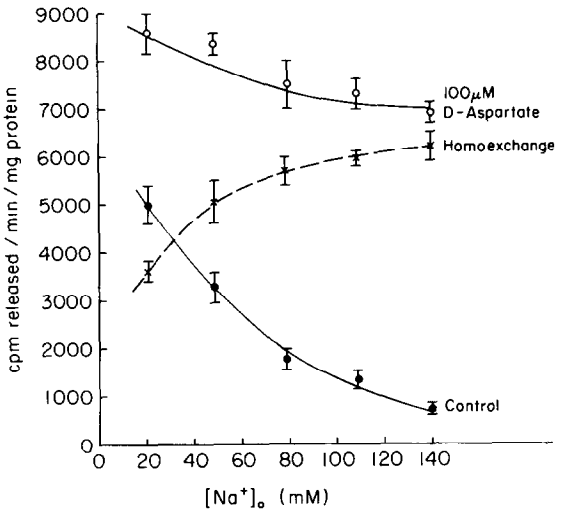

Fig.2. The dependence of aspartate homoexchange on the $[Na^+]_c$. Synaptosomes were preloaded with 2 μM D-[^{3}H]aspartate incubation for 15 min at 30°C and diluted 10–20-fold into media containing 5 mM potassium and various concentrations of sodium, with or without 100 μM unlabelled D-aspartate. The rates of radiolabel efflux were determined as given in section 2. Homoexchange was calculated as described in the legend to fig.1. Values are means ± SD for 4 experiments.

3.2. *Dependence of exchange on $[Na^+]$ and $[K^+]$*

The dependence of D-aspartate-stimulated D-[^{3}H]aspartate efflux on the concentrations of external sodium and potassium ion was determined by measuring the number of counts released from synaptosomes preloaded with the radiolabel and diluted into media containing 100 μM D-aspartate (i.e. a concentration which saturates the exchange

Table 1

Kinetic constants for D-aspartate homo- and heteroexchange

Amino acid	K_m (μM)	V_{max} (nmol/min per mg protein)
D-Aspartate	14.65 ± 0.98 (n = 4)	4.06 ± 0.58 (n = 4)
L-Aspartate	8.00 ± 1.50 (n = 3)	2.16 ± 0.29 (n = 3)
L-Glutamate	22.31 ± 1.62 (n = 3)	3.37 ± 0.32 (n = 3)
L-Cysteate	6.76 ± 0.30 (n = 3)	2.89 ± 0.45 (n = 3)
L-Cysteinesulphinate	7.89 ± 1.23 (n = 3)	3.13 ± 0.58 (n = 3)

D-Aspartate homo- and heteroexchange were measured after preloading the synaptosomes with radiolabelled D-aspartate as described in section 2. The results were plotted as $1/v$ vs $1/S$ (Lineweaver-Burk plots such as those presented in fig.1) and kinetic constants calculated by linear regression analysis. Values are means ± SD for the number of experiments indicated. The velocities were calculated from the number of counts accumulated in the synaptosomal pellet after loading and the concentration of aspartate inside the synaptosomes, 25.65 ± 3.23 nmol/mg protein, measured by HPLC. In these calculations the assumption was made that the preloaded [^{3}H]aspartate was equilibrated fully with all endogenous pools of this amino acid

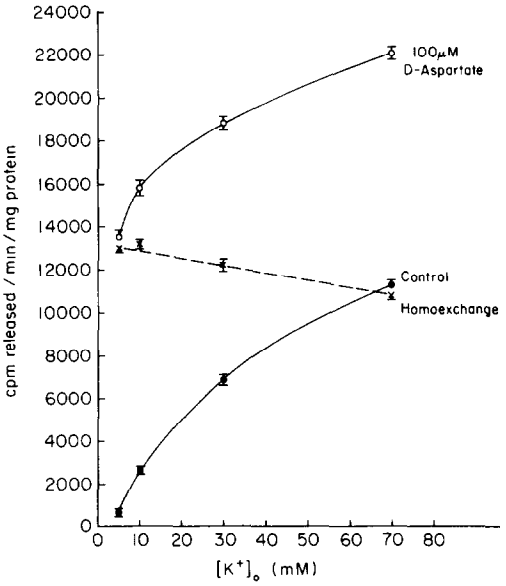

Fig.3. The dependence of aspartate homoexchange on the $[K^+]_e$. Synaptosomes were preloaded with radioactive D-aspartate as described in the legend to fig.2 and diluted into media containing 125 mM Na^+ and the indicated concentrations of potassium, with or without 100 μM unlabelled D-aspartate. The rates of efflux were determined as described in section 2, and homoexchange was calculated as given in fig.1. Values are means ± SD for 3 experiments.

reaction) at various $[Na^+]_e$ and $[K^+]_e$. The results obtained for sodium are shown in fig.2. It can be seen that the efflux of labelled D-aspartate in the absence of cold D-aspartate (control) was markedly enhanced by lowering the concentration of sodium in the external medium. By contrast, the velocity of D-aspartate-stimulated efflux (homoexchange) declined under the same conditions: the rate was reduced by about 50% when $[Na^+]_e$ was decreased from 140 to 15 mM. Similar results were obtained when synaptosomes were diluted to media containing saturated concentrations of other amino acids listed in table 1.

The dependence of the exchange reaction on $[K^+]_e$ is shown in fig.3. It can be seen that an increase in potassium concentration from 5 to 70 mM enhanced the control release of D-aspartate 15-fold. Under the same conditions the D-aspartate-stimulated efflux of the radiolabelled amino acid (homoexchange) was reduced, but only by about 20%.

4. DISCUSSION

A central finding of this paper is that the so-called high-affinity transporter for acidic amino acid neurotransmitters mediates an exchange process, in addition to the uptake reaction. This contention is supported by the following lines of evidence. First, the amino acids that compete with D-aspartate for high-affinity transport also induce its release. Second, the apparent K_m values for D- and L-aspartate, L-glutamate, L-cysteate and L-cysteinesulphinate exchange are strikingly similar to the respective K_m values for their influx [1–5,12,15–17]. Third, both processes follow the same time course and occur with similar velocities [12,15]. Finally, both processes are dependent on the concentration of external Na^+. The demonstration of a substantial exchange component in the transport of acidic amino acids in synaptosomes provides strong support for the suggestion [18] that, at least in this preparation, influx and efflux of neurotransmitter amino acids occur via a reversible carrier protein.

In evaluating the characteristics of the exchange process we have noticed several interesting properties of the acidic amino acid transporter. The first concerns its dependence on K^+ and Na^+. Unlike uptake and release, exchange of aspartate is only altered to a small extent by changes in the external K^+ concentration. Na^+, on the other hand, has a marked effect not only on uptake and release, but also on exchange. The second interesting property of the aspartate transporter is the marked stimulation of amino acid release at low $[Na^+]_e$. This would be expected in a reversible, carrier-mediated system because the efflux of the Na^+-aspartate complex occurs against the transmembrane sodium concentration gradient. When the latter is decreased by lowering the $[Na^+]_e$, release of sodium and the amino acid are favored. It was, however, noted that this increase in D-aspartate release at low concentration of sodium in the medium was much greater than that of GABA (Troeger, unpublished). Since both systems show a second power dependence on the transmembrane Na^+ concentration gradient [12,19] the difference may be attributed to the fact that the affinity of the GABA transporter for Na^+ is much lower (K_m about 70 mM [1,6,20–22]) than that of the acidic amino acid carrier (K_m <20 mM [1,2,17]). At

intrasynaptosomal concentrations of sodium observed under most experimental conditions (17–35 mM depending on the $[Na^+]_e$ [19,24]) the aspartate transporter will be more saturated with sodium and hence be able to release aspartate at higher velocities than the GABA carrier.

The third intriguing observation is that D-glutamate at millimolar concentrations also caused the release of radioactive D-aspartate. Since D-glutamate is not a substrate for the high-affinity transporter this may suggest that the low-affinity uptake system mediates likewise an exchange reaction in addition to net uptake.

ACKNOWLEDGEMENT

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