High affinity proline uptake in rat brain synaptosomes

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The uptake of L-proline by synaptosomes isolated from different regions of the brain was investigated. The highest rates of transport and the largest accumulation ratios were found in synaptosomes from the midbrain, striatum, hippocampus and hypothalamus, with lower activity in the cortex and medulla (+ pons) and lowest in the cerebellum. The high affinity, Na^+-dependent proline uptake had a K_m of 12 μM, a V_{max} of 0.6 nmol . min^{-1} . mg $protein^{-1}$ and the maximum accumulation ratio ($[proline]_i/[proline]_o$) at 2 μM added radioactive amino acid was 40–50. Our results suggest that: (1) only 5–10% of the synaptosomal population accumulates proline through the high-affinity uptake; (2) the characteristics of the proline uptake system into the prolinergic nerve endings are remarkably similar to those of other amino acid neurotransmitters (GABA, aspartate and glutamate); (3) the behavior of proline is consistent with this amino acid being a neurotransmitter in the central nervous system.

Synaptosome *Proline* *Neurotransmitter* *High affinity transport*

1. INTRODUCTION

Circumstantial evidence exists that L-proline may function as an inhibitory neurotransmitter in the mammalian central nervous system [1,2]. A sodium-dependent, high affinity uptake has been described for this amino acid in rat brain slices [3] and in synaptosomes [4–6] and potassium-stimulated release of proline from brain slices has been observed [3,7]. Based on measurements of tissue–medium ratios, the uptake of proline was found to be most efficient in slices of cerebral cortex and hypothalamus and least efficient in slices of cerebellum [3]. The accumulation ratios (tissue–medium) at 0.006 μM radioactive proline added to the incubation medium were found to be generally very low, 6.5 in the cerebral cortex and 1.4 in the cerebellum [3], far below those of other putative amino acid neurotransmitters [8,9].

In continuation of our ongoing studies on the mechanism(s) of accumulation of amino acid neurotransmitters [8,9] we re-examine here the kinetics and thermodynamics of proline transport. Since the systems which mediate the high affinity uptake of neurotransmitters are located in nerve-endings, we have utilized preparations of synaptosomes isolated from rat brain. Because of the known correlation between the high affinity uptake of neurotransmitters and their physiological function, comparative studies were carried out on synaptosomes isolated from seven regions of the brain: cortex, striatum, mid-brain, hippocampus, hypothalamus, medulla (+ pons) and cerebellum.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats were used throughout. Seven regions of the brain (cortex, striatum, midbrain, hippocampus, hypothalamus,

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medulla + pons and cerebellum) were dissected as in [10]. The crude synaptosomal fraction (P_2) and the purified synaptosomes were isolated as in [11]. The final pellet of each preparation was washed once in a modified Krebs-Henseleit buffer (NaCl, 140 mM; KCl, 5 mM; $NaHCO_3$, 5 mM; $MgSO_4$, 1.3 mM; Na-phosphate, 1 mM; Tris–Hepes, 10 mM) (pH 7.4) and finally suspended in the same medium at 3–6 mg protein/ml.

In all experiments synaptosomal suspensions were subjected to 5 min preincubation with 10 mM glucose and 2.5 mM $CaCl_2$ at 27°C. Protein concentration was determined as in [12] using bovine serum albumin as standard.

2.1. *Uptake of proline*

The synaptosomes of the P_2 fraction were diluted to 1–2 mg protein/ml and uptake measurements were started by the addition of proline (L-[2,3-^{3}H]proline, spec. act. 40.0 Ci/mmol, New England Nuclear, Boston MA). Samples (200 μl) of the incubation mixture were withdrawn at the time intervals indicated in the figures and rapidly centrifuged (Beckman microfuge) through a layer of silicone oil (specific gravity 1.03, General Electric, Waterford NY). Radioactivity of the pellets and the supernatants was measured in a Searle Delta 300 liquid scintillation counter using aqueous scintillant (ACS II, Amersham, Arlington Heights IL).

2.2. *Measurement of intracellular water*

Tritiated water and polyethylene [^{14}C]glycol (M_r 4000) were added to the suspensions of synaptosomes and, following centrifugation of the synaptosomes through silicone oil, using a Beckman microfuge, the total water of the pellet was determined from the content of ^{3}H and the extrasynaptosomal water was calculated from the content of ^{14}C. The intrasynaptosomal water was found to be 5.0 ± 0.5 μl/mg protein.

3. RESULTS

Fig.1 shows the rates of uptake of 2 μM L-proline by the crude synaptosomal fractions (P_2) isolated from different regions of the brain. The highest accumulation was seen in the midbrain, striatum, hippocampus and hypothalamus, intermediate activity was observed in the cortex and

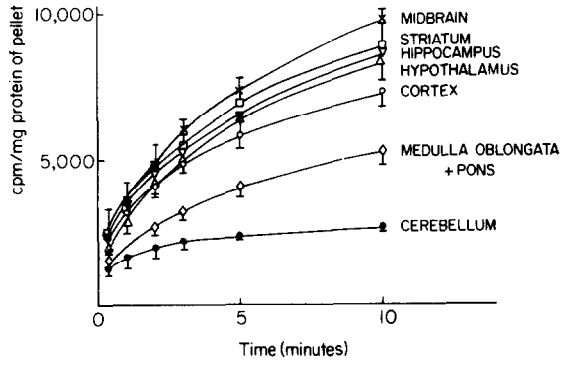

Fig.1. Uptake of proline by the P_2 fraction from various regions of the brain. The P_2 fractions were suspended to a final protein concentration of about 2 mg/ml in Krebs-Henseleit buffer (pH 7.4) and preincubated with 10 mM glucose and 2.5 mM $CaCl_2$ for 5 min at 27°C. At the end of the preincubation period, 20 μM L-[^{3}H]proline was added, duplicate samples (200 μl) were withdrawn at the intervals indicated and rapidly centrifuged through silicone oil (see section 2). Each point represents a mean of 3 independent experiments ± SEM.

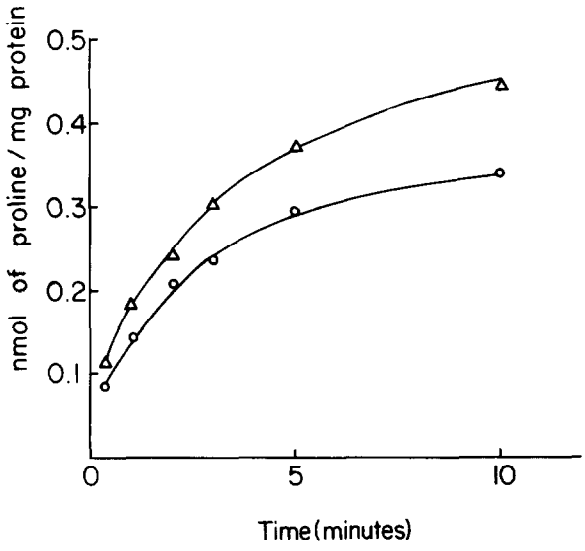

Fig.2. Uptake of proline by synaptosomes isolated from the cortex (o) and from the most active regions of the brain (Δ; striatum + midbrain + hypothalamus + hippocampus). Synaptosomes were suspended at a final protein concentration of about 1.2 mg/ml and treated as in fig.1. L-[^{3}H]Proline concentration was 2 μM. Values are means of two experiments which were within 5% of each other.

medulla + pons (25% and 50% lower, respectively), whereas the cerebellum exhibited only about 20–30% of the uptake measured in the midbrain. When much purer synaptosomes were prepared from the combined four regions of the brain which were most active in proline uptake and compared with those isolated from the cortex it was found (fig.2) that the cortical synaptosomes had somewhat slower rates of uptake and a lower final accumulation ratio: the $[\text{L-proline}]_i/[\text{L-proline}]_o$ was 40 ± 4 (mean $\pm$ SD, $n = 3$) in the latter as compared to 50 ± 3 (mean $\pm$ SD, $n = 3$) in the former. Since, however, these differences were not very large, in all subsequent experiments synaptosomes were isolated from the 5 regions most active in proline uptake, including the cortex, and used throughout.

The rates of proline uptake were linearly dependent over 0.5–2.2 mg protein/ml (fig.3) and markedly affected by the concentration of sodium in the external medium. Fig.4 shows that at 30 mM NaCl, the uptake of proline was very low but increased markedly with an increase in $[Na^+]$ from 30–140 mM. Kinetic analyses of the rates of uptake using Lineweaver-Burke plots ($1/v$ *vs* $1/S$, fig.5) revealed two components of transport, one with high and one with low affinity. At 140 mM sodium, the kinetic constants for the former were K_m, 12 μM and V_{max}, 0.6 nmol.min^{-1}.mg protein^{-1}; and for the latter, K_m, >0.5 mM and V_{max}, 2.5 nmol.min^{-1}.mg protein^{-1}. A sequential decrease in [sodium] to 70 mM and 30 mM increased the K_m to 33 μM and 60 μM, respectively,

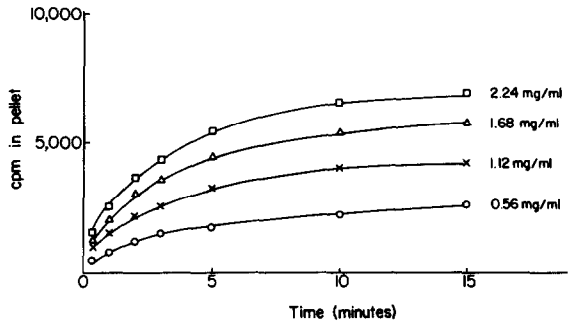

Fig.3. Dependence of proline uptake on protein concentration. Synaptosomes were diluted to the appropriate protein concentration in Krebs–Henseleit buffer (pH 7.4) supplemented with 10 mM glucose and 2.5 mM $CaCl_2$. Uptake measurements were done as in section 2.

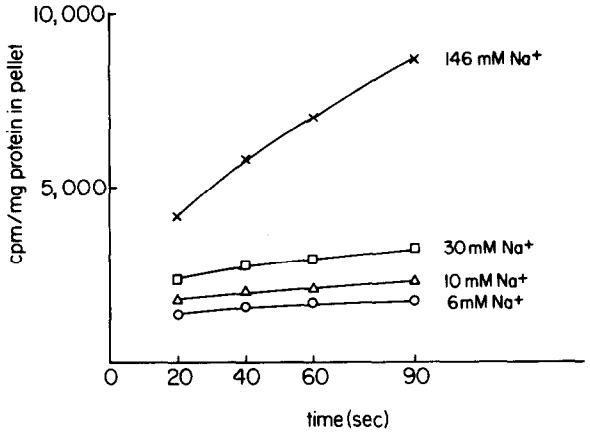

Fig.4. Dependence of proline uptake on $[Na^+]_e$. Synaptosomes (~1 mg protein/ml) were suspended and treated as in fig.1. When required, NaCl was replaced by equivalent concentrations of choline chloride. The uptake measurements were started by the addition of 2 μM L-[^{3}H]proline.

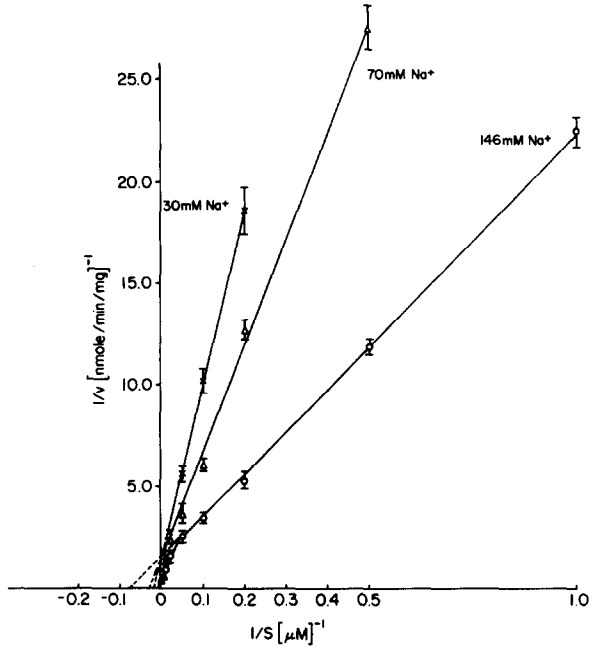

Fig.5. Double reciprocal plot analysis of proline uptake at different $[Na^+]_e$. Synaptosomes were treated as in fig.1 and finally suspended at 1 mg protein/ml. Uptake of proline was initiated by adding the ^{3}H-labeled amino acid at 1, 2, 5, 10, 20, 50, 100, 500 and 1000 μM and followed over the first 2 min by withdrawing aliquots at 20, 40, 60, 90 and 120 s. The initial (1.5–2 min) linear rates of uptake were used in calculations.

with no change in V_{max}. The low affinity uptake showed very little dependence on sodium concentration.

4. DISCUSSION

Our results show that the highest rates of uptake and largest accumulation ratios for L-proline, a putative inhibitory neurotransmitter in the mammalian CNS, are exhibited by synaptosomes isolated from the midbrain, with considerably lower activity in the medulla (+ pons) and lowest in the cerebellum. If one accepts the positive correlation between the high affinity uptake and the level of neurotransmitter in a defined region of the brain as a valid criterion for its physiological function, our results suggest that the proline-secreting pathways are more abundant in the midbrain, (as well as in striatum, hypothalamus and hippocampus) than in the cerebellum.

The high-affinity, sodium-dependent proline uptake system had a K_m of 12 μM and a V_{max} of 0.6 nmol . min^{-1} . mg protein^{-1}, the former being very close to the values in [5,6]. It is interesting to note that the K_m-values for the uptake of γ-aminobutyric acid [13,14], aspartate and glutamate [9,15–18] fall within a similar range of values. On the other hand, the maximum velocity is about 6–7-fold lower than that for aspartate or glutamate uptake [9,15–18]. If one assumes that the actual maximum rates for the various amino acid neurotransmitters (i.e., turnover/carrier protein) are similar, then the difference in the observed velocities may mean that proline is a neurotransmitter in 1/6–1/7 of that nerve-ending population which accumulates glutamate and aspartate. Since the latter was calculated to constitute 60–80% of our synaptosomal preparation, a rough estimate would yield a value of 6–8% for the prolinergic nerve endings.

The marked sodium-dependence of proline uptake observed here and in [4–6] and a stimulation of release of this amino acid upon depolarization [3,7] may suggest that the driving forces for accumulation of proline are very similar to those responsible for the uptake of other amino acid neurotransmitters [8,9]. These forces consist of a combination of the electrical potential and the sodium concentration gradient ($[Na^+]_o/[Na^+]_i$). In agreement with this suggestion is the observation [4] that a Hill plot of the Na^+ activation of proline uptake was linear with a slope of 2, identical to that for aspartate uptake. Hence, the neutral proline molecule may be co-transported with 2 Na^+, which would be an electrogenic process. Independent evidence for the electrogenic character of proline transport was provided in [19] where, in plasma membrane vesicles isolated from rat brains, the uptake of proline was stimulated by valinomycin and inhibited by the proton ionophore, carbonyl cyanide *m*-chlorophenylhydrazone. If the driving forces for proline uptake were the same as those for aspartate transport then the proline gradients ($[proline]_i/[proline]_o$) at 140 mM Na^+ and 5 mM K^+ should be 600–800, whereas the values measured were 10–20-fold smaller. Such calculations are based, however, on the assumption that all of the synaptosomes in our preparation are taking up proline by the high affinity system. However, if only 5% of the synaptosomal population actively accumulates proline, the proline gradient which is maintained by these synaptosomes would be 20-fold higher than the measured value and not significantly different from those of aspartate, glutamate and GABA.

In summary, proline is transported into the synaptosomal fraction of rat brain by a Na^+-dependent, high affinity uptake system. The maximal accumulation ratios for the radioactive amino acid at 2 μM added L-[2,3-^{3}H]proline is measured to be 40–50. The activity of the uptake system in different areas of the brain varies by a factor of 4, which indicates regional specificity. The properties of the proline uptake system are remarkably similar to those of other putative neurotransmitter amino acids, (GABA, aspartate and glutamate) with respect to Na^+-dependence, K_m, V_m and maximal accumulation ratio, the last two parameters calculated taking into account that only 5–10% of the synaptosomes accumulate proline. All of this evidence is consistent with proline functioning as a neurotransmitter in the central nervous system.

ACKNOWLEDGEMENT

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