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ATP-Dependent Glutamate Uptake into Synaptic Vesicles from Cerebellar Mutant Mice

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Abstract: The ATP-dependent glutamate uptake system in synaptic vesicles prepared from mouse cerebellum was characterized, and the levels of glutamate uptake were investigated in the cerebellar mutant mice, staggerer and weaver, whose main defect is the loss of cerebellar granule cells, and the nervous mutant, whose main defect is the loss of Purkinje cells. The ATP-dependent glutamate uptake is stimulated by low concentrations of chloride, is insensitive to aspartate, and is inhibited by agents known to dissipate the electrochemical proton gradient. These properties are similar to those of the glutamate uptake system observed in the highly purified synaptic vesicles prepared from bovine cortex. The ATP-dependent glutamate uptake system is reduced by 68% in the staggerer and 57–67% in the weaver mutant; these reductions parallel the substantial loss of granule cells in those mutants. In contrast, the cerebellar levels of glutamate uptake are not altered significantly in the nervous mutant, which has lost Purkinje cells, but not granule cells. In view of evidence that granule cells are glutamatergic neurons and Purkinje cells are GABAergic neurons, these observations support the notion that the ATP-dependent glutamate uptake system is present in synaptic vesicles of glutamatergic neurons. **Key Words:** Glutamate—Synaptic vesicle—Staggerer—Weaver—Nervous—Granule cell. **Fischer-Bovenkerk C. et al.** ATP-dependent glutamate uptake into synaptic vesicles from cerebellar mutant mice. *J. Neurochem.* **51,** 1054–1059 (1988).

A growing body of evidence strongly supports the concept that glutamate serves as a major excitatory neurotransmitter in the vertebrate central nervous system (Cotman et al., 1981, 1987; Watkins and Evans, 1981; Crunelli et al., 1984; Fonnum, 1984; Fagg, 1985; Ueda, 1986; Cotman and Iversen, 1987). However, whether the synaptic vesicle plays a role in glutamate synaptic transmission remains unclear. Supporting the argument for a role of synaptic vesicles in glutamate transmission is the demonstration of an ATP-dependent and specific uptake of glutamate into synaptic vesicles that are highly purified by the use of immunoprecipitation in the final step of purification (Naito and Ueda, 1983, 1985). This observation is in accord with the immunocytochemical studies by Storm-Mathisen et al. (1983), suggesting that glutamate is stored in certain synaptic vesicles. More recently, Nicholls and Sihra (1986) have provided evidence that glutamate is released in a calcium-dependent manner from a noncytoplasmic pool.

In an attempt to provide evidence for the notion that ATP-dependent glutamate uptake observed in highly purified vesicles reflects the vesicular uptake system in vivo in glutamatergic neurons, we have studied the uptake system in mouse cerebellar mutants. The cerebellar granule cells are considered to be glutamatergic neurons (Hackett et al., 1979; Messer, 1980), extending axons to the molecular layer to make numerous synaptic contacts with the dendrites of the Purkinje cells, which are GABAergic neurons (Obata et al., 1967; Curtis et al., 1971). The staggerer and weaver mutant mice are deficient in granule cells, whereas the nervous mutant mouse is deficient in Purkinje cells and has a near normal complement of granule cells (Sidman et al., 1962, 1965; Sidman and Green, 1970). We have analyzed the cerebella of these mutants and their age-matched littermate controls for the ATP-dependent glutamate uptake into synaptic vesicles. This study was prompted by the development of a simplified procedure for preparing from small tissues synaptic

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Abbreviations used: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; GABA, γ-aminobutyric acid; *nr*, nervous; *sg*, staggerer; *wv*, weaver.

vesicles which are crude, yet sufficiently pure to demonstrate highly ATP-dependent glutamate uptake (Kish and Ueda, 1989). A preliminary account of these results has been reported previously (Fischer-Bovenkerk et al., 1986).

MATERIALS AND METHODS

Materials

L-Glutamic acid, ATP, and dithiothreitol were purchased from Sigma. Potassium isethionate and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were generously provided by Dr. Ronald Holz (Department of Pharmacology, University of Michigan). L-[2,3-^{3}H]Glutamic acid (20 Ci/mmol) and scintillation cocktail (ACS) were obtained from Amersham.

Mutant mice were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The mutants used were staggerer (*sg;* strain B6C3Fe-a/a), weaver (*wv;* strain B6CBAw-j/A), and nervous (*nr;* strain C3/HeB/FeJ); *sg, wv,* and *nr* are autosomal recessive mutations (Roffler-Tarlov and Sidman, 1978). The homozygous mutants were identified by their abnormal gait and by their smaller size compared to their littermates (McBride et al., 1976*a*). The animals used as controls were the littermates and could not be distinguished by any behavioral means; they are collectively termed +/?. To obtain optimal assay conditions, wild type C57BL/6J mice were used.

Preparation of synaptic vesicles

Synaptic vesicles were prepared by the simplified procedure described by Kish and Ueda (1989). Mice were killed by cervical dislocation, and their cerebella were dissected at 0°C. In some cases, cerebella were frozen at −80°C for up to 3 weeks. Freezing for up to 5 weeks had no significant effect on the ATP-dependent glutamate uptake activity in the synaptic vesicle preparations. Pooled cerebella (10 for staggerer, four for weaver, and three for nervous) were homogenized in 10 volumes of a 0.32 *M* sucrose solution containing 1 m*M* magnesium acetate, 0.5 m*M* calcium acetate, and 1 m*M* $NaHCO_3$ (solution A), using a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min; the resulting pellet was subjected to osmotic shock by suspending in 20 volumes of 6 m*M* Tris-HCl (pH 8.1) for 45 min, followed by centrifugation at 43,000 *g* for 15 min. The resulting supernatant was centrifuged at 150,000 g_{av} for 50 min to pellet synaptic vesicles. The pellets were homogenized in 0.32 *M* sucrose solution containing 1 m*M* $NaHCO_3$ and 0.2 m*M* dithiothreitol (solution B), frozen, and stored in liquid nitrogen. There was no significant loss of glutamate uptake activity under these storage conditions for at least 3 months. This vesicle fraction was used as the synaptic vesicle preparation in all the experiments described here. Although this is not a highly purified synaptic vesicle preparation, it exhibits sufficiently high ATP-dependent glutamate uptake.

Assay of ATP-dependent glutamate uptake

The uptake of glutamate into synaptic vesicles was performed by a slight modification (Kish and Ueda, 1989) of the method by Naito and Ueda (1983). The standard uptake medium (final volume, 100 μl) contained 0.25 *M* sucrose, 4 m*M* $MgSO_4$, 5 m*M* Tris maleate (pH 7.4), 4 m*M* KCl, 2 m*M* aspartate, 0 or 2 m*M* ATP (neutralized with Tris base), 50 μ*M* [^{3}H]glutamate (0.4 Ci/mmol), and appropriate amounts of the synaptic vesicle fraction (35 or 40 μg). After preincubation at 30°C for 5 min, the uptake was initiated by the addition of a mixture of [^{3}H]glutamate and ATP (20 μl). Following incubation at 30°C for an appropriate period (usually 10 min), the uptake was stopped by the addition of 2 ml of ice-cold 0.15 *M* KCl and immediate filtration through a Millipore HAWP (24 mm, 0.45 μm) filter. The test tube was washed with 2 ml of KCl solution three more times and the filters washed an additional four times with the same solution. Radioactivity retained on the filters was determined by using a liquid scintillation spectrophotometer. The amount of nonspecific binding of [^{3}H]glutamate to the filter was determined by filtering the incubation mixture without synaptic vesicles. The difference between the uptake in the presence of ATP and that in the absence was taken to represent the ATP-dependent glutamate uptake. Each sample was assayed in triplicate. Three separate vesicle preparations were obtained from each mutant and littermate control source. The relative standard deviation was ±1.5% when the uptake activity was in the range of 16–30 pmol/10 min/40 μg of protein.

Protein determination

Protein of the synaptic vesicle fraction was determined according to Lowry et al. (1951) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The optimal conditions for the standard assay were determined using the synaptic vesicle preparation from wild-type mouse cerebellum. The time course of the vesicular uptake of glutamate is shown in Fig. 1. The ATP-dependent glutamate uptake reached a maximal level after 5 min of incubation. There was a 27-fold stimulation of glutamate uptake by ATP in the pres-

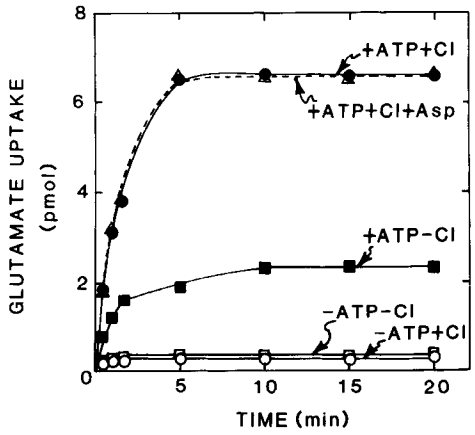

FIG. 1. Vesicular uptake of glutamate as a function of time. Synaptic vesicles (40 μg protein) were incubated in the sucrose medium described in Materials and Methods for various periods of time as indicated, in the absence (□) or presence (■) of 2 m*M* ATP, in the absence (○) or presence (●) of ATP with 4 m*M* KCl, and in the presence of ATP, KCl, and 2 m*M* aspartate (△). Each point is the mean of three determinations. In each case, the SD was ±0.10 pmol of [^{3}H]glutamate.

ence of 4 m*M* chloride at 10 min. The ATP-dependent uptake was potentiated substantially by the presence of 4 m*M* chloride, a physiologically relevant concentration. This uptake system was insensitive to aspartate; this property renders the vesicular glutamate translocator distinct from the sodium-dependent plasma membrane transporter, which does not distinguish between glutamate and aspartate (Naito and Ueda, 1985; Ueda, 1986; Kish and Ueda, 1989).

To ensure that the radioactivity accumulated in the vesicle after 10 min of incubation was indeed [^{3}H]glutamate and not some metabolite, we performed HPLC analysis on standard incubation mixtures containing 50 μg of synaptic vesicle protein. After 30 min of incubation, uptake was stopped by the addition of ice-cold KCl, and the vesicular contents were extracted by ethanol and subjected to analysis by HPLC and by liquid scintillation spectrophotometry. The majority (>95%) of radioactivity in the vesicles was eluted as a single peak with a retention time identical to that of glutamate.

The ATP-dependent glutamate uptake was linear when assayed with 10–50 μg of synaptic vesicle protein (Fig. 2). There was a small amount of uptake when ATP was absent, and this amount was subtracted from the total uptake to determine the ATP-dependent uptake activity.

The effects of various concentrations of chloride and isethionate on the ATP-dependent vesicular uptake of glutamate in mouse cerebellum are shown in Fig. 3. Low concentrations of chloride (1–4 m*M*) caused a marked potentiation of glutamate uptake, whereas concentrations higher than 10 m*M* attenuated the stimulation. In contrast to the permeant ion chloride,

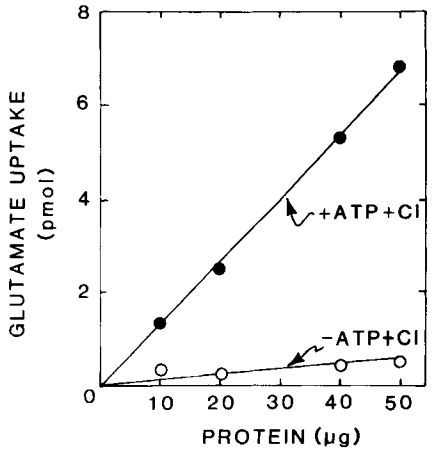

FIG. 2. Vesicular uptake of glutamate as a function of the amount of protein. Various amounts of synaptic vesicle protein were incubated for 10 min for glutamate uptake in the sucrose medium in the absence (○) or presence (●) of 2 m*M* ATP under the standard assay conditions described in Materials and Methods. Each point is the mean of three determinations. In each case, the SD was ±0.10 pmol of [^{3}H]glutamate.

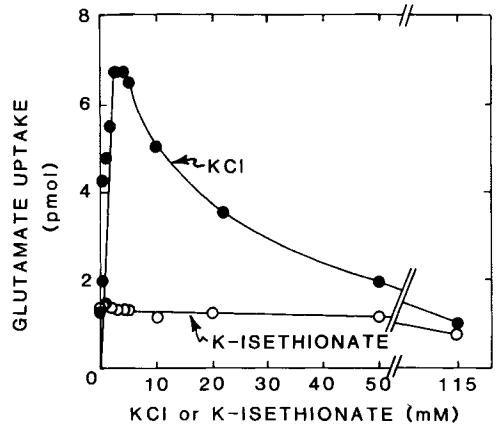

FIG. 3. Effects of various concentrations of chloride and isethionate on the ATP-dependent vesicular uptake of glutamate. Glutamate uptake was determined after 10 min of incubation. Synaptic vesicles (40 μg protein) were incubated in the presence of various concentrations of KCl (●) or K-isethionate (○) in the sucrose/5 m*M* Tris-maleate (pH 7.4) medium described in Materials and Methods. Sucrose concentration was varied accordingly to maintain the isoosmolarity. Each point is the mean of three determinations. In each case, the SD was ±0.10 pmol of [^{3}H]glutamate.

the impermeant monovalent anion isethionate exhibited little effect at all the concentrations tested.

The ATP-dependent uptake was inhibited markedly by FCCP; 5 μ*M* FCCP caused an 80% reduction of glutamate uptake. This sensitivity to FCCP suggests that the vesicular glutamate uptake in mouse cerebellum is driven by an electrochemical proton gradient, as in the case of catecholamine uptake into chromaffin granules (Holz, 1978; Johnson et al., 1979; Njus et al., 1986).

Thus, the ATP-dependent glutamate uptake system observed in crude synaptic vesicles prepared from mouse cerebellum has properties similar to those previously reported for highly purified bovine cortex synaptic vesicles (Naito and Ueda, 1983, 1985) and for crude vesicles from rat cortex (Kish and Ueda, 1989). This suggests that the ATP-dependent glutamate uptake observed in the present preparation, although not highly purified, represents a process that occurs in the synaptic vesicle membrane.

Using this type of vesicle preparation, we analyzed the cerebella of the mutant mice, i.e., staggerer, weaver, and nervous, for ATP-dependent glutamate uptake. The vesicular glutamate uptake in staggerer and weaver mutants lacking granule cells is given in Table 1. The weight of the staggerer cerebellum was only 17% of that of the normal littermate control. When corrected for the cerebellar weight loss, the amount of glutamate uptake per cerebellum was 32% of control. Likewise, when corrected for the corresponding weight loss, the amount of glutamate uptake per cerebellum in the 60- and 75-day-old weaver mice was 33% and 43% of that of their respective littermate controls. In contrast, after such a correction for cerebellar weight loss, there

TABLE 1. *Vesicular glutamate uptake in mouse cerebellar mutants*

Strain	Postnatal age (day)	Genotype	Specific activity (pmol glutamate/mg synaptic vesicle protein)	Total activity (pmol glutamate/ cerebellum)	Mutant cerebellar weight (% of +/?)
Staggerer	30	*sg/sg*	15.1 ± 3.7[a]	0.24 ± 0.1[a]	17
		+/?	32.8 ± 6.8	4.54 ± 0.48	
Weaver	60	*wv/wv*	16.6 ± 1.6[a]	0.80 ± 0.23[a]	52
		+/?	24.7 ± 2.8	4.67 ± 0.53	
	75	*wv/wv*	14.3 ± 1.7[a]	0.72 ± 0.10[a]	46
		+/?	26.9 ± 2.2	3.65 ± 0.72	
Nervous	50	*nr/nr*	17.3 ± 1.4	2.07 ± 0.21	65
		+/?	18.0 ± 2.7	2.80 ± 0.41	
	60	*nr/nr*	10.4 ± 1.4	0.95 ± 0.11	61
		+/?	11.1 ± 1.7	1.54 ± 0.38	
	80	*nr/nr*	10.5 ± 1.6	1.01 ± 0.36	70
		+/?	12.3 ± 3.3	1.71 ± 0.46	

The ATP-dependent glutamate uptake was determined using 35 μg (staggerer) and 40 μg (weaver and nervous) synaptic vesicles in the standard assay medium containing 50 μ*M* [^{3}H]glutamate, as described in Materials and Methods. The data represent the means ± SD of three separate preparations, each determination done in triplicate.

[a] The mutant values are not significantly different from their littermate controls ($p < 0.05$, Student's *t* test).

was little or no difference in the ATP-dependent glutamate uptake between the Purkinje cell-deficient nervous mutant and its age-matched littermate control (Table 1).

The specific activity of glutamate uptake is 50% less than the littermate controls for both the staggerer and weaver mutants, whereas there is little or no change in the specific activity of glutamate uptake in the nervous mutant when compared to the littermate control (Table 1). The decrease in specific activity of glutamate uptake could be due to (a) some change in the number or property of the glutamate translocator protein in the synaptic vesicle membrane or (b) a proportional increase in contamination of nonvesicular membrane in the mutant synaptic vesicle preparation.

A correlation of decreased levels of endogenous glutamic acid with a decrease in the number of cerebellar granule cells could provide one line of evidence to support the notion that glutamate is an excitatory neurotransmitter in the granule cell. McBride et al. (1976*a*) reported that the glutamate levels in staggerer and weaver mutants were decreased by about 30–45% when compared to their littermate controls, whereas those in the nervous mutant were unchanged. In addition, McBride et al. (1976*b*) and Valcana et al. (1972) have observed a reduction of endogenous glutamate content in the granule cell-depleted cerebellum. Roffler-Tarlov and Sidman (1978) found that, in staggerer and weaver mutants, the glutamate concentration was reduced by about 33% of control values in both the cortex and the deep nuclei of the cerebellum. They suggested that endogenous glutamate reduction in the cerebella of these mutants could not be taken as sufficient evidence in favor of identifying glutamate as an excitatory transmitter in the granule cell, because the reduction was not specific to those areas containing the granule cells. Dolphin and Greengard (1981) reported that the cerebellar levels of synapsin I, a neuronal phosphoprotein highly concentrated on the synaptic vesicle (Bloom et al., 1979; Ueda et al., 1979; DeCamilli et al., 1983), were also reduced by about 66% in staggerer and weaver mutants, but only 12% in the nervous mutant. This suggests that synapsin I is largely present in the granule cells, but only to a small extent in the Purkinje cells.

The existence of a sodium-dependent, high-affinity uptake system for glutamate has been demonstrated in cerebellar isolated granule cells, tissue slices, and isolated synaptosomes (Kuhar and Snyder, 1970; Balcar and Johnston, 1972; Logan and Snyder, 1972; Campbell and Shank, 1978; Gordon and Balazs, 1983). Young et al. (1974) reported a 65–70% reduction in the high-affinity uptake of glutamate into synaptosomes from hamsters with a virally depleted granule cell population. Rhode et al. (1979) reported a similar reduction in the high-affinity uptake of glutamate in the granule cell-depleted cerebellum. However, it may be noted that this sodium-dependent cellular uptake system does not distinguish between glutamate and aspartate (Logan and Snyder, 1972), in contrast to the more specific ATP-dependent vesicular glutamate uptake system (Naito and Ueda, 1983, 1985).

Stimulation-coupled release of glutamate has also been demonstrated in cerebellar synaptosomal and tissue slice preparations (Foster and Roberts, 1980; Levi et al., 1982; Bosley et al., 1983). Sandoval and Cotman (1978) and Flint et al. (1981) have observed that the synaptic release is reduced after depletion of the cerebellar granule cells.

In this study, we have observed a similar degree of reduction (57–68%) of the ATP-dependent glutamate uptake in the cerebellar synaptic vesicle fraction from staggerer and weaver mutants, but no significant change

in the nervous mutant. Our results suggest that the ATP-dependent glutamate uptake system is present in granule cells, but not in Purkinje cells. In view of the evidence that granule cells are glutamatergic and Purkinje cells are GABAergic, we suggest that the ATP-dependent glutamate uptake system is present in glutamatergic neurons, but not in GABAergic neurons. This is consistent with the hypothesis that whether or not a neuron is glutamatergic is determined by the presence or absence of the ATP-dependent glutamate-specific vesicular uptake system in the nerve terminal (Ueda, 1986). Thus, the ATP-dependent vesicular glutamate uptake system may serve an important function in glutamate synaptic transmission.

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