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$(Na^+ + K^+)$-Adenosinetriphosphatase in the Brain of Shiverer (Shi/Shi) Mice

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The myelin-deficient Shiverer (Shi/Shi) mutant mouse may be a useful model in assessing the dependence of brain $(Na^+ + K^+)$-ATPase concentration and composition on myelin membrane formation. Brain microsomal membranes from age-matched control (+/+) and Shiverer (Shi/Shi) mice were fractionated by differential centrifugation and sucrose gradient sedimentation. No reduction in $(Na^+ + K^+)$-ATPase specific activity was measured in whole homogenates, high- and low-speed fractions or gradient fractions from brains of Shi/Shi mice as compared to those of +/+ mice. In addition, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antisera specific for mouse brain $(Na^+ + K^+)$-ATPase revealed no significant difference in catalytic subunit composition between fractions of +/+ and Shi/Shi brains. The similar results obtained for both +/+ and myelin-deficient Shi/Shi mice suggest that myelin contributes little to total brain $(Na^+ + K^+)$-ATPase.

KEY WORDS: Shiverer; myelin; $(Na^+ + K^+)$-ATPase; brain; antibody; mouse.

INTRODUCTION

Homozygous Shiverer (Shi/Shi) mice exhibit tremors at about two weeks after birth and tonic seizures after one month (1). Biochemical and microscopic studies have shown that the CNS of Shi/Shi mice is severely reduced in myelin, myelin basic protein (MBP) and proteolipid protein (PLP) (2–5). However, certain membrane-bound enzyme activities have been found to be higher in this mutant. The specific activity of 2′:3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) was found to be normal or slightly elevated in Shi/Shi mouse CNS (4, 6), although the enzyme could not be localized immunocytochemically (7). The specific activity of carbonic anhydrase was slightly higher and 5′-nucleotidase was three times higher in spinal cord myelin fractions of Shi/Shi mice as compared to those of control mice (6).

$(Na^+ + K^+)$-ATPase is a membrane-bound enzyme that establishes concentration gradients for Na^+ and K^+ ions across plasma membranes in general (8, 9). The enzyme consists of a phosphorylatable catalytic polypeptide (α subunit, about 100 kDa) and a glycoprotein (β subunit, about 40 kDa) as shown by SDS-PAGE (8). Recently, it was found that the catalytic polypeptide can be resolved into two phosphorylatable species in brain (10) and other tissues (11–14). There is evidence that in the nervous system the larger polypeptide (α_1) is most prevalent in tissue containing myelinated axons and that the proportion of α_1 to α_2 in brain increases during maturation (10, 15). This raises the question as to whether α_1 is related to an early stage in myelin formation or to axons destined to be myelinated.

With respect to brain, the concentration or the subcellular distribution of $(Na^+ + K^+)$-ATPase may reflect differentiation of plasma membrane that

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is dependent on neuronal-glial interactions or myelination. For example, it has been reported that in myelinated axons of knifefish brain (16), goldfish optic nerve (17) and rat nervous system (18), (Na^+ + K^+)-ATPase immunoreactivity is localized to axolemma at nodes of Ranvier. Because of the virtual absence of myelin (2–5), the Shiverer brain is an interesting model for investigating the subcellular distribution of (Na^+ + K^+)-ATPase and possible isozymes in the CNS and, in comparison to normal brain, for indicating properties of the enzyme that may be related to glial function or myelin formation. Earlier data on (Na^+ + K^+)-ATPase in the Shiverer mutant CNS are sparse. (Na^+ + K^+)-ATPase specific activity was reported reduced by 11% in forebrain homogenates of 60 day-old myelin-deficient Shi/Shi mice, while specific activity in cerebellar homogenates was not reduced (19). However, studies of brain microsomal fractions enriched in (Na^+ + K^+)-ATPase have not been reported for Shiverer mutants.

The purpose of this investigation was to determine if a CNS myelin deficit in the Shiverer mouse mutant has an effect on the concentration or distribution of (Na^+ + K^+)-ATPase in microsomal fractions.

EXPERIMENTAL PROCEDURE

Materials. Polymerizing reagents and polyacrylamide for electrophoresis and nitrocellulose and Trans-blot apparatus used in immunoblotting were purchased from Bio-Rad. Diaminobenzidine (DAB), SDS, Coomassie blue, ATP salts, EDTA and gel buffer (Trizma, pH 8.8) were obtained from Sigma Chemical Co. Goat antirabbit IgG-horseradish peroxidase (HRP) and normal goat serum were purchased from Miles Laboratories. Prestained protein molecular weight standards were obtained from Bethesda Research Lab.

Control (+/+) and Shiverer (Shi/Shi) male mice (CBA/J strain) used in this study were 60 ± 5 days old and were offspring of +/+ × +/+ or Shi/+ × Shi/+ matings, respectively. At this age the normal mouse cerebrum, brain stem and cerebellum are heavily myelinated (20), while the Shiverer CNS contains little myelin (2). Shi/Shi mice exhibit a shivering motion at about 14 days, thus being distinguished easily from their +/+ or Shi/+ littermates. Experimental animals were selected on the basis of their distinctive behavior. The +/+ mice were selected from breeding pairs of +/+ mice as determined by several brother/sister matings of the same strain showing no shivering after several generations. The breeding stock of Shiverer mice was the kind gift of Dr. T. J. Sprinkle (Augusta, GA). NIH standard practices for laboratory animals were observed.

Methods

Antigen and Antisera Preparation. The procedures for the preparation of mouse brain (Na^+ + K^+)-ATPase catalytic subunit antigen (combined α_1 and α_2) and characterization of the antisera raised in rabbits have been reported (21, 22).

Sucrose Gradient Fractionation Procedure. Mice were decapitated and the cerebrum, cerebellum and medulla were excised and either used immediately or frozen in liquid nitrogen. Brain microsomal membranes enriched in (Na^+ + K^+)-ATPase (P2 pellet), were sedimented from pools of 5 to 7 +/+ and Shi/Shi brains in parallel, using procedures described previously (21, 23). Three separate fractionation experiments, with a total of 19 control and 16 Shiverer mice, were carrried out.

The 30,000 *g* (P2) pellet was suspended in an incubation mixture (4 mg protein/ml, 1 mM EDTA-Tris, 3 mM Na_2ATP and 30 mM imidazole-HCl, pH 7.4, 0.15% (w/v) SDS, 10% (w/w) sucrose) as described by Siegel and co-workers (21). However, the sucrose gradient was modified as follows (top to bottom): 8 ml P2 incubation mixture in 10% sucrose (designated Fraction A prior to centrifugation), 20 ml 20% sucrose, 8 ml 32% sucrose and 2 ml 64% sucrose. The gradients were centrifuged in an SW-28 rotor in the Beckman L5-50 at 27,000 rpm (100,000 *g*) for 4 hours. The fractions were aspirated from above and designated as follows: B (10% sucrose), C (10–20% interface), D (20% sucrose), E (20–32% interface) and F (32% sucrose). The fractions were diluted 10 fold in water, centrifuged at 39,000 *g* (#19 rotor) overnight and the final pellets were resuspended in water. Protein contents in the final pellets were determined by the method of Lowry et al. (24).

(Na^+ + K^+)-ATPase Activity. The pellets, supernatant portions and the pellets from the sucrose gradient fractions were assayed for (Na^+ + K^+)-ATPase specific activity according to the procedure described by Bertoni and Siegel (25). (Na^+ + K^+)-ATPase specific activity was determined by subtracting Mg^{2+}-ATPase from (Mg^+ + Na^+ + K^+)-ATPase activity. The units of specific activity were μmoles Pi released/minute/mg protein. Each assay was performed in triplicate for 10 minutes at 37°C with 2 μg of membrane protein in media containing 3 mM Tris-ATP, 65 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$ with or without 80 mM NaCl and 10 mM KCl (21).

Electrophoresis and Immunoblotting. Samples containing 50 μg protein were applied to two gels and electrophoresed in a Laemmli 6% polyacrylamide slab-gel system (26). One gel was stained with Coomassie blue while the companion gel was electroblotted to nitrocellulose according to the procedure of Towbin et al. (27) as previously described (21, 23).

After incubation in a BLOTTO (bovine lacto transfer technique optimizer) blocking solution (28) for 60 minutes at 37°C, the nitrocellulose sheets were incubated in primary antiserum (27C) diluted 1:500 in a medium consisting of 5% nonfat dry milk solids and 10% normal goat serum in 0.1 M Tris-buffered saline (TBS), pH 7.4, for 60 minutes at 37°C. After rinsing in TBS for 30 minutes, the nitrocellulose sheets were incubated with goat antirabbit IgG-HRP (1:2000) for 60 minutes at 37°C. For color development the sheets were treated with 0.05% DAB, 1% acetone and 0.03% hydrogen peroxide in TBS for 2 minutes at room temperature.

RESULTS

(Na^+ + K^+)-ATPase Activity. No significant difference in protein concentration or in total (Na^+ + K^+)-ATPase activity in the sedimentable frac-

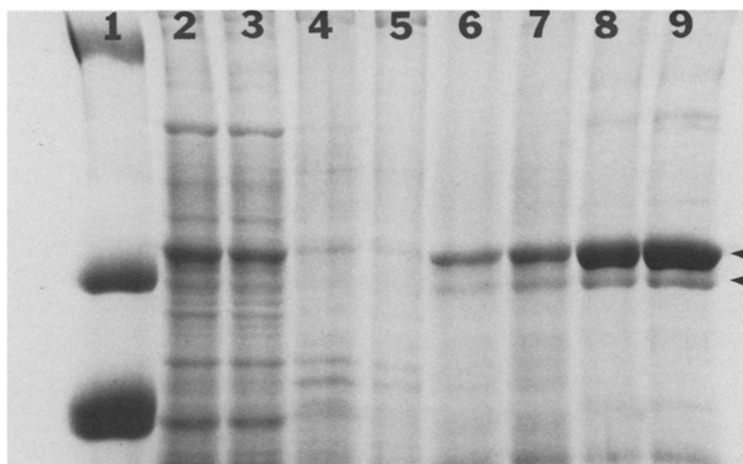

Fig. 1. Coomassie blue-stained 6% polyacrylamide gel of sucrose gradient fractions A, B, C and D of +/+ and Shi/Shi mouse brain. Each sample contained 50 μg of protein. Lane 1: Pre-stained protein molecular weight standards (top to bottom) are myosin (200 kDa), phosphorylase B (97.4 kDa) and bovine serum albumin (67 kDa); Lane 2: +/+ A; Lane 3: Shi/Shi A; Lane 4: +/+ B; Lane 5: Shi/Shi B; Lane 6: +/+ C; Lane 7: Shi/Shi C; Lane 8: +/+ D; Lane 9: Shi/Shi D. The arrowheads indicate the (Na^+ + K^+)-ATPase catalytic subunit doublet (α_1 and α_2).

tions (P1 and P2) is found between the Shiverer and control mice (Table I). Further, the ratios of protein concentration and of (Na^+ + K^+)-ATPase activity in the P1 and P2 pellets relative to respective contents in the total homogenates are similar in +/+ and Shi/Shi mice (Table I).

Table II shows that the distributions of specific enzyme activity in the various sedimentable and sucrose gradient fractions of control and Shiverer brain are similar. The highest specific activity is present in the microsomal gradient fractions containing 20% and higher sucrose (Fractions D–F).

Electrophoresis and Immunoblotting. Coomassie blue-stained gels and immunoblots show no difference between +/+ and Shi/Shi mice in the staining patterns in the various brain fractions (Figures 1 and 2). Myelin basic proteins (14 to 21.5 kDa) are not resolved in the 6% gel system used in this

Table I. Total Protein Content and (Na^+ + K^+)-ATPase Activity Recovered in Sedimentable Fractions from Brains of Control and Shiverer Mice

A.	Protein (mg) per brain		Protein in fraction/Protein in TH	
	+/+	Shi/Shi	+/+	Shi/Shi
TH	49.7 ± 2.6	47.1 ± 6.6		
P1	3.6 ± 0.6	3.7 ± 1.1	0.07	0.09
P2	24.4 ± 2.4	19.3 ± 2.2	0.49	0.43
B.	**Activity (U) per brain**		**Activity in fraction/Activity in TH**	
	+/+	Shi/Shi	+/+	Shi/Shi
TH	17.0 ± 3.1	15.0 ± 0.38		
P1	1.3 ± 0.35	1.7 ± 0.69	0.08	0.13
P2	10.3 ± 0.18	8.7 ± 0.58	0.61	0.61

TH = Total homogenate; P1 = Low-speed (8500 *g*) pellet; P2 = High-speed (30,000 *g*) pellet. Values are averages ± standard errors. A, Protein (mg). B, (Na^+ + K^+)-ATPase activity (U = μmol Pi released/min at 37°C). The values for brain protein and specific (Na^+ + K^+)-ATPase activity of Shiverer mice do not differ significantly from those of control mice ($P > 0.05$).

Table II. Comparison of (Na^+ + K^+)-ATPase Activity in Fractions of Brains From +/+ and Shi/Shi Mice

	Control (+/+)		Shiverer (Shi/Shi)	
Fraction	Activity U/mg	Distribution of activity (U) %	Activity U/mg	Distribution of activity (U) %
TH	0.34 ± 0.03		0.31 ± 0.03	
S1	0.29 ± 0.06		0.30 ± 0.02	
S2	0.13 ± 0.02		0.13 ± 0.03	
P1	0.34 ± 0.03		0.42 ± 0.06	
P2	0.43 ± 0.03		0.46 ± 0.03	
A	0.24 ± 0.06		0.31 ± 0.03	
B	0.18 ± 0.03	4.1	0.32 ± 0.06	6.1
C	0.31 ± 0.09	4.9	0.54 ± 0.17	4.4
D	1.06 ± 0.13	71.8	1.31 ± 0.20	75.6
E	0.84 ± 0.17	13.3	1.02 ± 0.17	6.7
F	1.61 ± 0.26	5.8	1.66 ± 0.13	7.2

TH = Total homogenate; S1 = Supernatant after low-speed centrifugation; S2 = Supernatant after high-speed centrifugation; P1 = Low-speed pellet; P2 = High-speed pellet; A = P2 + incubation mixture; B = 10% sucrose layer; C = 10–20% sucrose interface; D = 20% sucrose layer; E = 20–32% sucrose interface; F = 32% sucrose layer. The unit of (Na^+ + K^+)-ATPase activity (U) is μmole Pi released/min at 37°C. The values for specific activity are means ± standard errors. The distribution of activity (U) of each fraction is represented as a percentage of the total units recovered from all the gradient fractions (B–F). The specific activity of Shiverer mice does not differ significantly from that of control mice ($P > 0.05$).

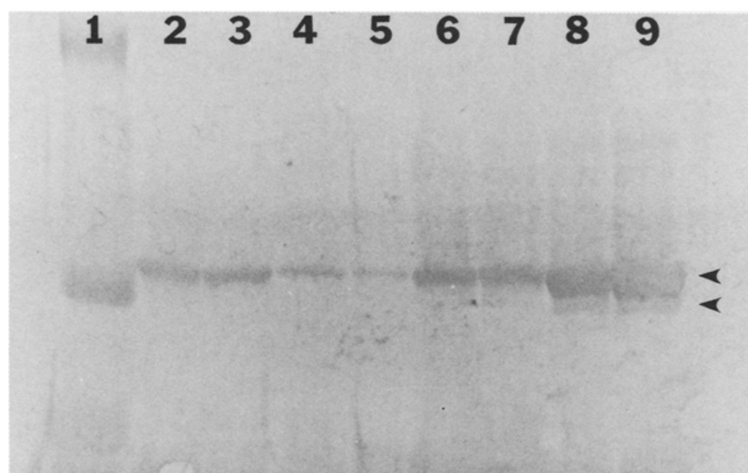

Fig. 2. Immunoblot analysis of sucrose gradient fractions of +/+ and Shi/Shi mouse brain. The immunoblot was prepared, as described in Methods, from a companion gel as in Fig. 1. Each sample contained 50 μg protein. The lanes are as indicated in Figure 1. The pre-stained 97.4 kD protein standard (phosphorylase B) is shown in lane 1. The α_1 and α_2 subunits of (Na^+ + K^+)-ATPase are indicated by arrowheads.

study. The (Na^+ + K^+)-ATPase catalytic subunit doublets (α_1, 110 kDa and α_2, 100 kDa) are most prominent in the fractions of 20% sucrose (D) from both +/+ and Shi/Shi mice as visualized by staining with Coomassie blue (Figure 1). The staining proportions are the same with predominance of α_1 in both species.

In the immunoblot (Figure 2), α_1 and α_2 subunits in fraction D are stained also with similar intensity in both +/+ and Shi/Shi mice.

DISCUSSION

(Na^+ + K^+)-ATPase Activity. Previous studies have shown that the CNS of Shiverer mice is severely reduced in myelin (2–5). The major conclusion from the present data is that specific (Na^+ + K^+)-ATPase activity is not reduced in the Shiverer brain despite the near absence of myelin formation. Thus, no significant change in specific or total (Na^+ + K^+)-ATPase activity in the total homogenate or in the crude low- or high-speed sedimentable or supernatant portions of Shiverer mouse brains as compared to those of control mice (Table I) was observed. Among these fractions, P1 contains the bulk of myelin constituents as shown by immunostaining for myelin-associated glycoprotein (MAG) (23). As shown by immunoblotting, MAG in the P1 and P2 fractions of Shi/Shi mice is significantly reduced (~50%) when compared to corresponding fractions of +/+ mice (23). However, P1 and P2 obtained from Shiverer brains showed no significant difference in (Na^+ + K^+)-ATPase activity when compared to the same fraction from control brains (Table II). The present data also indicate no significant difference ($P > 0.05$) in enzyme specific activity in various sucrose gradient fractions of microsomal membranes from Shiverer compared to control brains (Table II). With regard to the other fractions containing the bulk of (Na^+ + K^+)-ATPase activity, there is no change in the total (Na^+ + K^+)-ATPase activity recovered or in the gradient distribution of the activity (Tables I, II).

The question of how much of normal brain (Na^+ + K^+)-ATPase activity may be attributed to myelin membranes has been discussed. Bourre et al. (19) estimated that, assuming 50% loss of myelin during myelin isolation, myelin accounts for approximately 25% of the (Na^+ + K^+)-ATPase in mouse brain. Reiss et al. (29) concluded from studies of myelin and microsomal fractions that "(Na^+ + K^+)-ATPase may be a myelin-associated enzyme", although they found it difficult to assess the extent of axolemmal contamination in myelin fractions. In contrast, Zimmerman and Cammer (30) found that less than 2.6% of (Na^+ + K^+)-ATPase activity in rat brain homogenate was recovered from myelin and that oligodendrocytes from rat brain had less enzyme activity than had myelin. Our finding of no reduction in Shiverer brains is consistent with the results of Zimmerman and Cammer (30) indicating very little (Na^+ + K^+)-ATPase activity in myelin from rodent CNS.

Catalytic Subunits of (Na^+ + K^+)-ATPase. Specht (15) reported that synthesis of the larger (α_1) polypeptide of (Na^+ + K^+)-ATPase precedes myelination in rat brain. Inspection of the Coomassie blue-stained gel (Figure 1) and of the immunoblot (Figure 2) reveals that the predominance of α_1 in

relation to α_2 is almost identical in brain fractions from adult +/+ and myelin-deficient Shi/Shi mice. Therefore, these data provide more evidence that the synthesis of α_1 or the ratio of α_1 to α_2 is not dependent on normal myelination.

Conclusion. The Shiverer gene codes for an MBP which has a large segment deleted (31, 32). The reduction in PLP (5) and the pronounced increase in 5′-nucleotidase activity (6) may be secondary effects. It appears that the synthesis of CNP (4) and carbonic anhydrase (6) and the synthesis and distribution of (Na^+ + K^+)-ATPase in subcellular fractions (this study) in the CNS are not affected by the Shiverer gene or altered indirectly by reduced myelin formation in Shiverer mice. Ultrastructural studies of (Na^+ + K^+)-ATPase in the Shiverer nervous system may help determine if the reported nodal localization (16–18) is related to myelin formation.

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