IT IS KNOWN THAT hyponatremic encephalopathy leads to death or permanent brain damage most frequently in pubescent children (2, 4, 20) and in women of child-bearing age (7, 11). In adults, the risk of death and permanent brain damage from hyponatremic encephalopathy decreases with advancing age and with the gender of the patient. As a result, postmenopausal women have a reduced risk of dying or developing permanent brain damage from hyponatremic encephalopathy (7), although they are far from immune from the development of serious complications (5). The situation is somewhat different in small children, where there is no gender difference, but infants appear to be far more susceptible to hyponatremic brain damage than do pubescent children (20, 27).

Brain damage resulting from hyponatremic encephalopathy implies failure of adaptive volume regulatory mechanisms. Hyponatremia may lead to osmotic brain swelling such that if the brain is unable to adapt, pressure develops within the rigid skull that can lead to brain damage and brain herniation (3). There are two basic mechanisms by which the brain can adapt to hypoosmotic stress. These include 1) extrusion of osmotically active solutes (cations and organic molecules) from the brain during conditions of plasma hypoosmolarity (3, 13, 25) and 2) decrease in the blood volume and/or cerebrospinal fluid (CSF) in the brain to facilitate changes in brain cell volume due to hyponatremia (23, 24). The effectiveness of the latter mechanism is dependent on physical factors (i.e., the relationship between intracranial volume and brain volume). In the elderly, brain volume as a percentage of intracranial volume is substantially less than in children, thus allowing more space for the brain to swell without being physically compromised by the rigid skull (8, 10, 19). On the contrary, the increased susceptibility of small children to brain damage from hyponatremia appears to be due primarily to the markedly decreased intracranial space that restricts brain expansion should cerebral edema develop (2, 3). One of the most important mechanisms by which intracellular brain solute is regulated during hypoosmotic stress is the extrusion of sodium from brain cells in exchange for potassium by the Na-K-ATPase pump (13, 28).

The increased morbidity and mortality from hyponatremia in young women is considered to be due in part to the inhibitory actions of the female sex hormones on the Na-K-ATPase pump. Both estrogen and progesterone exhibit significant inhibitory actions on the Na-K-ATPase pump in brain and in a number of other tissues as well (9, 13, 17, 21, 26). With the development of hyponatremia, the Na-K-ATPase pump actively extrudes sodium from the relatively hypertonic intracellular space to the hypotonic extracellular environment to prevent brain swelling. Thus, when the Na-K-ATPase pump is activated, the risk of developing central nervous system (CNS) complications from hyponatremia is substantially reduced (17, 18).

The relative roles that physical factors and the Na-K-ATPase pump play in brain adaptation to hyponatremia are unknown. In particular, the relative importance of each of these mechanisms in brain adaptation...
to hyponatremia in postmenopausal women has not been determined. In this study, we decided to investigate whether increased age affected the function of the brain Na-K-ATPase pump and whether this might help to explain why elderly subjects are better able to tolerate hyponatremia than younger ones.

METHODS

General. Experiments were performed on age-matched (2, 12, and 19 mo old) female Sprague-Dawley rats that were obtained from Fisher. The 19-mo-old rats were of the same strain as the 2- and 12-mo-old animals and were supplied by Fisher to the National Institute of Aging. To isolate synaptosomes, rats were killed by decapitation, and their forebrains were removed and immediately placed in 10 ml of ice-cold isolation media (320 mM sucrose, 0.2 mM K-EDTA, 5 mM Tris·HCl, pH 7.4, at 0–4°C). Brains from the age groups were processed simultaneously but kept separated in distinctly marked containers (13). The supernatant S1 was poured off and saved, whereas the pellet P1 was resuspended in 15 ml of isolation media to remove any blood. The extract was then brought up to a volume of 15 ml with isolation media, poured into a glass Dounce homogenizer (clearance 1 mm), and homogenized with 15 evenly pressured up-and-down strokes, pausing for 10 s after every three strokes to prevent the generation of heat by friction. The suspension was then brought up to 30 ml with isolation media, divided equally into two centrifuge tubes, and spun at 1,300 g for 3 min with a centrifuge (model J21B; Beckman Instruments, Fullerton, CA). The supernatant S1 was poured off and saved, whereas the pellet P1 was resuspended in 15 ml of isolation media by homogenization. The suspension was again centrifuged at 1,300 g for 3 min, and both supernatants S1 and S2 were combined. The combination was centrifuged at 1,300 g for 3 min, the pellet P2 was discarded, and the supernatant S2 was spun at 18,000 g for 10 min to obtain the crude synaptosomal-mitochondrial pellet. The pellet was resuspended in 15 ml of isolation media by homogenization with three strokes. Aliquots of 7.5 ml were then layered on a discontinuous Ficoll gradient consisting of 13 ml of 11% Ficoll at the bottom of the centrifuge tube, on which was layered 13 ml of 7.5% Ficoll. Ficoll was dialyzed for 5 h before use. The gradient was then ultracentrifuged at 100,000 g for 70 min with an ultracentrifuge (model L-880, SW28 rotor; Beckman Instruments). On completion of the spin, the purified synaptosomes were located at the interface between the 11 and 7.5% Ficoll layers. The brown mitochondrial pellet was adherent to the bottom of the centrifuge tube, and a small band of myelin was found at the interface of the isolation media and 7.5% Ficoll layer. The synaptosomes were removed by suction with a plastic pipette, and the pellet was brought up to 20 ml with ice-cold isolation media. They were then spun at 17,000 g for 10 min, and the pellet was brought up to 15 ml with isolation media and divided into 0.5-ml aliquots (15).

Na-K-ATPase enzyme assay. Na-K-ATPase activity was determined as previously described (13). Enzyme activity was measured in synaptosomes isolated from groups of rats that were either 2, 6, or 19 mo old. Cells were suspended by addition of protein to 0.5 ml of Hanks' buffer (5 mM MgCl2, 100 mM NaH2BO3, 150 mM NaCl, and 100 mM imidazole at pH 7.3) in the presence and the absence of 1 mM ouabain at 25°C. To permeabilize the membranes, the suspension was sonicated in an ice bath (0–4°C) for 90 s and vortexed until completely suspended. The reaction was initiated by adding 0.1 ml of a working solution containing 100 mM imidazole buffer, pH 7, 0.162 mM disodium-NADH, 4.2 mM disodium-ATP, 16.6 mM trisodium phosphosolopyruvate, 4 units pyruvate kinase, and 13 units lactate dehydrogenase to two cuvettes containing 30 μl of the protein suspension. After ouabain was added to one cuvette, both cuvettes were incubated at 37°C for 15 min before enzyme activity was read on a Beckman recording spectrophotometer (model DUS050) at 25°C and a wavelength of 340 nm. The difference between the total hydrolytic activity and non-ouabain-inhibitable hydrolytic activity represents the Na-K-ATPase activity (12). Na-K-ATPase assay was also used to determined sidedness of synaptosomes, as we previously described (12, 14). Approximately 35% of the vesicles are inside out, and the remaining 65% are right side out (14). No difference in vesicle orientation was found among the three age groups.

Potassium uptake assay. In this experiment, the synaptosomes were loaded with sodium by incubation for 10 min at 37°C in four times its volume with preequilibrium media (150 mM NaCl, 1 mM MgCl2, and 5 mM MOPS-Tris, pH 7.4). Synaptosomes were then centrifuged at 20,000 g for 5 min, and the pellet was brought up in 400 μl of preequilibrium media and placed on ice. Sodium transport was initiated by the addition of 5 μl of synaptosomal protein (~50 μg of protein) to 95 μl of external media [140 mM choline chloride, 5 mM MgCl2, 0.2 mM EGTA, 1 mM KCl, 5 mM MOPS-KOH, pH 7.4, and 0.5 μCi 86Rb+ (70,000 counts/min) at 25°C (13, 15)]. Transport was terminated at the prescribed time of incubation by immediate addition of 2 ml of ice-cold stop solution to 100 μl of the transport media. The mixture was then filtered through a 0.45-μm-pore-size cellulose acetate membrane. The filters were washed twice with 2 ml of cold stop solution (150 mM choline chloride) and dissolved in phase combining system II scintillation fluid as previously described. Potassium uptake was then calculated on a 1:1 ratio with rubidium (15).

Protein determination. Protein concentration was determined as described (22). The extraction method yielded ~100 μg of synaptosomal protein/4 g of rat brain cortex. For the presentation of results, protein concentration was measured from the final synaptosomal suspension used in the experiments.

Statistical analysis. Significant differences between the different groups were determined by two-way ANOVA with Fisher’s post hoc comparisons. A probability of P < 0.05 was considered to be significant. All data unless otherwise stated are expressed as means ± SE.

Materials. The chemicals used in these experiments were obtained from the following sources. Ficoll, ouabain octahydrate, rotenone, EGTA, and EDTA were obtained from Sigma Chemical (St. Louis, MO). Cellulose acetate filters, type SM 111-P12, were obtained from Sartorius Filters (Hayward, CA). Sprague-Dawley rats were obtained from Bainton Kingman (Fremont, CA). All other chemicals were of reagent grade and obtained from Sigma Chemical.

RESULTS

Measurements of Na-K-ATPase activity were carried out as described in METHODS. Studies were carried out in synaptosomes isolated from brains of female rats that were either 2, 12, or 19 mo of age. As shown in Fig. 1, Na-K-ATPase activity progressively decreased with the increasing age of the animals. Enzyme activity decreased from 0.416 ± 0.011 μmol·min⁻¹·mg protein⁻¹ in 2-mo-old rats and to 0.345 ± 0.014 μmol·
Na-K-ATPase activity decreased significantly from 2- to 19-mo-old rats and further decreased to 0.274 ± 0.017 μmol·min⁻¹·mg protein⁻¹ in 19-mo-old animals (Table 1). Compared with 2-mo-old animals, enzyme activity decreased by 17% in 12-mo-old animals and was further reduced by an additional 34% in the 19-mo-old age group. Differences in Na-K-ATPase activity between each age group were highly significant (P < 0.0026 in 2- vs. 12-mo-old, P < 0.0001 in 2- vs. 19-mo-old, and P = 0.0023 in 12- vs. 19-mo-old rats). When Na-K-ATPase enzyme activity between male and female rats was compared, activity was significantly greater in males than in females, as we previously described (13). In 2-mo-old rats, enzyme activity in males was 0.440 ± 0.013 vs. 0.416 ± 0.011 nmol/mg protein in females. Activity was 0.611 ± 0.018 nmol/mg protein in 12-mo-old males and 0.592 ± 0.015 nmol/mg protein in 19-mo-old males. In all age groups, Na-K-ATPase activities were greater in males than in the corresponding female groups but were only significantly different at 12 and 19 mo.

As a result of these findings, we decided to investigate whether the transport properties of the Na-K-ATPase pump were affected by the age of the rats from which synaptosomes were isolated. We determined potassium transport by the sodium pump, using rubidium ((98)Rb⁺) as tracer, as was previously described (13). Rubidium uptake in the three groups was studied in the absence and in the presence of 2.5 mM ouabain (Fig. 2). In the absence of ouabain, potassium uptake in the 2-mo-old group was 51.89 ± 2.20 nmol/mg protein but decreased to 35.72 ± 1.45 nmol/mg protein in the presence of 2.5 mM ouabain. In the 12-mo-old group, potassium uptake decreased from 44.97 ± 2.56 to 35.18 ± 3.98 nmol/mg protein and from 41.97 ± 4.93 to 35.84 ± 4.49 nmol/mg protein in the 19-mo-old group with ouabain. The ouabain-sensitive component of potassium uptake in the three groups was 16.18 ± 1.31 vs. 9.79 ± 1.44 vs. 6.12 ± 1.05 nmol/mg protein in the 2-, 12-, and 19-mo-old rats, respectively. Thus, compared with the 2-mo-old rats, potassium uptake was reduced by 39% at 12 mo of age and by 62% in 19-mo-old females. As shown (Fig. 3), differences between 2- and 12-mo-old rats were highly significant (P = 0.0063); they were also highly significant (P = 0.0003) between the 2- and 19-mo-old rats as well.

**DISCUSSION**

These data provide evidence to support the idea that adaptation of the elderly female rat brain to hyponatremia is largely dependent on physical factors (i.e., brain-to-skull size ratio). The Na-K-ATPase pump is one of the major biochemical mechanisms by which the brain adapts to hyponatremia, to prevent brain edema. Thus, because elderly females have decreased morbidity and mortality from hyponatremia, one might have speculated that the Na-K-ATPase pump function...
would be increased with advancing age. Moreover, because it is known that estrogen and progesterone inhibit the sodium pump (9, 17, 18, 21, 29) and that these hormones are decreased in the elderly, the assertion was that these factors alone were sufficient to explain the decreased mortality and morbidity from symptomatic hyponatremia seen in elderly females subjects. The results of this study, however, suggest that the Na-K-ATPase pump function is significantly decreased in female rats with advancing age (Table 1), and, as such, survivability of hyponatremia in elderly females may be independent of the sex hormones and the Na-K-ATPase activity.

The reason for this age-related decrease in the sodium pump function is unclear. Factors such as the female sex hormones, which are known to inhibit the Na-K-ATPase pump (9, 13, 17, 29), are significantly diminished after menopause. As such, estrogen and progesterone would be unlikely to contribute to the age-related decrease in sodium pump function that was observed in the older age group. Additionally, if the age-dependent decrease in the sodium pump function was primarily mediated by the sex hormones, one would expect pump activity to increase with advancing age, coincident with the lower circulating levels of estrogen and progesterone. Because the Na-K-ATPase activity decreases with age, it is quite likely that other factors may play a more dominant role in elderly subjects regarding brain adaptation to hyponatremia.

Those factors, which are known to play important roles in brain adaptation to hyponatremia in postmenopausal females, include an age-related decrease in estrogen and progesterone and increased intracranial space (physical factors) due to naturally occurring age-related brain atrophy. On the basis of the results of these studies, it appears that physical factors may be the more dominant factor of the two in ensuring brain survival during hyponatremia in elderly female subjects. These findings suggest that even in the presence of cerebral edema, if the brain is able to swell without generating a significant increase of intracranial pressure, the prospects for patient survival may be substantially improved. This observation is consistent with our recent work employing magnetic resonance imaging evaluation of the brain, which showed that elderly patients with severe hyponatremia (plasma sodium of ~108 mmol/l) and significant cerebral edema may exhibit only minimal symptoms of encephalopathy (5). As noted in RESULTS, the Na-K-ATPase activity in male rats did not decrease with age, as was the case with the female groups. It is unclear why this difference occurred between the sexes, but clinical observations and the availability of circulating testosterone until old age may contribute to this observation.

Adult males of any age generally survive hyponatremia without significant morbidity and mortality. One reason may be that adult males have high circulating levels of testosterone, even up to an age of 80 yr. Testosterone is known to stimulate the Na-K-ATPase pump in many tissues, including the brain (17, 18). Thus elderly males who develop hyponatremia are able to compensate more appropriately than are females, because of the ready availability of testosterone to stimulate the sodium pump (6). Additionally, elderly males also benefit from the cerebral atrophy of aging, as do elderly female subjects.

It has also been shown recently, in brain astrocytes in culture, that decreased Na-K-ATPase activity may result in failure of regulatory volume decrease (RVD) (17). This failure in RVD could lead to brain swelling, increased intracranial pressure, brain herniation, and death (1, 6, 11). Therefore, on the basis of a decrease in Na-K-ATPase activity alone, aging subjects should be more susceptible than younger ones to develop complications from hyponatremia. This, however, has not been the clinical experience, where elderly females (patients and animals) are better able to tolerate hyponatremia than are young ones (1, 5, 11). Thus the increased survival of elderly females from hyponatremia may be due in large part to the increase in intracranial space that results from cerebral atrophy during aging (21). Taken together, these findings suggest that brain swelling without the development of significant intracranial pressure to cause brain compression by the skull may result in reduced morbidity and mortality.

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