Phosphatidylserine (PS), a major acidic phospholipid in the brain, has been studied extensively in regard to its actions on brain functions. They are, for example, the enhancement of Na⁺, K⁺-ATPase activity in the brain (1), the increase of the brain glucose concentration (2) and recovering effects on age-associated memory impairment in experimental animals (3–5).

In 1986 Delwade et al. (6) reported that oral administration of bovine brain cortex-derived PS (BC-PS) to patients with senile dementia improved their cognitive disorders. This finding of an antidementia effect has been confirmed by a double-blind, placebo-controlled studies. Notably, a clinical trial on 494 elderly patients in Italy (7) demonstrated the benefit of taking BC-PS as an antidementia agent that can improve behavior and cognitive performance without side effects.

Because the effective dose of BC-PS ranges from 100 to 500 mg/d, which is not much higher than the estimated daily intake (80 mg) of PS (8), it is possible to use this phospholipid as a food supplement to improve and/or prevent senile dementia. However, for safety reasons, BC-PS is not suitable for use as a food supplement to improve and/or prevent senile dementia. This is because PS in brain cortex is not high enough (3 g per bovine brain cortex) (9) and no naturally abundant and safe source of PS has been found.

To overcome these problems, a transphosphatidylation reaction using phospholipase D (EC 3.1.4.4.) was applied to produce PS from soybean lecithin (10), where phosphatidylcholine or phosphatidylethanolamine is a donor of the phosphatidyl residue and l-serine is an acceptor.

Although the fatty acid composition of soybean lecithin transphosphatidylated phosphatidylserine (SB-tPS) is considerably different from that of BC-PS, whose structure is primarily confined to a 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-ethanolamine, our previous experiments demonstrated that SB-tPS restored scopolamine-induced memory impairment in rodents on intraperitoneal (11), oral (12) or intracerebroventricular (13) administration. Also, we demonstrated that oral administration of SB-tPS prevented ischemic damage in gerbil hippocampus (14).

In this study, the effect of continuous oral administration of SB-tPS on the spatial memory of aged memory-impaired rats was investigated, and some biochemical variables related to the synaptosomal functions, such as potassium-induced acetylcholine release or Na⁺, K⁺-ATPase activity of the synaptosomes, were examined to determine the nootropic mechanism of this phospholipid.

**MATERIALS AND METHODS**

Animals. Male Wistar rats, 24 to 25 mo old (aged) or 8 wk old (young), were purchased from CLEA (Tokyo, Japan). Aged rats were housed two to a cage and young rats three to a cage and consumed a commercially available nonpurified diet (MF diet; Oriental Yeast, Tokyo, Japan) and water ad libitum, under conditions of controlled temperature (24 ± 2°C), humidity (55 ± 10%) and lighting (0830–2030). The MF diet contained the following nutrients (g/kg): water, 76; protein, 246; lipid, 56; carbohydrate, 552; minerals, 63; and

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2 Abbreviations used: BC-PS, bovine brain cortex-derived phosphatidylserine; EL, escape latency; PS, phosphatidylserine; SB-tPS, soybean lecithin transphosphatidylated phosphatidylserine.
vitamins. The care and treatment of the rats complied with the guidelines for the ethical treatment of laboratory animals at the Yakult Central Institute for Microbiological Research (Tokyo, Japan).

Administration of SB-tPS. SB-tPS prepared from soybean lecithin and t-serine (14) was emulsified in water and administered orally in drinking water. The concentration of SB-tPS was adjusted to ensure an average daily intake of 60 mg/kg throughout the experiment. Tap water was given to the control group. The drinking water was replaced every 2 or 3 d.

Water maze test apparatus. The water maze test was adapted from Morris (13). The apparatus comprised a circular pool (150 cm in diameter and 45 cm in depth) and a circular invisible platform made of transparent acrylic resin (12 cm in diameter). The pool was filled with water up to 30 cm in depth and maintained at 23 ± 1°C. The platform was fixed in the middle of one of the four quadrants of the pool, 1 cm below the water surface. A video camera mounted in the center above the pool was connected to a data-analyzing system (equipment: AXIS-30; program: TARGET/2; Neuroscience, Tokyo, Japan) and the movements of rats were automatically recorded. Three light bulbs were arranged around the pool to give spatial clues.

Screening test. Rats were placed in water and the escape latency (EL), the time taken to recognize the platform in the third quadrant, was measured. The mean swimming velocity and a swim path were recorded by the data-analyzing system. If the rat left the platform within 90 s, it was placed on the platform for 15 s. The trial was performed continuously for 4 d. Eight of 42 aged rats whose average EL on d 4 was below the upper limit of the 99% confidence level for young rats (dotted line in Fig. 1B) were regarded as nonimpaired and excluded from subsequent experiments. The impaired aged rats were divided into a control and SB-tPS-administered group with the mean EL of each group almost the same (Fig. 1C).

Escape test 1. After administration of the sample solution for 60 d, the EL was measured for 4 d under the same conditions as the screening test.

Spatial probe test. On the day after the last trial of escape test 1, seven rats of the control group and six rats of the SB-tPS group were used for the spatial probe test. The platform was removed from the third quadrant, where it had been in escape test 1, and the total time spent swimming in the third quadrant was measured for 90 s.

Escape test 2. Six days after the last trial of escape test 1, the rats that had not been used for the spatial probe test (11 of the control group and 10 of the SB-tPS group) were subjected to the same EL of each group almost the same (Fig. 1).

After administration of the sample solution for 60 d, the EL was measured for 4 d under the same conditions as the screening test.

Dissection of rat brain. After escape test 2, the rats were killed by decapitation and the brains were immediately removed and dissected into eight parts (frontal cortex, striatum, cerebellum, medulla oblongata and pons, hypothalamus, hippocampus, parietal cortex, thalamus and midbrain). The brains of 10-wk-old male rats (Wistar strain) were also dissected for reference.

Brain lipid analysis. All parts of the brain were homogenized in 1.0 mL of 0.1 mol/L phosphate-buffered saline (pH 7.2) with sonication (Sonifer cell disruptor 200; Branson, Danbury, CT) and were extracted by the Bligh-Dyer method (16). The extracted lipid fractions were examined for cholesterol and phospholipid contents using Determiner TC555 (Kyowa Medex, Tokyo, Japan) and Phospholipid Test Wako (Wako Pure Chemical Industries, Osaka, Japan). The molar ratio of cholesterol and phospholipids was calculated assuming the molecular weight of phospholipid to be 800. For analyzing phospholipids composition, lipid extract of brain (containing 20 μg of phospholipid) was applied to a silica gel thin layer plate (Silica gel 60; Merck, Darmstadt, Germany) and developed using an acidic solvent system (chloroform:methanol:acetic acid = 13:5:2, v/v/v). After the coloring of phosphorus with Dittmer-Leiter reagent, the phospholipid composition was determined with an image analyzing system (Dr. GEL; Mitani, Fukuji, Japan).

Preparation of synaptosomes. Synaptosomes were prepared by Ficoll (Amersham Pharmacia Biotech, Little Chalfont, UK) discontinuous gradient centrifugation according to the method of Tanaka et al. (17).

Measurement of acetylcholine synthesis and release. The synthesis and release of acetylcholine and choline were measured according to the method of Tanaka et al. (17). For the determination of acetylcholine and choline synthesis, synaptosomes were suspended in low K+-Krebs-Ringer solution (4.7 mmol/L KCl, 10 mmol/L glucose and 0.1 mmol/L eserine, pH 7.4) and preincubated at 37°C for 30 min. The reaction was stopped by adding 0.1 mol/L of perchloric acid before centrifugation at 2250 × g for 3 min at 4°C, and the supernatants were collected for analysis by HPLC. To determine the K+-induced acetylcholine release, synaptosomes preincubated above were washed with the low K+ solution and resuspended (0.25 g of protein/L) in low or high K+ (50 mmol/L/KCl) Krebs-Ringer solution. After 5-min incubation at 37°C, the reaction mixtures were centrifuged at 2250 × g for 3 min at 4°C, and the supernatants were used for measuring the acetylcholine release. To quantify the acetylcholine content, a known amount of ethylhomocholine (EICOM, Kyoto, Japan) was added to the sample as an internal standard, and the mixture was centrifuged at 2,250 × g for 3 min at 4°C. Resulting supernatants were subjected to HPLC in a system equipped with an AC-GEL column (6 × 150 mm; EICOM), a postcolumn (AC-ENZ; EICOM) and an electrochemical detector (ECD-300; EICOM). The postcolumn reactor composed of immobilized acetylcholine esterase and choline oxidase was used to produce hydrogen peroxide from acetylcholine and hydrogen peroxide was measured by the amperometric method.

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acetylcholine, choline and ethylhomocholine. The hydrogen peroxide generated was detected by an electrochemical detector equipped with a platinum electrode (WE-PT; EICOM). The reaction mixture containing 115 mmol/L Tris-HCl buffer (pH 7.6), 5 mmol/L MgCl₂, 100 mmol/L NaCl and 20 mmol/L KCl, whereas the basal Mg²⁺ component was measured by omitting both Na⁺ and K⁺ from the reaction mixture. The difference of these activities was regarded as Na⁺, K⁺-ATPase activity. 

**Measurement of Na⁺, K⁺-ATPase activity.** Na⁺, K⁺-ATPase activity was determined according to the methods of Huang et al. (18). Total ATPase activity was measured in a 1.0-mL reaction mixture containing 115 mmol/L Tris-HCl buffer (pH 7.6), 5 mmol/L MgCl₂, 100 mmol/L NaCl and 20 mmol/L KCl, whereas the basal Mg²⁺ component was measured by omitting both Na⁺ and K⁺ from the reaction mixture. The difference of these activities was regarded as Na⁺, K⁺-ATPase activity. After the synaptosome suspension (~1.0 mg of protein) with the reaction mixture was preincubated at 37°C for 10 min, the reaction was started by adding ATP (final concentration, 5.0 mmol/L) and terminated by adding trichloroacetic acid at a final concentration of 50 g/L. Finally, the released inorganic phosphorus was measured by using the Fiske and Subbarow reducer (Sigma, St. Louis, MO).

**Statistical analysis.** For statistical analysis, Yukms Statistical Library I, Version 3.0 (Yukms, Tokyo, Japan) was used. Behavioral data were analyzed by nonparametric Mann-Whitney U test for the comparison between two groups. To compare the data among three groups, the Kruskal-Wallis test with Schef’s posthoc test was done. Biochemical data were analyzed by the two-tailed Student’s t test or two-tailed Welch test after ANOVA. All data were expressed as means ± SEM. Differences were considered significant at P < 0.05.

**RESULTS**

**Screening test.** The EL for the aged group on d 4 was significantly longer (P < 0.01, Mann-Whitney U test) than that of the young group (Fig. 1A). EL of the aged nonimpaired control and aged impaired SB-tPS group are shown in Figure 1C. The EL of the nonimpaired group was significantly shorter than that of the aged impaired control (P < 0.01) or aged impaired SB-tPS groups (P < 0.01).

**Escape test 1.** The EL of the SB-tPS group was significantly shorter than that of the control group (Fig. 2) on d 3 (P < 0.05) and d 4 (P < 0.01), whereas swimming velocity did not differ between the two groups.

**Spatial probe test.** The time spent in the third quadrant for the SB-tPS group was significantly longer (P < 0.01) than that for the control group (Fig. 3A). Figure 3B shows a typical swim path of a SB-tPS-administered aged rat.

**Escape test 2.** Six days after the last trial of escape test 1, EL was measured with the platform transferred to the fourth quadrant. The EL of the SB-tPS group tended to be shorter (P = 0.057) on d 1 than that of the control group (Fig. 4), whereas the swimming velocity did not differ between the two groups.

**Effects on the synaptosomal acetylcholine dynamics.** The high K⁺ (50 mmol/L) depolarization-induced acetylcholine release from synaptosomes of the aged impaired control rats was approximately one-half of that of young rats (Table 1), whereas it was 45% greater in the SB-tPS administered group than in the control group (P < 0.05). However, neither the basal acetylcholine release (4.7 mmol/L KCl) from synaptosomes nor the synthesis of acetylcholine or choline was affected by SB-tPS administration.

**Improvement of Na⁺, K⁺-ATPase activity.** The Na⁺, K⁺-ATPase activity of the synaptosomes was significantly increased in aged rats by the administration of SB-tPS (Table 2, P < 0.01).

**Brain lipid analysis.** In the SB-tPS group, the cholesterol/phospholipid molar ratio (Table 3) and phosphatidylcholine content (Table 4) in the thalamus and midbrain were signific-
significantly higher ($P < 0.05$), whereas the phosphatidylethanolamine content (Table 4) in this region was significantly lower ($P < 0.05$) than those of the control group. Groups did not differ in the other seven regions examined.

**DISCUSSION**

To estimate the effects of SB-tPS on age-related memory impairment, the Morris water maze test was applied to aged rats. Sixty days of oral administration of SB-tPS restored performances in the escape test and the spatial probe test. Therefore, SB-tPS can improve age-related spatial memory impairment in rats.

The present findings are consistent with Zanotti et al. (5) who demonstrated an improvement of Morris water maze performance in aged impaired rats (21–24 mo) with chronic oral administration of BC-PS (50 mg kg$^{-1}$ d$^{-1}$ for up to 12 wk). Recently, the effect of three types of PS (PS from bovine cortex, soybean, and egg) on the learning ability of middle-aged rats was reported (19). In this experiment, contrary to our results, no improvement in Morris water maze performance was observed, but the age of the rats (12 mo) and the administration route (intraperitoneal) were different (24–25 mo, oral).

In the Morris water maze test, the scores for escape test 1 and the spatial probe test are considered to reflect primarily the long-term spatial memorizing ability. The results that SB-tPS shortened EL without affecting swimming velocity and extended the time spent in the place where the platform had existed previously suggest that the administration of SB-tPS could improve long-term spatial memory.

Escape test 2 was performed to confirm the result of escape test 1 by changing the position of the platform. However, unexpectedly, the EL of the SB-tPS group tended to be shorter than that of the control group from the first trial. This seems contradictory to the result obtained in the spatial probe test, where the SB-tPS-administered rats showed a preference for the third quadrant. This discrepancy can be explained as follows: there are possibly two methods for memorizing a specific spatial location (20). One is to use a cognitive map that encodes information about the geometric relationship between the object and several landmarks (the cognitive map method). The other is to use a heading vector that specifies the direction and distance from a single landmark to the object (the heading vector method).

In the present case, the rats could find the platform from the placement of light bulbs around the pool (the cognitive map method) and/or from the distance and direction relative to the wall (a likely heading vector method). In escape test 1

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Release</th>
<th>Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$ Basal</td>
<td>$n$ Induced</td>
</tr>
<tr>
<td><strong>Young Aged</strong></td>
<td>3 119.8 ± 8.7</td>
<td>3 31.2 ± 10.5</td>
</tr>
<tr>
<td>Control</td>
<td>15 95.2 ± 5.6</td>
<td>15 16.6 ± 1.7</td>
</tr>
<tr>
<td>SB-tPS</td>
<td>16 95.9 ± 5.2</td>
<td>16 24.0 ± 2.7*</td>
</tr>
</tbody>
</table>

* $P < 0.05$ vs. control (two-tailed Welch test).
and the spatial probe test, cognitive ability due to both methods contributed to the score. But in escape test 2, cognitive ability due to the heading vector method mostly affected the initial score, because the distance from the wall was not changed. In addition, short-term memory (working memory) to effectively explore the pool by memorizing the area already explored could also contribute to the improved score in escape test 2. Thus, PS may improve not only reference memory, but also working memory.

SB-tPS also ameliorated the acetylcholine release in aged rats to the level in young rats as in the microdialysis study on BC-PS (21). In addition to the finding that the in vitro treatment of mouse synaptosomes with SB-tPS increased high K+-induced acetylcholine release (22), the present result suggests involvement of acetylcholine dynamics in the nootropic actions of PS. Because the activity of acetylcholine synthesis was nearly the same in the control and the SB-tPS groups, SB-tPS may increase acetylcholine release by affecting some metabolic events other than acetylcholine synthesis.

The transmitting activity of nerve cells is regulated by the membrane potential. According to Tanaka and Ando (23), the resting membrane potential of aged mice is lower than that of young mice. This is partly explained by the depression of Na+, K+-ATPase, a pump acting to maintain the membrane potential. Therefore, it is possible that an enhancement of acetylcholine release through the restoration of Na+, K+-ATPase activity contributes to the improvement of learning impairment due to SB-tPS.

According to Pepeu et al. (24), decreased 45Ca2+ uptake into K+-depolarized cortical synaptosomes and acetylcholine release from an electrically stimulated cortical slice of aged rats were restored by an intraperitoneal administration of BC-PS. Considering that the synaptic Ca2+ uptake is an important event for triggering neurotransmitter release, SB-tPS may also restore acetylcholine release through this mechanism and improve the learning impairment of aged rats.

PS can also affect exocytosis of neurotransmitters by interacting with membrane-binding proteins. For example, synaptic vesicle-associated proteins, such as synaptotagmin (25), rabphilin-3A (26) and double C2 protein (27), or neuronal plasma membrane proteins, such as annexin (28), can bind to PS liposomes in a Ca2+-dependent manner. Because the binding of the synaptic vesicle proteins to the inner surface of the neuronal plasma membrane is an essential event for Ca2+-triggered exocytosis of neurotransmitters, it is possible that SB-tPS incorporated into the membranes enhances the fusion between synaptic vesicles and membranes to release neurotransmitters.

The present study showed that continuous oral administration of SB-tPS improved Morris water maze performance in aged rats as did BC-PS (5), which strongly supports the functional similarity of SB-tPS to BC-PS, whose efficacy against senile dementia or age-associated memory impairment was demonstrated in human clinical studies.

ACKNOWLEDGMENTS

We thank K. Shimizu for excellent technical assistance. We also thank H. Shishido and S. Tanabe for helpful discussions.

LITERATURE CITED


TABLE 4
Phospholipid composition of the young rats and control and soybean lecithin transphosphatidylated phosphatidylserine (SB-tPS)-treated impaired aged rats used in the Morris water maze test 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SB-tPS</th>
<th>Young</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>40.8 ± 1.9</td>
<td>42.2 ± 2.7</td>
<td>40.0 ± 1.9</td>
</tr>
<tr>
<td>Striatum</td>
<td>18.0 ± 1.8</td>
<td>17.8 ± 1.6</td>
<td>18.5 ± 1.1</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>41.3 ± 3.1</td>
<td>40.1 ± 3.2</td>
<td>41.4 ± 1.5</td>
</tr>
<tr>
<td>Medulla oblongata and Pons</td>
<td>42.0 ± 3.4</td>
<td>41.7 ± 3.3</td>
<td>40.9 ± 4.6</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>17.7 ± 1.3</td>
<td>17.3 ± 1.3</td>
<td>17.9 ± 3.0</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>40.3 ± 3.5</td>
<td>41.1 ± 3.7</td>
<td>41.2 ± 2.8</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>40.0 ± 1.4</td>
<td>41.0 ± 1.3</td>
<td>41.3 ± 0.4</td>
</tr>
<tr>
<td>Thalamus and Midbrain</td>
<td>18.4 ± 0.9</td>
<td>18.1 ± 0.8</td>
<td>17.5 ± 0.2</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM.
2 Includes all phospholipids other than phosphatidylethanolamine (PE) and phosphatidylserine (PS).
3 P < 0.05 vs. control (two-tailed Student’s t test).

TABLE 3
Cholesterol/phospholipid molar ratio of the young rats and control and soybean lecithin transphosphatidylated phosphatidylserine (SB-tPS)-treated impaired aged rats in the Morris water maze test 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SB-tPS</th>
<th>Young</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.50 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.58 ± 0.01</td>
<td>0.66 ± 0.08</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.49 ± 0.02</td>
<td>0.48 ± 0.02</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>Medulla oblongata and Pons</td>
<td>0.50 ± 0.01</td>
<td>0.50 ± 0.01</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.51 ± 0.02</td>
<td>0.49 ± 0.02</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.51 ± 0.02</td>
<td>0.50 ± 0.01</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>0.51 ± 0.02</td>
<td>0.50 ± 0.02</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td>Thalamus and Midbrain</td>
<td>0.51 ± 0.02</td>
<td>0.50 ± 0.02</td>
<td>0.49 ± 0.01</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM.
2 P < 0.05 vs. control (two-tailed Student’s t test).