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Phospholipid Translocation from the Outer to the Inner Leaflet of Synaptic Vesicle Membranes Isolated from the Electric Organ of Japanese Electric Ray *Narke japonica*[1]

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The phospholipid translocation from the outer to the inner leaflet of synaptic vesicles isolated from the electric organ of the Japanese electric ray, *Narke japonica*, was measured using fluorescent phospholipid probes. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS) with a fluorescent NBD-labeled short acyl chain at the *sn*-2 position was mixed with purified synaptic vesicles and the probe in the outer leaflet of the membranes was reduced with dithionite to quench the fluorescence from time to time. The percentage of fluorescence remaining after the dithionite treatment served as an index for the phospholipid translocation. The results obtained indicated that about 30, 13, and 9% of NBD-PE, NBD-PS, and NBD-PC, respectively, were translocated from the outer to the inner leaflet in 3 h. Thus, the translocation activity in synaptic vesicle membranes was much higher for PE than for PS, in contrast to the previous results obtained with plasma membranes, including synaptosomal membranes. The percentages of the phospholipid in the inner leaflet at equilibrium were estimated to be 41, 31, and 14% for PE, PS, and PC, respectively. The translocation was inhibited by pretreatment with an SH reagent, iodoacetamide, indicating the involvement of a proteinaceous translocator. These data may provide a biochemical basis for elucidating the mechanisms of membrane fusion and exocytosis at nerve endings.

Key words: asymmetric phospholipid distribution, electric organ, NBD-labeled phospholipid, phospholipid translocation, synaptic vesicle.

The plasma membrane phospholipids of various cells are asymmetrically distributed between the two leaflets of bilayers (*1-5*). In many types of cells, aminophospholipids including phosphatidylserine (PS) and phosphatidylethanolamine (PE), reside mostly in the inner (cytoplasmic side) leaflet, whereas neutral polar phospholipids such as phosphatidylcholine (PC) and sphingomyelin mainly exist in the outer leaflet of plasma membrane bilayers. The creation and maintenance of this asymmetric distribution of phospholipids require ATP and an aminophospholipid-specific translocator, aminophospholipid translocase (*3, 5-8*). The asymmetric distribution of phospholipids between the two leaflets of membrane bilayers of intracellular organelles has also been reported in mitochondria (*9*), endoplasmic reticulum (*10*), chromaffin granules (*11, 12*), and synaptic vesicles (*13, 14*), although the asymmetric distribution seems less specific and less prominent compared to that in plasma membranes. The distribution and translocation of phospholipids in secretory vesicles such as chromaffin granules and synaptic vesicles is especially interesting because the loss and restoration of the asymmetric distribution of the plasma membrane lipids have been implicated in membrane fusion and exocytosis (*5, 15-18*). Since we found the PS-specific translocation from the outer to the inner leaflet, and its enrichment in the inner leaflet of the plasma membrane of the presynaptic terminals isolated from the electric organ of the Japanese electric ray, *Narke japonica* (*19*), we next became interested in the translocation and equilibrium distribution of phospholipids between the two leaflets of the synaptic vesicle membranes in this animal. The electric organ contains a single type (cholinergic) of presynaptic nerve terminal at high density and hence purified synaptic vesicles can be prepared from it. The high purity of a preparation is a prerequisite for measurement of the asymmetric distribution of membrane lipids.

The most successful and widely used method for monitoring phospholipid translocation across a bilayer is the so-called "back-exchange" method (*3*). For this method,

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Abbreviations: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; IAA, iodoacetamide; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine.

the outer leaflet of the cell membrane is first labeled with a spin-labeled or fluorescent phospholipid analog as a probe. Probes are allowed to be translocated for some time and then any probes remaining at the outer leaflet are extracted with BSA. After separation of membranes from BSA/probe complexes by centrifugation, the amount of probes bound to BSA is determined. We previously developed radioisotope (RI)-labeled phospholipid probes (*20*), which assure high sensitivity, for use with the back-exchange method. However, in a preliminary study with synaptic vesicles, we found that measurement with the back-exchange method using the RI-labeled phospholipid probes was hampered by incomplete extraction with BSA of the probes residing at the outer leaflet of the synaptic vesicle membranes.

In the present study, therefore, we used another method for the measurement of phospholipid translocation, which involves fluorescence-labeled phospholipid probes, together with a membrane-impermeant reducing reagent (*21*). The reducing reagent reduces the fluorophore of the probes located on the outer leaflet of the bilayer, producing non-fluorescent derivatives. The persistent fluorescence of the membrane after the addition of the reducing agent represents phospholipid probes translocated to the inner leaflet of the membrane. This method does not need an extraction procedure and thus is free from the problem of incomplete extraction. We employed this method to determine whether or not the membrane of the electric organ synaptic vesicles has a phospholipid translocation system for the three major phospholipids, PC, PE, and PS. This is the first report on measurement of the phospholipid translocation across synaptic vesicle membranes.

MATERIALS AND METHODS

Chemicals—1-Palmitoyl-2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl-*sn*-glycero-3-phosphocholine (NBD-PC), 1-palmitoyl-2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), and 1-palmitoyl-2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl-*sn*-glycero-3-phosphoserine (NBD-PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, disodium salt) and luminol were from Sigma Chemicals (St. Louis, MO, USA). Sodium dithionite ($Na_2S_2O_4$) and acetylcholine esterase (from bovine erythrocytes) were purchased from Wako Pure Chemicals (Osaka). Choline oxidase was from Funakoshi (Tokyo) and peroxidase (from *Arthromyces*) was obtained from Nakalai Tesque (Kyoto). All other chemicals were of the highest grade available and used without further purification.

Preparation of Purified Synaptic Vesicles—Synaptic vesicles were prepared from the electric organ of an electric ray, *Narke japonica*, according to the methods of Tashiro and Stadler (*22*), and Rahamimoff *et al.* (*23*), with slight modifications as follows. All procedures were performed at 4°C unless otherwise noted. The electric organ was dissected and frozen with liquid nitrogen. The frozen tissue (totally about 180 g) was put into a plastic bag, placed on a dry ice-cooled chopping board made of bronze, and then crushed with a hammer. The resulting small pieces of tissue were ground into powder in a mortar cooled with dry ice. A buffer solution [0.4 M NaCl, 3.5 mM EGTA, 10 mM Hepes-NaOH, pH 7.4, and a protease inhibitor mixture comprising 0.3 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin and 0.5 μg/ml pepstatin A] was added to the powder (1-1.25 v/w), and then the mixture was squeezed through 4 layers of cotton gauze. The extract (amounting to 60-70% of the tissue weight) was centrifuged at 10,000 × *g* for 30 min. The supernatant was placed on a discontinuous sucrose gradient (0.2 M sucrose/0.3 M NaCl; 0.6 M sucrose/0.1 M NaCl) and then centrifuged at 67,700 × *g* for 3 h. Crude synaptic vesicles that sedimented at the interface were collected and diluted twice with a buffer (1 M sucrose, 10 mM Hepes-NaOH, pH 7.4, and the protease inhibitor mixture) to a final sucrose concentration of 0.7-0.8 M. The crude vesicles were further purified by flotation in a discontinuous sucrose gradient (8 ml each from the top: 0.2 M sucrose/0.3 M NaCl, 0.45 M sucrose/0.175 M NaCl, 0.65 M sucrose/0.075 M NaCl, layered on the sample) at 100,000 × *g* for 16 h. Fractions (1.5 ml each) were collected from the top, and the contents of ATP and acetylcholine were measured by a chemiluminescence method (see below). Fractions No. 4-7 containing ATP and acetylcholine peaks were collected (Fig. 1), diluted twice with buffer A (0.3 M NaCl, 0.2 M sucrose, 0.3 mM EGTA, 10 mM Hepes-NaOH, and the protease inhibitor mixture, pH 7.4), and then centrifuged at 100,000 × *g* for 3 h. The pellet was resuspended in buffer A and used for the experiments within 9 h.

Measurement of the ATP Content—The ATP content of the synaptic vesicles was measured by a chemiluminescence method (*24*). A suspension of synaptic vesicles (*ca.* 0.1 mg protein/ml, 40 μl) was heat-treated at 100°C for 3 min to break the membranes. After cooling down to room temperature, 25 μl of the suspension was mixed with 465 μl of a buffer solution (0.15 M NaCl and 10 mM Tris-HCl, pH 7.4) and 10 μl of an ATP assay mixture (FL-AAM; Sigma Chemicals, St. Louis, MO, USA) in a test tube. Ten seconds later, the chemiluminescence was measured with a photometer (Lumicounter 1000; Niti-on, Funabashi).

Measurement of the Acetylcholine Content—The acetylcholine content of the synaptic vesicles was measured by a chemiluminescence method, *i.e.*, the modification by Schweitzer (*25*) of the procedure of Israël and Lesbats (*26*). A suspension of synaptic vesicles (*ca.* 0.1 mg protein/ml, 100 μl) was heat-treated at 100°C for 3 min to break the membranes. After the centrifugation at 17,800 × *g* for 10 min at 4°C, 40 μl of the supernatant was mixed with 110 μl of a buffer solution comprising 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM luminol, 10 μg of peroxidase, 1 unit of choline oxidase, and 5 units of acetylcholine esterase in the cuvette of a photometer to measure the chemiluminescence.

Measurement of the Protein and Phospholipid Concentrations—Protein concentrations were determined by dye-binding (*27*) using a protein assay kit from Bio-Rad (Hercules, CA, USA) and BSA as a standard. The phospholipid content was determined by extracting the phospholipids by the Bligh-Dyer method (*28*) and measuring the total phosphorus (*29*).

Measurement of the Particle Size Distribution—The size of the synaptic vesicles was determined by a dynamic laser light scattering method with a NICOMP (Silver Spring, MD, USA) Model 370. The sample volume was 0.6 ml for each of the synaptic vesicle suspensions (0.005 mg protein/

ml in 0.4 M NaCl, 0.3 mM EGTA, and 10 mM Tris-HCl, pH 7.4).

Measurement of Phospholipid Translocation Using Fluorescence-Labeled Probes—A chloroform solution of NBD-labeled phospholipids (0.60 nmol) was evaporated to dryness under Ar gas, and then the solid film formed was resuspended in 6 ml of buffer A with vigorous vortexing for 10 min and with 20 s sonication in ice-cooled water. The suspension of NBD-PLs was brought to 25°C and then mixed with 6 ml of a synaptic vesicle suspension (6.0 μmol phospholipid in buffer A), which had been incubated at 25°C for 10 min with 0.5 mM PMSF [an inhibitor of the hydrolysis of the probes (*30, 31*)]. After an appropriate incubation period at 25°C, a 2 ml aliquot was placed in the cuvette of a fluorescence spectrophotometer (model F-2000; Hitachi, Tokyo), which was equipped with a stirring device for the contents of the cuvette. One minute after the addition of 1 μl of 100 mM DIDS in distilled water, recording of the fluorescence intensity was started. Ten seconds later, 20 μl of 1 M dithionite in 100 mM Tris-HCl buffer (pH 10.0) was injected through a rubber septum into the cuvette. The fluorescence measurement was carried out with excitation at 470±10 nm and emission at 540±10 nm, and recorded for at least 5 min.

RESULTS

Purity of the Synaptic Vesicles—Measurement of the ATP and acetylcholine contents confirmed that the peak fraction containing ATP and acetylcholine, which corresponds to that of synaptic vesicles, resided at the interface of 0.2 and 0.45 M sucrose on flotation sucrose gradient centrifugation (Fig. 1). The yield of the synaptic vesicles was 0.4-0.5 mg protein from 100 g of the electric organ, and the ATP and acetylcholine contents were respectively about 120 and 320 nmol/mg protein, which are comparable to the values previously reported for synaptic vesicles isolated from *Torpedo marmorata* (*32*). An electron micrograph shows synaptic vesicles with a diameter of 70-100 nm (Fig. 2). The purity of the sample was estimated to be about 85% based on the area on the micrograph. The hydrodynamic size of the synaptic vesicles measured by dynamic laser light scattering was 127±39 nm.

Translocation of NBD-PL from the Outer to the Inner Leaflet of Synaptic Vesicles—The method involving fluorescent NBD-PL probes together with dithionite anions as a fluorescence-quenching agent can measure phospholipid translocation without the need of a separation procedure, thus providing good time-resolution. This method relies on the facts that dithionite reduces the NBD groups of NBD-PLs to make the probes non-fluorescent and that biomembranes are impermeable to dithionite anions in the presence of DIDS, an inhibitor of an anion transporter (*21*). Accordingly, when NBD-PLs are incorporated into membranes and treated with dithionite, only the probes residing in the outer leaflet of the membranes lose their fluorescence.

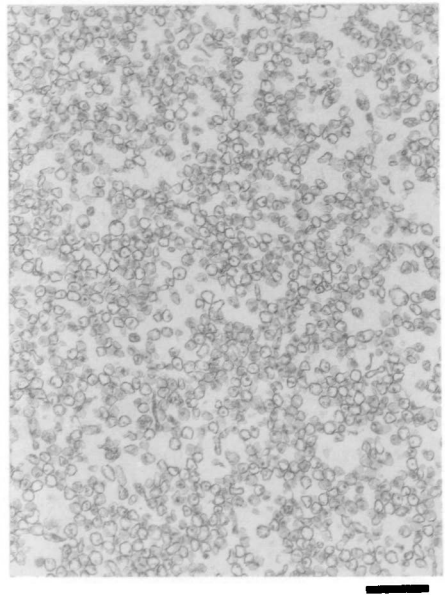

Fig. 2. **An electron micrograph of purified synaptic vesicles from the electric organ of *Narke japonica*.** Scale bar, 500 nm.

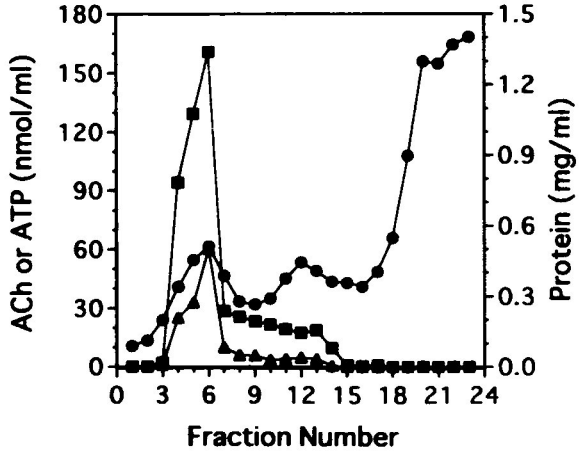

Fig. 1. **Purification of synaptic vesicles by flotation sucrose density gradient centrifugation.** Fractions of 1.5 ml were taken from the top of the centrifugation tube and then acetylcholine (ACh, ■), ATP (▲), and protein (●) were determined.

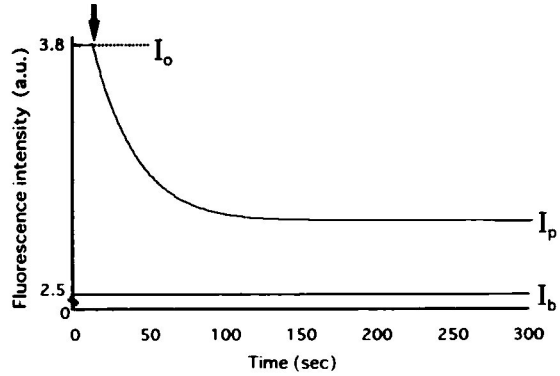

Fig. 3. **A trace of the decrease in the fluorescence intensity of NBD-PE after the addition of dithionite (arrow).** Synaptic vesicles were mixed with NBD-PE (0.01 mol% of the total membrane phospholipid) and then incubated at 25°C. At the time indicated by an arrow, dithionite (final, 10 mM) was added. I_p is the fluorescence intensity at the plateau level, I_o the fluorescence just before the addition of dithionite, and I_b the background signal intensity.

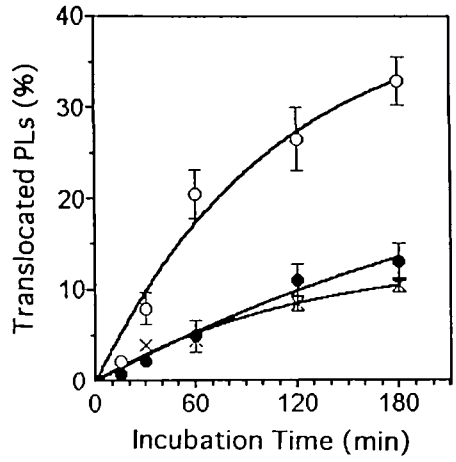

Fig. 4. **Time courses of outside-to-inside translocation of NBD-PLs in synaptic vesicles at 25°C.** The fluorescence intensity after the addition of dithionite was plotted as a function of the time of incubation of synaptic vesicles at 25°C. PE (○), PS (●), PC (×). Each point represents the mean±SD ($n=3$).

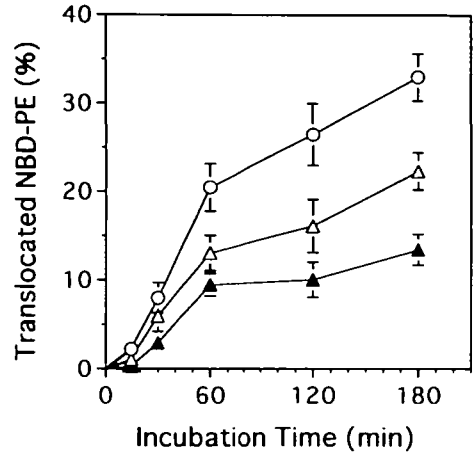

Fig. 5. **Effect of pretreatment of synaptic vesicles with IAA on the translocation of NBD-PE.** The time course of translocation of NBD-PE was plotted as a function of the time of incubation of synaptic vesicles at 25°C. Synaptic vesicles were treated with IAA for 30 min at 25°C before the incorporation of NBD-PE. ○, control (no IAA added); △, 10 mM IAA; ▲, 20 mM IAA. Each point represents the mean±SD ($n=3$).

Synaptic vesicles were mixed with one of the NBD-PLs (0.01 mol% of the total membrane phospholipid) and then incubated at 25°C. After an appropriate time, DIDS (final, 50 μM) was added and 70 s later dithionite (final, 10 mM) was added. A typical trace of the fluorescence intensity as a function of time is shown in Fig. 3. The fluorescence intensity decreases upon the addition of dithionite and reaches a plateau within 200 s. The background level is due to light scattering and obtained as the signal intensity with synaptic vesicles without an NBD-PL added. The fraction (%) of NBD-PLs in the inner leaflet of the bilayer can be obtained as $(I_P - I_b)/(I_o - I_b) \times 100$, where I_P is the fluorescence intensity at the plateau level, I_o the fluorescence just before the addition of dithionite, and I_b the background signal intensity. The value obtained with equation represents the fraction (%) of NBD-PLs translocated from the outer to the inner leaflet.

Figure 4 shows the time courses of the fractions (%) of NBD-PLs translocated to the inner leaflet of the bilayer. In 3 h, about 30% of NBD-PE was translocated to the inner leaflet, while only 13 and 9% of NBD-PS and NBD-PC, respectively, were translocated across the bilayer. Each curve can be fitted with an exponential function. Extrapolation of time to infinity gives equilibrium values for the fractions of the NBD-PLs in the inner leaflet of 41, 31, and 14% for PE, PS, and PC, respectively. The times to reach half the equilibrium values were estimated to be 76, 219, and 84 min for NBD-PE, -PS, and -PC, respectively.

Inhibitory Effect of Iodoacetamide—The effect of iodoacetamide (IAA), a reagent which modifies the function of a protein by reacting with SH groups of the protein, on the translocation of NBD-PLs was examined. As shown in Fig. 5, IAA inhibited the translocation of NBD-PE in a dose-dependent manner. The treatment of synaptic vesicles with 10 and 20 mM IAA reduced the translocation of NBD-PE at 3 h from 33 to 22.4 and 13.5%, respectively. The translocation of NBD-PS and NBD-PC was similarly inhibited by IAA. The inhibition by 20 mM IAA of the translocation at 3 h was 59, 55, and 30% for NBD-PE, NBD-PS, and NBD-PC, respectively. This indicates that the translocation of NBD-PLs is mediated by a protein molecule, such as a translocase.

Effect of MgATP in the Medium—The time course of translocation of NBD-PE was obtained by the addition of 3 mM MgATP to the suspending medium. However, the time course was little changed from that without the addition of MgATP (data not shown). Therefore, if the translocation depends on a kind of ATPase, ATP should be provided from the intravesicular lumen as synaptic vesicles contain plenty of ATP inside them.

DISCUSSION

In order to obtain good data through experiments involving subcellular membrane fractions, the purity of the sample is generally important. In the present study, we prepared highly purified synaptic vesicles. The preparation was homogeneous and satisfactory not only regarding the shape and size of the vesicles but also the internal transmitter.

Although the asymmetrical distribution of phospholipids between the outer and inner leaflets of synaptic vesicle membranes has been measured using a chemical modification method (*13*) or phospholipase C digestion (*14*), the measurement of translocation activity has not been performed. In the present study, the translocation of the major phospholipids, *i.e.*, PC, PE, and PS, in synaptic vesicles was investigated using NBD-PL probes coupled with fluorescence quenching by dithionite.

As shown in Fig. 3, data with a good signal-to-noise ratio were obtained despite the low concentration of the probe (0.01 mol% of the membrane lipid). The time resolution was good and interpretation of the obtained data was straightforward. Extrapolation of the time course of the phospholipid translocation (Fig. 4) gave equilibrium distribution ratios of the phospholipid in the outer and inner leaflets of 59:41 for PE, 69:31 for PS, and 86:14 for PC. These values are within the range (60:40-85:15 for PE and 40:60-100:0 for PS) reported by Deutsch and Kelly (*13*) based on the results of chemical modification experiments

involving synaptic vesicles isolated from *Narcine brasiliensis*. Our value for PE is also close to the value of 77:23 of Michaelson *et al.* (*14*) obtained on phospholipase C digestion of synaptic vesicle membranes from *Torpedo ocellata*, although the values for PC are somewhat different: 86:14 in our case and 58:42 in theirs. Although the reason for the difference is not clear, one possibility is the different electric ray species used.

The curve fitting in Fig. 4 gives times to reach half the equilibrium value of 76, 219, and 84 min for NBD-PE, -PS, and -PC, respectively, indicating that the rate of the translocation is in the order, PE>PC≫PS. Thus, the present study has shown that PE is translocated more preferentially than PS in synaptic vesicle membranes. This is different from the results reported for cell membranes previously. In erythrocyte plasma membranes, PS and PE are translocated with almost equal specificity (*6, 33*), leading to the assumption of the presence of an aminophospholipid translocase. In synaptosomal plasma membranes from *Narke japonica*, we found PS-specific translocation (*19*).

The present study has also shown that the translocation of phospholipids in synaptic vesicles is inhibited by treatment with IAA, indicating that the translocation may be mediated by a protein. It did not depend on MgATP added outside the synaptic vesicles, but was assumed to depend on ATP inside the vesicles. Since the measurement of phospholipid translocation in a synaptic vesicle preparation with intralumenal ATP depleted is not practical, it is difficult to confirm this assumption. Since the lumen of such vesicles is topologically equivalent to the extracelluar space, the direction of the phospholipid translocation (from the outer to the inner leaflet, *i.e.*, from the cytoplasmic to the lumenal face) in synaptic vesicle membranes appears different from that in cell membranes (from the outer to the inner leaflet, or from the extracellular to the cytoplasmic face). However, they are in fact the same seen from another point of view. Under the experimental conditions used, both are in the direction from the leaflet which is not in contact with ATP to the other leaflet in contact with ATP.

In conclusion, phospholipid translocation activity was first observed in the membranes of purified synaptic vesicles. The translocation was mediated by a protein, most likely a translocase dependent on ATP inside the vesicles. The extent and rate of the translocation were greatest for PE. The translocation of PS was very slow. Most PC and PS exist in the cytoplasmic face of a membrane bilayer at equilibrium, while the distribution of PE is almost equal between the two leaflets. It would be most interesting to further explore if and how the asymmetric distribution of phospholipids of synaptic vesicles as well as plasma membranes contributes to membrane fusion and to exocytosis of the synaptic transmitters, although there is no evidence at present that the asymmetric distribution of PS and PC or the relatively symmetric distribution of PE, and the translocation of these phospholipids in synaptic vesicle membranes are involved in membrane fusion. However, the present findings should provide a basis for further studies on the relationship of the asymmetric distribution and translocation of membrane phospholipids with the membrane fusion and synaptic transmission at nerve endings.

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