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Lipids of Synaptic Vesicles: Relevance to the Mechanism of Membrane Fusion†

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ABSTRACT: Synaptic vesicles from the electric organ of the marine ray *Narcine brasiliensis*, purified to at least 90% homogeneity, were analyzed for the lipid and fatty acid content of their membranes. The major lipids (mol %) were phosphatidylcholine (32.3%), phosphatidylethanolamine (20.5%), phosphatidylserine (6.1%), sphingomyelin (3.0%), and cholesterol (33.3%), a composition which did not differ greatly from that of the parent electric organ. While the number of double bonds per fatty acid molecule was similar for both synaptic vesicle and whole electric organ phospholipids, the vesicles were highly enriched in docosahexenoic acid (22:6). Reaction with the amine labeling reagents isethionylacetimidate and trinitrobenzenesulfonic acid indicated that 40% of the phosphatidylserine and 60% of the phosphatidylethanolamine are present on the external (cytoplasmic) surface of the synaptic vesicle. These data on a natural fusing membrane have relevance to models of membrane fusion, which have been based largely on studies of in vitro fusion using synthetic membranes.

In many types of secretory cells the secretory product is contained in membrane vesicles or granules and the release of the secretory product involves the fusion of the vesicle with the cell plasma membrane. The vesicle membrane constituents are at least transiently incorporated into the plasma membrane, and the contents are released into the extracellular space. In general this process of exocytosis is triggered by entry of calcium into the cell (Douglas, 1968; Llinas & Heuser, 1977; Holtzman, 1977). Exocytosis in vivo may involve proteins of the vesicle membrane and plasma membrane or the carbohydrate moieties of their glycoproteins and glycolipids. In vitro, however, model membrane studies [review, Papahadjopoulos et al. (1979)] have shown that membranes composed solely of purified lipids are capable of undergoing fusion. It has been

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argued that at least for release of neurotransmitter from nerve terminals, the final step of fusion need involve only calcium–phospholipid interactions (Kelly et al., 1979). Whether this is true or not, it is very likely that lipid interaction plays a crucial role in the fusion event. A first step then in understanding the molecular mechanism of membrane fusion is to identify the lipids of the secretory vesicle and the plasma membrane.

We have chosen to study the synaptic vesicle. Although preparations enriched in synaptic vesicles have been available for several years [review, Kelly et al. (1979)], only recently has their purity been established with sufficient precision (Carlson et al., 1978) to warrant biochemical analysis. These vesicles are derived from purely cholinergic nerve endings of the electric organs of marine rays. By isopycnic and velocity sedimentation and by electrophoresis of the purified vesicles, their contamination with nonvesicular lipid was estimated to be less than 10% (Wagner et al., 1978). Although a phospholipid composition has been published for marine ray synaptic vesicles (Nagy et al., 1976), the specific activity of the material analyzed (287 nmol/mg of protein) was more than 10-fold less than that of more recent preparations (Carlson et al., 1978). We therefore decided to repeat the phospholipid analysis on the more highly purified material. In addition, the fatty acid composition of each phospholipid was determined, and the amounts of phosphatidylethanolamine and serine in the cytoplasmic monolayer were estimated. The lipid composition of synaptic vesicles is similar in some respects to that of artificial membranes that have been shown to undergo Ca^{2+}-facilitated fusion in vitro.

Materials and Methods

Narcine brasiliensis were shipped live from Turtle Cove Laboratory, Port Aranses, TX. Phospholipid standards were purchased from Avanti Biochemicals, Birmingham, AL (egg phosphatidylcholine (PC),[1] and lysophosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylethanolamine transphosphatidylated from egg PC, bovine braine phosphatidylserine, and sphingomyelin) or from Serdary Research Laboratories, London, Ontario (porcine liver phosphatidylinositol, bovine heart cardiolipid, phosphatidic acid, and phosphatidylglycerol from transphosphatidylated egg PC). Lysophospholipids were also generated from the above diacyl compounds by treatment with snake venom phospholipase A_2 (Sigma) (Hubbell & McConnell, 1971). Salts and solvents were all reagent grade. Chloroform and methanol were distilled in glass; water was deionized and double distilled. Thin-layer chromatography plates were precoated with silica gel 60, layer thickness 0.25 mm (EM Laboratories, Elmsford, NY). The antioxidants used were BHT from Sigma Chemical Co., St. Louis, MO, and BHBE from ICN–K&K Laboratories. DNFB and TNBS were purchased from Sigma and IAI from Pierce Chemical Co., Rockford, IL. [^{3}H]TNBS (30 Ci/mol) was from Amersham, Amersham, U.K. The phosphate assay employed 70% perchloric acid, double distilled from Vycor (G. Frederick Smith, Columbus, OH), L-ascorbic acid (Sigma), and ammonium molybdate (J. T. Baker Chemical Co., Phillipsburg, NJ). Boron trichloride in methanol (12% w/v), (trimethylsilyl)imidazole, cholesterol, and 5α-cholestane were obtained from Supelco, Inc., Bellefonte, PA, and fatty acid methyl ester standards from Supelco and Applied Science Laboratories, Inc., State College, PA. Aquasol was from New England Nuclear, Boston, MA.

Synaptic Vesicle Preparation. Synaptic vesicles were prepared as described previously (Carlson et al., 1978) from perfused *Narcine brasiliensis* electric organ which had been frozen and stored in liquid nitrogen. Purification involved homogenization of the organ, differential centrifugation, flotation to equilibrium on a sucrose density gradient, and permeation chromatography on a Controlled Pore Glass (CPG) column of 3000-Å average pore size. The last step yielded two protein-containing fractions, an excluded peak ("Excluded Material") and the synaptic vesicle peak, which contains the acetylcholine and ATP. After CPG chromatography the highest specific activity fractions, measured as acetylcholine to protein ratio, were pooled. The pool was concentrated prior to lipid extraction either by pressure dialysis (Amicon X M-100A membrane) or by first diluting with sucrose-free buffer to reduce the density of the medium and then centrifuging 6 h or more at 30000 rpm in a Beckman 30 rotor. The pellet was resuspended to a concentration of 0.1–1 mg of protein/mL in a small volume of 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA, and 0.02% NaN_3, pH 7.4. All operations were carried out at 0–4 °C and all buffers were saturated with the antioxidant BHBE, about 5 mg/L.

Lipid Extraction. Lipids were extracted using three different methods. The first was that of Bligh & Dyer (1959) as modified by Rothman & Kennedy (1977) to acidify the medium and increase the recovery of acidic phospholipids. The second method was that of Folch et al. (1957). The third involved extracting a synaptic vesicle pellet with 1 mL of methanol and 2 mL of chloroform and filtering on glass wool. The glass wool was then washed with 3 mL of chloroform–methanol (1:1) followed by 3 mL of chloroform–methanol (1:2). All solvents contained 1 mg of BHT/L, and solvents were bubbled with argon when samples were destined for fatty acid analysis. Extracts were evaporated under a stream of nitrogen and were taken up in chloroform–methanol (2:1).

Thin-Layer Chromatography. The phospholipid compositions of the lipid extracts were analyzed by either one- or two-dimensional thin-layer chromatography (TLC). The former employed 5 × 10 or 5 × 20 cm EM silica gel 60 plates and one of the following solvents: (1) chloroform–methanol–water 65:65:4, (2) chloroform–methanol–acetic acid–water 65:50:5:3, (3) chloroform–methanol–7 N NH_4OH 55:35:5, (4) hexane–2-propanol–water 6:8:1. The two-dimensional system, optimized for separation of PC, PS, PE, and SM on the silica gel 60 plates, employed chloroform–methanol–7 N NH_4OH 55:35:5 (first dimension) and chloroform–methanol–acetic acid–water 65:50:5:3 (second dimension). It was crucial that all traces of NH_4OH be removed prior to chromatography in the second dimension. The chromatography chamber was lined with paper saturated with solvent, and the plates used were 10 × 10 cm. Migration distances were compared with those of authentic standards on the same or parallel plates.

Neutral lipids were analyzed by one-dimensional TLC in chloroform–acetone 9:1 on 10 × 10 cm plates. The presence of most other sterols or of cholesterol ester was ruled out by argentation chromatography or comparison of saponified and control samples. All plates were prerun in solvent followed by activation, and all chromatography solvents contained 50 mg of BHT/L.

[1] Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LPE, lysophosphatidylethanolamine; BHT, butylated hydroxytoluene; BHBE, 3,5-di-*tert*-butyl-4-hydroxybenzyl ether; DNFB, 2,4-dinitrofluorobenzene; DNP, dinitrophenyl; SV, synaptic vesicle; EM, excluded material; TNBS, 2,4,6-trinitrobenzenesulfonic acid; IAI, isethionylacetimidate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ATP, adenosine triphosphate; TLC, thin layer chromatography; CPG, controlled pore glass.

Quantitation of Lipid, Protein, and ATP. Lipid spots were detected in an iodine chamber or (for samples for fatty acid and cholesterol analysis) with 2′,7′-dichlorofluorescein (0.4% in methanol); it was found, however, that brief (10 s) exposure to iodine vapor gave better detection of spots with no loss of polyunsaturated fatty acids. For phosphorus analysis, plates were charred with 50% H_2SO_4 at 180 °C for 30 min. Cholesterol was quantitated either by the densitometric method of Fisher (1976) on charred plates or by gas chromatography. The cholesterol was extracted from silica gel and with an internal standard of 5α-cholestane was reacted with (trimethylsilyl)imidazole. The trimethylsilyl derivative was then analyzed by using a 3 ft SP-2250 column at 250 °C in a Hewlett-packard 5830A gas chromatograph (Koblin et al., 1979).

Lipid phosphorus was determined on scraped chromatogram spots by the method of Rouser et al. (1970). Molar ratios of cholesterol to phospholipid phosphorus were determined by quantitating both cholesterol and phospholipid phosphorus in the same sample after chromatography in chloroform–methanol–water 65:25:4. Protein was determined by Amido Schwarz staining (Schaffner & Weissmann, 1973) using bovine serum albumin as standard. ATP was assayed by a modification of the method of Stanley & Williams (1969) described by Carlson et al. (1978).

The phospholipid fatty acids were transesterified in the presence of the thin-layer chromatography adsorbent in 12% BCl_3 in methanol at 90 °C for 30 min, a modification of the method of Morrison & Smith (1964). The products were analyzed in a Hewlett-Packard 5830A gas chromatograph on a 6-ft glass column packed with 10% SP-2330 cyanosilicone over a 180–230 °C region with a temperature increase of 2 °C/min (Koblin et al., 1979).

While the two-dimensional TLC system described separated all authentic standards, the PS, lysoPE (LPE), and phosphatidylinositol (PI) all migrated in close proximity. Fatty acid chain length influences the chromatographic behavior of the parent lipid (Renkonen, 1967); because of the uncertainty in comparing R_f's of lipids of unknown fatty acid composition with standards from other sources, it was decided to effect a further separation of the lipids in the PS region. This was accomplished by reacting extracted lipids (≤0.4 μmol of phospholipid) with 2,4-dinitrofluorobenzene (DNFB) (12.5 μmol) in 0.55 mL of chloroform–methanol–water 69:37:4 with 1 μg of BHT/mL and an additional 10 μL of 5% $NaHCO_3$ adjusted to pH 9.4 with NaOH. The reaction was carried out in a sealed vessel under argon at 28 °C for 3–4 h. The reaction medium was then partitioned against 0.4 mL of 0.1 M KCl–0.1 N HCl and the upper phase washed with synthetic Bligh-Dyer lower phase. The combined lower phases were evaporated under a stream of N_2 and redissolved in a small amount of chloroform–methanol (2:1). The reaction products were separated by two-dimensional TLC. The separation of the nonreacting lipids, PC, SM, and PI, and the reaction products dinitrophenyl-PS (DNP-PS), DNP-LPE, and DNP-PE was greatly improved. Alternatively, the lipid extract was first separated by two-dimensional TLC and the entire PS region then extracted from the silica gel with chloroform–methanol–acetic acid–water 65:50:5:3 followed by methanol. This extract was then reacted with DNFB and separated as above.

Reaction of Intact and Disrupted Synaptic Vesicles with Trinitrobenzenesulfonic Acid and Isethionylacetimidate. The vesicles (final concentration approximately 0.15 mM lipid phosphorus were diluted in 3 mL of reagent plus buffer. The reactions were carried out at 4 °C with either [^{3}H]TNBS (1 Ci/mol, 3 mM final concentration) in 0.4 M NaCl, 10 mM EGTA, 20 mM Hepes, pH 7.6, or with IAI (40 mM final concentration) in 0.35 M NaCl, 10 mM EGTA, 50 mM sodium pyrophosphate, pH 8.05. A 20-fold concentrated solution of IAI was made and adjusted to pH 8.05 with NaOH immediately prior to dilution to initiate the reaction. The pH of the reaction was maintained at 8.05–8.15 with 1 N HCl. In each case, half of the reaction mixture was sonicated to disrupt synaptic vesicles. This was accomplished by applying the flat end of a 1.25-cm diameter probe of a Heat Systems-Ultrasonics Sonifier (Plainview, NY) to the side of an Eppendorf 1.5-mL polypropylene micro test tube in ice water and delivering 10-s 10-W pulses, separated by 15-s intervals for cooling, until all of the intravesicular ATP had been liberated (3 min total). At various times during the reactions 250-μL aliquots were removed and precipitated with trichloroacetic acid, and the phospholipid was subsequently extracted (Rothman & Kennedy, 1977). The amidinated samples were reacted in organic solvent with [^{3}H]TNBS (Rothman & Kennedy, 1977) to determine the amount of unreacted amino-containing lipid; the trinitrophenyl derivatives were then separated by thin-layer chromatography on silica gel 60 by using chloroform–methanol–water 65:25:4, and the radioactivity of the scraped spots was determined by liquid scintillation counting in Aquasol.

Table I: Lipid to Protein Ratios (Weight/Weight)[a]

	phospholipid[b]/ protein	(phospholipid + cholesterol)/protein
synaptic vesicles[c]	5.32 ± 0.53 (5)	6.69 ± 0.54
excluded material	0.98 ± 0.23 (2)	1.37 ± 0.32
electric organ[d]	0.30 ± 0.08[d] (2)	0.36 ± 0.09[d]

[a] Values are mean ± standard deviation followed in parentheses by the number of preparations used for the determination. [b] Assume 750 g/mol phospholipid. [c] Wagner et al. (1978). [d] Based on the value of 10 mg of protein/g of tissue (Deutsch & Raftery, 1979).

Results

To allow us to interpret phospholipid and fatty acid compositions of the synaptic vesicles, we have simultaneously determined the phospholipid and fatty acid compositions of whole electric organ. We have also examined a membrane fraction which copurifies with the vesicles during differential centrifugation, has the same buoyant density on isoosmotic sucrose gradients, and is only separated from the vesicles at the last step of the purification, permeation chromatography on a glass bead column (Carlson et al., 1978). These membrane fragments, the "excluded material", are excluded from the column presumably because they have larger dimensions than vesicles. We have studied this membrane fraction because, although it has been subjected to the same purification steps as synaptic vesicles, it is biochemically different since it does not contain synaptic vesicle-specific antigens (Carlson & Kelly, 1980) and has a different protein composition (Wagner & Kelly, 1979).

The phospholipid/protein ratio of synaptic vesicle is 5.3:1 by weight (Carlson et al., 1978). Thus synaptic vesicle membranes are richer in lipid than is myelin (Ansell et al., 1973). The corresponding ratio for homogenates of the whole electric organ (which of course include cytoplasmic protein) is 0.30, while that for "excluded material" is 0.98 (Table I). The reason that the excluded material and the vesicles have the same buoyant density in sucrose gradients despite their differing protein to lipid ratio is probably the difference in size (Wagner et al., 1978).

Table II: Phospholipid Compositions of Synaptic Vesicles (SV) Excluded Material (EM) and Electric Organ[a]

	SV	EM	organ
phosphatidylcholine	48.2 ± 2.4 (6,11)	22.3 ± 3.1 (3,3)	38.5 ± 3.5 (2,4)
phosphatidylethanolamine	30.6 ± 2.6 (6,16)	30.1 ± 1.8 (3,3)	24.5 ± 5.9 (2,4)
sphingomyelin	4.5 ± 1.3 (6,11)	21.9 ± 1.5 (3,3)	12.7 ± 1.2 (2,4)
phosphatidylserine	9.2 ± 1.1 (1,4)	16.8[c] (1,1)	ND[d]
phosphatidylinositol[b]	2.6 ± 0.4 (1,4)	2.6[c] (1,1)	ND
lysophosphatidylethanolamine	3.0 ± 1.3 (1,4)	6.9[c] (1,1)	ND
total PS + PI + LPE[e]	15.9 ± 1.52 (6,11)	23.4 ± 2.6 (3,3)	21.9 ± 7.9 (2,4)
other	0.42 ± 0.59 (6,11)	2.3 ± 2.5 (3,3)	2.4 ± 1.4 (2,4)

[a] Values are mean ± standard deviation of mole percent. Numbers in parentheses denote the number of preparations assayed and total number of determinations respectively. [b] By inference only. Could be unreacted PS or LPE. [c] Numbers are for a single preparation, and in this case do not add up to the "total PS + PI + LPE", which is the average of three preparations. [d] ND, not determined. [e] See abbreviations.

Table III: Cholesterol to Phospholipid Ratios[a]

	mol/mol	w/w[b]
synaptic vesicle	0.50 ± 0.043 (4,8)	0.26 ± 0.022
excluded material	0.77 ± 0.043 (2,4)	0.40 ± 0.022
electric organ	0.38 ± 0.034 (1,4)	0.20 ± 0.017

[a] Data presented as in Table II. [b] Calculated from molar ratio by assuming an average molecular weight of 750 for phospholipid.

The major phospholipids of synaptic vesicles are PC, PE, SM, and a component which cochromatographs with bovine brain PS and stains with ninhydrin. Reaction of the lipid extract with DNFB forms dinitrophenyl (DNP) derivatives of the primary amine containing lipids. After derivatization, only part of the material in the PS region migrates as DNP-PS. Two additional minor components are revealed, one which cochromatographs with DNP-LPE and the other which is not reactive with DNFB since it remains in the original position. This material is presumably PI, although it may include some unreacted PS and LPE. The small amount of LPE may be the breakdown product of ethanolamine plasmalogen as a result of exposure to the acidic medium in the course of extraction (Rothman & Kennedy, 1977). It is also possible that the LPE is the result of hydrolysis in the course of purification of PE by phospholipase A. The fact that no other lyso compounds are found makes this less likely unless the phospholipase is specific for PE.

The phospholipids of the synaptic vesicles can be quantitated as described under Methods. The mole percentages of the various phospholipid classes of synaptic vesicles and, for comparison, excluded material and intact electric organ are listed in Table II. Because the PI spot might contain unreacted PS and LPE, the PI values are maximum estimates. The ratios of PC to SM are quite different for the synaptic vesicles and the excluded material, strongly suggesting that the vesicles are not heavily contaminated with membranes similar to the excluded material.

The molar ratios of cholesterol to phospholipid phosphorus are summarized in Table III. The synaptic vesicle ratio, 0.50, is quite high for an intracellular membrane, slightly higher than that of intact organ, 0.38. The ratio for synaptic vesicles, is, however, lower than that of excluded material membranes (0.77). Again the dissimilarity between two membranes of similar buoyant density is striking.

Table IV: Fatty Acid Compositions of Total Lipid Extracts (mol %)

fatty acid	synaptic vesicles $N = 7$[a]	excluded material $N = 2$	electric organ $N = 2$
16:0	31.2 ± 1.5[b]	23.3 ± 5.1	23.9 ± 1.3
16:1	5.56 ± 0.34	6.55 ± 0.07	6.75 ± 0.07
17:0	0.86 ± 0.53	1.15 ± 0.92	1.55 ± 0.21
18:0	12.7 ± 1.2	17.4 ± 2.1	12.7 ± 0.9
18:1	10.8 ± 1.05	16.8 ± 2.6	13.9 ± 0.35
18:2	0.3 ± 0.4	0.1 ± 0.1	0.35 ± 0.07
20:1	0.1 ± 0.1	0.1 ± 0.1	0.45 ± 0.07
20:3 ($n-6$)	0.1 ± 0.1	0.3 ± 0.4	0.65 ± 0.2
20:4	3.54 ± 0.6	10.4 ± 3.8	6.5 ± 0.7
20:5	0.59 ± 0.45	1.6 ± 1.9	2.55 ± 0.9
24:1/22:4	1.74 ± 0.24	8.35 ± 0.49	5.65 ± 0.78
22:5 ($n-6$)	0.49 ± 0.34	1.8 ± 2.5	2.25 ± 0.35
22:5 ($n-3$)	3.1 ± 0.81	3.25 ± 0.35	7.5 ± 0.14
22:6	29.1 ± 1.6	8.95 ± 0.49	15.4 ± 0.49

[a] N = number of different preparations examined. [b] Mean ± standard deviation.

The total fatty acid compositions determined on synaptic vesicle, excluded material, and whole electric organ are summarized in Table IV. The most striking difference in composition between the synaptic vesicles and the other preparations is in the amount of the polyunsaturated acid, docosahexenoic acid (22:6). While vesicles have 29 mol % of their fatty acids as docosahexenoic acid, excluded material and the whole organ have only 9 and 15 mol %, respectively. However, the average number of double bonds per fatty acid molecule is approximately the same in each case (2.3, 1.9, and 2.3, respectively; Table VI).

The fatty acids of the major individual phospholipid classes of synaptic vesicles and electric organ are listed in Table V. Note that the PS + LPE + PI in synaptic vesicles includes three components: PS 62.1 mol %, LPE 20.3%, and PI 17.6% (Table II). However, because 1 mol of LPE has only 1 mol of fatty acid, the contributions to the fatty acid composition are 69.1, 11.3, and 19.6 mol %, respectively. Thus PS contributes the majority of fatty acid to the total PS + LPE + PI pool. Synaptic vesicle PC, PE, and PS are all enriched in 22:6 compared to electric organ and excluded material, but when the average degree of unsaturation is considered (Table VI), the differences are less pronounced. Synaptic vesicle PE and PS contain significantly higher proportions of 22:6 (45% and 33%, respectively) as well as of total polyunsaturates (56% and 46%) than does synaptic vesicle PC (17% 22:6, 26% polyunsaturates). This relative unsaturation of PE and PS compared with PC has been found in a number of other membranes (White, 1973). The composition of LPE and SM is difficult to determine because they are present in small amounts and have only one fatty acid chain per molecule. Synaptic vesicle SM appears to contain primarily palmitic (16:0) and stearic (18:0) acids. The major fatty acids of LPE are 18:1, 16:0, 18:0, and 22:6.

Presumably lipids on the cytoplasmic face of the synaptic vesicle are those most immediately involved in fusing with the plasma membrane. We have attempted to determine the presence of amino lipids in the cytoplasmic layer by using membrane-impermeable amine-labeling reagents. IAI (Figure 1B) reacts with 50% of the PE within 40 min and with an additional 12% in the next 145 min. It reacts with about 40%

Table V: Fatty Acid Composition of Phospholipid Classes (mol %)

fatty acid	PC[a]			PE			PS			PS + LPE + PI		LPE		
	SV (5)[b]	EM (1)	organ (2)	SV (5)	EM (1)	organ (2)	SV (1)	EM (1)	organ (1)	SV (3)	organ (2)	SV (1)	EM (1)	organ (1)
16:0	45.7 ± 2.6[c]	46.2	52.8 ± 1.8	20.1 ± 1.7	10.9	11.8 ± 1.1	4.4	6.6	6.3	4.8 ± 0.3	8.2 ± 2.1	21.6	7.8	1.8
16:1	10.1 ± 0.4	8.6	11.4 ± 0.5	2.5 ± 1.5	10.0	3.6 ± 0.6	1.1	3.0	5.2	1.8 ± 0.15	4.15 ± 1.3	3.3	12.8	9.3
17:0	0.92 ± 0.2	1.0	1.0 ± 0.3	0.9 ± 0.8	1.6	1.3 ± 0.1	0.9	2.2	1.7	1.4 ± 0.1	2.2 ± 0.8		0.5	0.5
18:0	3.28 ± 0.21	2.7	2.4 ± 0	14.2 ± 1.0	11.2	14.3 ± 1.6	34.8	35.4	27.1	33.7 ± 3.1	29.0 ± 5.4	20.1	8.1	1.2
18:1	14.4 ± 0.8	14.6	19.4 ± 1.6	6.3 ± 1.3	16.7	11.4 ± 2.5	12.8	25.1	14.7	8.2 ± 2.7	12.7 ± 1.0	32.9	25.5	20.8
18:2	0.3 ± 0.2	0.1	0.2 ± 0	0	0.4	0.05 ± 0.05	0	0	1.2	0	0.35 ± 0.35		1.8	
20:1	0.23 ± 0.05	0.5	0.4 ± 0.1	0.1 ± 0.07	0.6	5.9 ± 6.6	0.2	1.4	0.5	0.4 ± 0.2	0.5 ± 0.1			0.2
20:3 ($n-6$)	0.23 ± 0.05	0.2	0.6 ± 0.6	0.04 ± 0.05	0.1	0.15 ± 0.07	0	0.6	0.8	0.17 ± 0.06	0.7 ± 0			0.1
20:4	1.85 ± 1.1	2.85	1.6 ± 0.1	4.1 ± 0.4	14.4	11.1 ± 0.1	2.7	5.8	7.8	4.3 ± 1.5	8.15 ± 2.1	7.0	17.4	13.1
20:5	1.0 ± 0.2	0.97	0.7 ± 0	0.6 ± 0.2	3.2	2.2 ± 0.1	0	0.4	1.2	2.5 ± 2.3	1.4 ± 0		0.9	1.9
22:4	1.15 ± 0.1	1.54	1.05 ± 0.1	1.5 ± 0.3	7.9	7.2 ± 0.28	5.7	8.2	6.9	3.0 ± 0.6	6.9 ± 0		10.8	9.1
22:5 ($n-6$)	0.63 ± 0.1	0.29	0.3 ± 0	0.5 ± 0.3	2.2	1.4 ± 0.14	0.8	1.3	2.0	0.77 ± 0.1	2.2 ± 0.1		1.1	2.1
22:5 ($n-3$)	3.2 ± 0.6	2.05	2.4 ± 0.2	3.3 ± 0.6	7.2	10.0 ± 0.1	4.0	4.8	9.5	4.33 ± 0.95	9.2 ± 1.4		5.5	12.3
22:6	17.1 ± 1.9	18.6	6.35 ± 0.1	45.4 ± 1.6	13.4	25.2 ± 1.0	32.7	4.6	15.1	37.0 ± 6.19	14.2 ± 2.6	15.0	7.5	27.4

[a] See abbreviations. [b] Numbers in parentheses indicate number of different preparations examined. [c] Mean ± standard deviation.

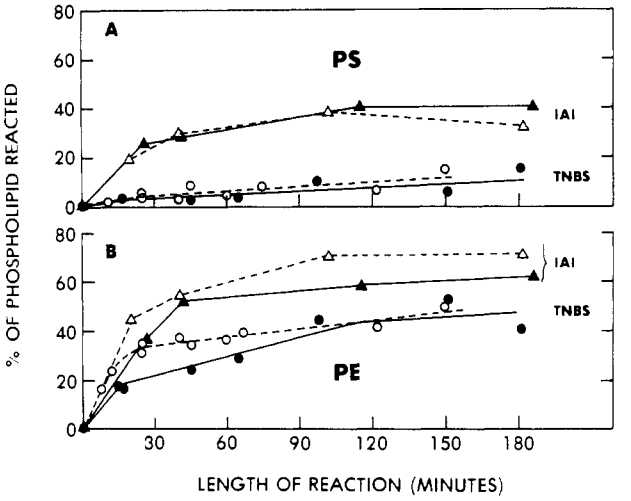

FIGURE 1: The reaction of synaptic vesicle (A) phosphatidylserine and (B) phosphatidylethanolamine with isethionylacetimidate (IAI) and trinitrobenzenesulfonic acid (TNBS). (▲—▲) IAI + intact vesicles; (△---△) IAI + sonicated vesicles; (●—●) TNBS + intact vesicles; (○---○) TNBS + sonicated vesicles. The TNBS data are combined from two separate experiments on two separate preparations carried out under identical conditions.

of the PS of intact vesicles in 145 min (Figure 1A). These values would be underestimates if the reactions do not go to completion, for example, because of the bulkiness of the *N*-acetimidoyl group introduced by the IAI or because of hydrolysis of the reagent, which is significant in the time course of the experiment. The reagent TNBS which introduces the much bulkier trinitrophenyl derivative shows even less reactivity, labeling eventually only 40–45% of the PE and 10–15% of the PS (Figure 1). The reaction of TNBS with membrane PE and especially PS has often been found to be incomplete [review: Op den Kamp (1979)], even in pure lipid monolayers (Bishop et al., 1979).

Limited labeling with IAI would also be expected if significant amounts of PE and PS are on the noncytoplasmic lumenal monolayer of the vesicle membrane. To expose the lumenal monolayer the vesicles can be lysed by sonication such that all of their intracellular contents are released as measured by access of ATP to luciferase. Lysis increases the extent of labeling of PE by 8%, from 62% to 70%, and increases the rate of labeling 1.5-fold. The 30% which remains inaccessible to the IAI could be either sterically protected or concealed within a small population of resealed vesicles. The initial rate of labeling of PE with TNBS is also faster with lysed vesicles than with intact ones, but the final extents are similar, perhaps because of slow TNBS leakage into intact vesicles.

Lysis of vesicles, however, has little or no effect on either the extent or rate of IAI labeling of PS. Thus under conditions where more PE becomes accessible to reagent, no increase in PS labeling is found. This result could be explained if a large fraction of the PS is in the cytoplasmic monolayer. However, since the maximum extent of labeling is only 40%, other interpretations are possible, including the low reactivity of the internal PS to IAI. Nontheless, these experiments suggest that at least 60% of the PE and 40% of the PS are in the cytoplasmic monolayer. The upper limit on the amount of PE and PS in the cytoplasmic monolayer would be 85% and 100%, respectively, if sonication were assumed to expose all the amino lipids capable of reacting with IAI and the reactivity were equal for external and internal monolayer phospholipids.

Precise estimates of membrane asymmetry are difficult to make with impermeant organic reagents. One possible problem is lysis of vesicles during the labeling procedure. This

Table VI: Degree of Unsaturation of Fatty Acids

	total			PC			PE			PS		
	SV	EM	organ	SV	EM	organ	SV	EM	organ	SV	EM	organ
average[a] degree of unsaturation	2.34	1.87	2.26	1.65	1.70	0.99	3.26	2.61	3.14	2.23	1.47	2.38
% saturated	44.8	41.9	38.1	49.9	49.9	56.2	35.2	23.7	25.9	40.1	44.5	35.1
% monounsaturated	16.5	23.5	21.1	24.7	23.7	31.2	8.9	27.3	19.8	14.1	29.7	20.4
% polyunsaturated	39.0	34.7	40.8	25.5	26.4	13.2	55.6	48.8	54.3	45.9	25.9	44.5
minimum % phospholipid with two unsaturated chains	10.4	16.2	23.8	0.1	0.2		29.6	52.6	48.2	17.8	11.0	29.8

[a] $\Sigma_{j=1}^{6} A_j(j)$, where j = the number of double bonds in a given fatty acid and A_j = mole fraction of fatty acids with j double bonds.

is unlikely, since in three different preparations the intravesicular ATP, measured as the difference between total and unbound ATP (Carlson et al., 1978), decreases by only 4–8%. The presence of significant quantities of lysed vesicles in the preparation prior to labeling is unlikely to be a significant problem since studies on vesicle membrane proteins (Wagner & Kelly, 1979) have revealed a population concealed from impermeable protein reagents until the vesicles are lysed. This study also showed that the vesicles do not reseal in the original orientation after lysis. Another possible problem is reagent penetration into the vesicle, although the reagents used have been found to permeate membranes slowly if at all at 4 °C, the temperature used in this study (Op den Kamp, 1979). Due to the absence of a reactible internal marker, permeation could not be directly determined. Finally, we have no measure of phospholipid flip-flop rates either during the experiment or during the preparation of the vesicle. These reservations must be borne in mind in interpreting our conclusion that a significant fraction of the amino lipids are exposed on the cytoplasmic surface of the vesicle.

Discussion

In the present study purified synaptic vesicles from *Narcine brasiliensis* are analyzed for their lipid and fatty acid content. For biochemical characterization, this preparation of secretory vesicles offers two advantages over most other such preparations. They have been extensively purified, as demonstrated by rigorous biophysicla criteria of purity; they appear to be at least 90% homogeneous (Carlson et al., 1978). A second advantage is that the phenomenon of neurotransmitter release has been characterized in detail both electrophysiologically and morphologically (Llinas & Heuser, 1977; Kelly et al., 1979). In particular, the rapidity of the exocytotic event implies that extensive enzyme-mediated catalysis does not occur during the fusion of vesicle and plasma membrane. Lipid–lipid interactions remain feasible. Before speculating on what sort of lipid–lipid interactions might occur, it was necessary to know the lipid composition of both synaptic vesicle and plasma membrane.

Synaptic vesicles appear to contain no unusual phospholipids. The major phospholipids of electric organ synaptic vesicles, separated by two-dimensional thin-layer chromatography, are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM). When the PS is further fractionated by dinitrophenylating amino groups, two additional minor components are revealed: lyso-PE (LPE) and a component presumed by its lack of reaction to be phosphatidylinositol (PI) (Table II).

PI represents no more than 2.6 mol % of the phospholipids. Hawthorne & Pickard (1979) have proposed that in the course of neurotransmitter release PI is enzymatically degraded to diglyceride, a fusogen. These data suggest that such a diglyceride would be present in amounts too small to bring about fusion (Cullis & Hope, 1978). Similarly, the amount of lysophospholipid appears to be too low to be effective as a fusogen. Baker et al. (1975) found 1% lysophosphatidylcholine in their less pure preparation of synaptic vesicles from either Torpedo electric organ or guinea pig brain. Since there is no time for extensive phospholipid hydrolysis during exocytosis (Kelly et al., 1979) and we can find little naturally occurring lysophospholipid, it is unlikely that such fusogens are involved in exocytosis.

A number of studies of Ca^{2+}-induced fusion in model systems have revealed a requirement for negatively charged lipid; fusion in vitro has been achieved with artificial membranes containing several varieties [review: Papahadjopoulos et al. (1979)]. Synaptic vesicle membranes do contain the negatively charged phospholipids PS and PI, but they are not enriched compared to the whole electric organ (Table II). In fact, their total concentration (11.8%) is rather low compared to the 50% PS required for Ca^{2+}-induced fusion to occur in mixed PS–PC liposomes (Papahadjopoulos et al., 1979). However recent studies indicate that liposomes composed of PS and PE, rather than PC, fuse at much lower concentrations of PS (Düzgüneş et al., 1980). Apposition of patches of negatively charged lipid in the presence of calcium (Portis et al., 1979) is thus a feasible intermediate in the fusion event if significant amounts of PS and PE are present in the cytoplasmic monolayers of fusing membranes.

We have used membrane-impermeable reagents to detect the presence of PE and PS in the cytoplasmic monolayer of the vesicle membrane. The data suggest that 60–85% of the PE and 40–100% of the PS are on the cytoplasmic surface and so could be involved in fusion events. The numbers must be interpreted cautiously, however, because of uncertainties introduced by flip-flop, permeation, resealing, and unreactive phospholipids. More quantitative measures of membrane asymmetry could be made by using phospholipases and phospholipid-exchange proteins in conjunction with chemical labeling (Rothman & Lenard, 1977; Op den Kamp, 1979).

A second lipid rearrangement postulated to occur in fusion of artificial membranes is a Ca^{2+}-induced fluid to solid-phase transition (Papahadjopoulos et al., 1979). Such a mechanism is feasible in natural membranes only if they are fluid at ambient temperatures. The lipids of synaptic vesicles are highly unsaturated (Table IV) and thus presumably fluid at ambient temperature. The lipids of fish are generally highly unsaturated (White, 1973). This can be seen by comparing the vesicle phospholipids with those of excluded material and total electric organ which, except in the case of PC, have similar degrees of unsaturation (Table VI). Significant proportions of PE and PS have two unsaturated chains per molecule (at least 30% and 18%, respectively; Table VI). If the majority of the PS and PE were in the cytoplasmic mon-

olayer, another implication would be that this monolayer would be more unsaturated than the lumenal monolayer.

Although the amino phospholipid composition of vesicle membranes and their fluidity are consistent with a Ca^{2+}-induced phase transition mechanism of fusion, there are two difficulties in applying this model of fusion to synaptic vesicles. The first is that the vesicles contain high concentrations of the 22:6 fatty acid. A similarly high concentration has been found in rod outer segments (Sklar et al., 1979; Giusto & Bazan, 1979). In this case Ca^{2+}-induced lateral phase separation of outer segment PS was not observed (Sklar et al., 1979). It was proposed that 22:6 in high concentrations prevents the formation of solid phases and indeed may be present for exactly such a purpose in cells whose cytoplasmic Ca^{2+} varies. Secondly, the concentration of cholesterol (33 mol % of the total lipid; Table III) is sufficient to abolish phase transitions in artificial lipid bilayers (Hinz & Sturtevant, 1972). A simple model of a phase boundary as a nucleation site for fusion is difficult to reconcile with these data.

An alternative for membrane fusion recently suggested by Cullis, deKruijff, and co-workers is that transitory nonbilayer configurations of membrane lipid might be involved (Cullis & de Kruijff, 1979). The suggestion stems from findings that unsaturated PE readily forms the hexagonal phase and that this process is facilitated by Ca^{2+} in mixtures of PS and PE and by cholesterol in mixtures of PE and PC. In addition, freeze–fracture studies indicate, at least in the case of mixed cardiolipin–PC systems, that such hexagonal-phase regions might occur at regions of Ca^{2+}-induced fusion [review: Cullis & deKruijff (1979)]. Since synaptic vesicles contain both cholesterol and highly unsaturated PE, as well as PS, a fusion step involving a hexagonal phase is more attractive than one involving a solid–fluid phase boundary.

The above studies have looked at the bulk phospholipid composition of vesicles. During fusion only a small fraction of the synaptic vesicle membrane is in contact with the plasma membrane. It is possible that the concentration of fusogens, cholesterol, or phospholipids in the small regions of apposition might be quite different from the bulk composition. The present preparation of synaptic vesicles exhibits neither vesicle–vesicle aggregation nor fusion in the presence of Ca^{2+} as measured by either light scattering or sedimentation velocity (J. Deutsch and R. Fraley, unpublished results). Under similar conditions, vesicles of appropriate pure phospholipids both aggregate and fuse. Fusion of synaptic vesicles with each other is perhaps not to be expected. Although vesicle–vesicle aggregation and fusion (compound exocytosis) are frequently seen in electron micrographs of numerous secretory cells and in vitro (Gratzl & Dahl, 1978), such vesicle interactions are not seen in electron micrographs of nerve terminals. Haynes et al. (1979) have observed intervesicular aggregation in an electric organ synaptic vesicle preparation which is less pure in terms of acetylcholine to protein ratio. Aside from possible differences in experimental procedures, this disagreement with our results might be accounted for by the loss during purification by our procedure of some protein component which may or may not be biologically relevant and which facilitates aggregation in the presence of Ca^{2+}; such a role has been proposed for synexin (Creutz et al., 1978). Physiologically relevant fusion may be accessible to study only when both pure synaptic vesicles and pure presynaptic plasma membrane are available.

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Role of the β-Phosphate–γ-Phosphate Interchange Reaction of Adenosine Triphosphate in Amino Acid Discrimination by Valyl- and Methionyl-tRNA Synthetases from *Escherichia coli*†

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ABSTRACT: Aminoacyl-tRNA synthetases catalyze a novel amino acid dependent interchange reaction in which the β- and γ-phosphates of ATP are interchanged independent of the ATP–PP_i exchange reaction. This reaction is designated the direct interchange reaction. To assess the role of the direct interchange reaction in preventing misaminoacylation of tRNA, the reactions catalyzed by Val- and Met-tRNA synthetases from *Escherichia coli* were investigated with cognate and noncognate amino acids as substrates, and the relative contributions of the ATP–PP_i exchange reaction and the direct interchange reaction to the overall rate of interchange were evaluated. The kinetic parameters (with respect to amino acid) of the total interchange reaction were compared to those of the exchange reaction with cognate and noncognate amino acids. The latter included Thr, Ile, or α-aminobutyric acid for Val-tRNA synthetase and DL-homocysteine for Met-tRNA synthetase. In the interchange reaction, V_{max} values with noncognate amino acids were 4–6-fold higher than those with the cognate amino acid. Furthermore, the ratio V_{max}(interchange)/V_{max}(exchange) was 8–30-fold greater for the noncognate than the cognate amino acids. The addition of 10 μM to 1 mM PP_i increased the rate of total interchange with cognate amino acids 3–18-fold. In contrast, with noncognate amino acids, the total interchange rates increased slightly (less than 20%) at 10 μM PP_i and in fact decreased at higher PP_i concentrations. Thus, interchange with cognate amino acids is predominantly exchange mediated. In contrast, with noncognate amino acids, the exchange reaction does not play a significant role in interchange, and interchange results predominantly from the direct interchange reaction. The striking difference in the behavior of cognate and noncognate amino acids even under more physiological conditions (in the presence of appropriate amounts of tRNA, inorganic pyrophosphatase, and P_i) led to the conclusion that the direct interchange reaction provides a contributory mechanism for preventing the aminoacylation of tRNA by noncognate amino acids.

Most aminoacyl-tRNA synthetases carry out an amino acid dependent ATP–PP_i exchange as well as the aminoacylation of tRNA (Kisselev & Favorova, 1974). As shown in a previous report (Rossomando et al., 1979), Val- and Met-tRNA synthetases of *E. coli* can carry out an amino acid dependent reaction designated the direct interchange reaction with ATPβS[1] as substrate in which the β- and γ-P of ATPβS are interchanged without dissociation of SPP_i from the enzyme. This reaction was shown to be distinct from an ATPβS–SPP_i exchange reaction. As depicted in Scheme I in the form of minimal sequences, the total observed interchange of β-P and γ-P of ATP may be formulated as the sum of contributions from the direct interchange reaction (reaction 1) and the exchange-mediated reaction (reaction 2a). The exchange-mediated interchange reaction is identical with the ATP–PP_i exchange reaction in all respects except that the concentration of PP_i never exceeds the enzyme concentration and only half of the exchanges can be detected as net interchange (see reactions 2a and 2b). By use of ATPβS, it was possible to reduce greatly the contribution, if any, of the exchange-mediated reaction to the total interchange since the exchange rate is so drastically reduced with the thio analogue as substrate that only the direct interchange mechanism is responsible for the observed interchange reaction. The very slow exchange rate and the opposite stereospecificities of the (*R*)- and (*S*)-ATPβS diastereomers in the interchange and aminoacylation reactions clearly demonstrated that an interchange reaction distinct from the exchange-mediated reaction does exist with this substrate.

The present study was undertaken to determine the contributions of the exchange-mediated and direct interchange reactions to the observed interchange with ATP, the normal

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[1] Abbreviations used: AA-AMP, aminoacyl adenylate; Abu, α-aminobutyric acid; ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); BSA, bovine serum albumin; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Hcy, homocysteine; PEI–cellulose, poly(ethylenimine)–cellulose.

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