Asymmetry of lipid organization in cholinergic synaptic vesicle membranes

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The lipid composition of purified *Torpedo* cholinergic synaptic vesicles was determined and their distribution between the inner and outer leaflets of the vesicular membrane was investigated. The vesicles contain cholesterol and phospholipids at a molar ratio of 0.63. The vesicular phospholipids are (mol% of total phospholipids): phosphatidylcholine (40.9); phosphatidylethanolamine (24.6); plasmalogen ethanolamine (11.5); sphingomyelin (12); phosphatidylserine (7.3); phosphatidylinositol (3.7). The asymmetry of the synaptic vesicle membranes was investigated by two independent approaches: (a) determining accessibility of the amino lipids to the chemical label trinitrobenzenesulphonic acid (TNBS); (b) determining accessibility of the vesicular glycerophospholipids to phospholipase C (*Bacillus cereus*). TNBS was found to render the vesicles leaky and thus cannot be used reliably to determine the asymmetry of *Torpedo* synaptic vesicle membranes. Incubation of the vesicles with phospholipase C (*Bacillus cereus*) results in biphasic hydrolysis of the vesicular glycerophospholipids. About 45% of the phospholipids are hydrolysed in less than 1 min, during which no vesicular acetylcholine is released. In the second phase, the hydrolysis of the phospholipids slows down markedly and is accompanied by loss of all the vesicular acetylcholine. These findings suggest that the lipids hydrolysed during the first phase are those comprising the outer leaflet. Analysis of the results thus obtained indicate that the vesicular membrane is asymmetric: all the phosphatidylinositol, 77% of the phosphatidylethanolamine, 47% of the plasmalogen ethanolamine and 58% of the phosphatidylcholine were found to reside in the outer leaflet. Since phosphatidylserine is a poor substrate for phospholipase C (*B. cereus*), its distribution between the two leaflets of the synaptic vesicle membrane is only suggestive.

The protein and lipid constituents of most biological membranes are distributed asymmetrically between the outer and inner leaflets of the bilayer (for recent review, see Rothman & Lenard, 1977; Op den Kamp, 1979; Etemadi, 1980a,b). Almost every type of lipid present is on both sides of the membrane bilayer and its asymmetry is due to the unequal distribution of the lipids between the membrane surfaces (Rothman & Lenard, 1977). In contrast the protein and glycosphingolipids constituents of the two faces of the membrane are qualitatively different and their asymmetry is there-

fore absolute (Rothman & Lenard, 1977). In addition to lipid asymmetry due to the lipid head groups there may also be an unequal distribution based on the acyl chains of lipids with the same polar moiety. For example, in the membrane of the vesicular stomatitis virus almost all the polyunsaturated acyl chains are present in the inner leaflet (Patzner *et al.*, 1978). Similarly, in rat erythrocytes, arachidonate-labelled phosphatidylcholine and phosphatidylethanolamine are mainly constituents of the inner side of the membrane (Crain & Zilversmit, 1980).

The role of the asymmetric distribution of proteins between the two faces of the bilayer is easy to understand on a functional basis. The function of lipid asymmetry is less clear. It may be a result of the protein asymmetry. Alternatively it may have a physiological role. For example, studies with a membrane model system have shown that Ca^{2+}

Abbreviations used: TNBS, trinitrobenzenesulphonic acid; DPH, 1,6-diphenylhexa-3,5-triene; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; SPM, sphingomyelin.

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induces fusion between phospholipid vesicles via phase separation and that this effect depends on their lipid composition (Papahadjopoulos *et al.*, 1979).

Membrane fusion is believed to play a role in presynaptic neurotransmitter release. After stimulation of the nerve terminal there is a transient increase in the level of free cytoplasmic Ca^{2+} , which induces neurotransmitter release, presumably by exocytosis of the synaptic vesicles (Llinas & Heuser, 1977; Holtzman, 1977). In order to further our understanding of the mechanisms underlying neurosecretion it is of importance to analyse the lipid and protein constituents of the synaptic vesicles and of the presynaptic plasma membranes and their organization within these membranes.

In this work we characterized the membranes of cholinergic synaptic vesicles purified from the electric organ of *Torpedo ocellata* by determining their lipid composition, the lipid distribution between the inner and outer leaflets of the vesicular membrane and its degree of order. The results obtained are compared with those recently described for cholinergic synaptic vesicles purified from the electric organs of *Narcine brasiliensis* (Deutsch & Kelly, 1981) and the differences between the phospholipid distribution presently reported and that obtained by Deutsch & Kelly (1981) is discussed.

Materials and methods

Isolation and purification of *Torpedo* and rat brain synaptic vesicles

Torpedo ocellata were caught live off the coast of Tel Aviv. The fish were maintained in sea water aquaria for up to 4 months before use. The electric organs were excised from fish, which were pre-chilled at 4°C for 30 min. The tissue was homogenized in 0.8 M-glycine/1 mM-EGTA, pH 6.6, and the vesicles were purified by differential and sucrose-density-gradient centrifugation, followed by permeation chromatography on a controlled-pore glass-bead column (Michaelson & Ophir, 1980a,b). The purified vesicles in 5 mM-Tris/HCl, pH 7.4, which contained 0.3 M-NaCl, 0.2 M-sucrose and 0.2 mM-EGTA, were stored in liquid N_2 . The vesicles do not burst on either freezing or thawing and can be kept in liquid N_2 for at least several weeks without any detectable decrease in their acetylcholine content.

Measurement of vesicular acetylcholine release

The purified *Torpedo* synaptic vesicles contain residual acetylcholinesterase activity (Michaelson & Ophir, 1980a). Thus acetylcholine released from the vesicles into the external medium is rapidly hydrolysed. Accordingly the kinetics of vesicular acetylcholine release were monitored by withdrawing

samples at different times and measuring the amount of acetylcholine retained within the vesicles.

Extraction and analysis of lipids

Total synaptic vesicle lipids were routinely extracted by the method of Folch *et al.* (1957). Quantitative analysis of phospholipids was performed by using two-dimensional t.l.c. on silica gel G (Merck 5626) by the method of Yavin & Zutra (1977). The lipids were identified by short exposure to I_2 vapour, after which the spots were scraped off and their phospholipid content was determined by measuring their organic phosphorus content (Bartlett, 1959). The phospholipid/cholesterol molar ratio was determined from the total phospholipid content (Bartlett, 1959), and from the unesterified cholesterol content (Barenholz *et al.*, 1978) of the washed lower phase obtained after total lipid extraction (Folch *et al.*, 1957).

TNBS labelling

The total amino lipids of *Torpedo* synaptic vesicles and the relative fraction of their amino lipids exposed to TNBS were determined as described by Barenholz *et al.* (1977), except that the osmolarity of the medium was adjusted to that of the *Torpedo* vesicles (e.g. 0.4 M- NaHCO_3 , pH 8.5, and TNBS in 0.4 M-NaCl). The labelling with TNBS was performed at various temperatures and in some cases at a lower pH of 7.4. It was terminated by acidification, extraction of the lipids (Folch *et al.*, 1957) and determination of the trinitrophenyl-amino lipid content in the lower chloroformic phase (Folch *et al.*, 1957) spectrophotometrically at 350 nm. Under the same conditions, trinitrophenyl-phosphatidylethanolamine (derived from Supelco 4-6002 phosphatidylethanolamine) has $\epsilon = 14 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 350 nm.

Phospholipase C treatment

Partially purified phospholipase C from *Bacillus cereus* (EC 3.1.4.3) (Makor Chemicals, Jerusalem, Israel) was used without further purification. The enzyme was preserved in 50% glycerol at a concentration of 100 units/ml. This enzyme preparation used glycerophospholipids but not sphingomyelin as substrates (Roelofsen & Zwaal, 1976; also confirmed by us) and is widely used for membrane asymmetry studies (Roelofsen & Zwaal, 1976; Etemadi, 1980b). It is noteworthy that sphingomyelinase can also be isolated from *Bacillus cereus* (Ikezawa *et al.*, 1978). This sphingomyelinase is not yet commercially available and its activity towards intact membrane requires further investigation. The reaction was performed in 5 mM-Tris/HCl buffer, pH 7.4, which contained 0.2 M-sucrose, 0.3 M-NaCl and 0.2 mM-EGTA. The reaction mixture contained synaptic vesicles (2 μmol of vesicular phospholipid/ml) and phospholipase C (0.25 unit/ml), which

were incubated at 25°C for the appropriate time. The distribution of lipids between the two faces of the synaptic vesicles was determined as described by Patzer *et al.* (1978). By using two-dimensional t.l.c. (Yavin & Zutra, 1977) the enzymic specificity of the *Bacillus cereus* phospholipase C was determined by using liposomes (Barenholz *et al.*, 1977) and a diethyl ether/buffer biphasic system.

Assays

Acetylcholine was extracted from the synaptic vesicles by boiling the samples for 10 min at pH 4.0 (Whittaker, 1969). The acetylcholine content of the extracts was measured with the guinea-pig ileum bioassay (Barker *et al.*, 1967). ATP was determined by the firefly tail luciferin/luciferase method (Kim-mich *et al.*, 1975) and protein was determined by the method of Lowry *et al.* (1951).

Measurements of DPH fluorescence depolarization

Fluorescent labelling of synaptic vesicle membranes with DPH and measurement of steady-state fluorescence depolarization were performed as described previously (Barenholz *et al.*, 1976). The use of this technique for monitoring membrane properties was described by Shinitzky & Barenholz (1978), Barenholz *et al.* (1976) and Litman & Barenholz (1982). In short, the steady-state fluorescence anisotropy (r_s) was determined from the fluorescence emission intensities measured through an analyser oriented parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the plane of polarization of the excitation light. The data were corrected for the various effects of light scattering (Barenholz *et al.*, 1976; Lentz *et al.*, 1979; Litman & Barenholz, 1982).

$$r_s = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}$$

The limiting fluorescence anisotropy (r_{∞}), which is a measure of the order parameter (S_{DPH}), was calculated as $r_{\infty} = \frac{4}{3}r_s - 0.1$, for $0.13 < r_s < 0.28$ (Van Blitterswijk *et al.*, 1981).

Electron microscopy

Samples were fixed, in suspension, in 0.5M-cacodylate buffer, pH 7.4, which contained 2.5% glutaraldehyde. The material was post-fixed with OsO_4 , dehydrated and embedded in Epon. Sections were stained on the grids with uranyl acetate and lead citrate and observations were carried out on a JEOL 1009 electron microscope.

Results

Cholinergic synaptic vesicles were purified from fresh electric organs of *Torpedo ocellata* by dif-

ferential and sucrose-density-gradient centrifugation and by permeation chromatography on a controlled-pore glass-bead column as described previously (Michaelson & Ophir, 1980a,b). The purity of the vesicles was assayed by biochemical criteria and the homogeneity of the preparation was verified by electron microscopy (Fig. 1).

The total lipid composition of the purified synaptic vesicles is shown in Table 1. The vesicles contain cholesterol and phospholipids at a molar ratio of 0.63 ± 0.03 . The phospholipids are composed of choline-containing (PtdCho and SPM) and amino-containing (PtdEtn and PtdSer) phospholipids and of PtdIns (Table 1). The ratio of choline to amino-phospholipids is 1.2. It is worth noting that

Fig. 1. Electron micrograph of purified *Torpedo* synaptic vesicles (see the Materials and methods section) Scale bar = 1 μ m. Abbreviation used: Sv, synaptic vesicle.

Table 1. Lipid composition of *Torpedo* synaptic vesicle membranes

Phospholipid	Content (% of total phospholipids)
PtdCho	40.9 $\pm$ 2.5
SPM	12.0 $\pm$ 2
PtdEtn ester	24.6 $\pm$ 6
Plasmenylethanolamine	11.5 $\pm$ 1
PdtSer	7.35 $\pm$ 1.3
PtdIns	3.7 $\pm$ 0.7
Choline phospholipids/amino phospholipids molar ratio	1.22
Cholesterol/total phospholipid molar ratio	0.63

the PtdEtn is composed of two different types: plasmenylethanolamine and PtdEtn ester.

The fluidity (for further details of this concept, see the Discussion section) of the hydrophobic regions of the *Torpedo* vesicular membrane was investigated by using fluorescence depolarization of DPH embedded in the membrane (Shinitzky & Barenholz, 1978; Lakowicz *et al.*, 1979; Van Blitterswijk *et al.*, 1981). There was no detectable phase transition or phase separation at the temperature range of 10–50°C, as shown in Fig. 2 and other graphic procedures (not shown), which describe fluorescence-polarization data (Shinitzky & Barenholz, 1978).

The distribution of phospholipids between the inner and outer leaflets of the synaptic vesicles was investigated by determining the accessibility of amino lipids of intact synaptic vesicles to the non-permeant chemical label TNBS (Litman, 1973) and the accessibility of glycerolipids to digestion by phospholipase C. Incubation of *Torpedo* synaptic vesicles with TNBS, at 25°C and pH 8.5, resulted in formation of trinitrophenyl-phospholipids (approx. 70% of the total amino phospholipids). Treatment of the vesicles with the non-ionic detergent Triton X-100 (0.4%) increased the availability to TNBS to about 90% of the total amino phospholipids (Fig. 3). On the basis of the findings it is tempting to conclude that the distribution of the amino phospholipids across the membrane is very asymmetric with an 'outside/inside' ratio of about 3. This assertion is based on the assumption that TNBS does not permeate the synaptic vesicles and that they remain intact during the reaction. As shown in Fig. 3, TNBS had a major effect on the permeability barrier of the vesicular membrane, resulting in complete release of the vesicular acetylcholine, with

Fig. 2. The effect of temperature on the DPH steady-state fluorescence anisotropy (r_s) (○) and limiting fluorescence anisotropy (r_∞) (●) in cholinergic synaptic vesicles purified from the electric organ of *Torpedo ocellata*

kinetics that parallel those of the labelling of the lipids. Consequently, under these conditions TNBS cannot be employed to determine the lipid asymmetry of *Torpedo* synaptic vesicles. Labelling with TNBS at 4°C did not prevent the induced acetylcholine leak (60% of the total vesicular acetylcholine in 40 min). Performing the labelling reaction at lower pH values (7.4 and even down to pH 6) also resulted in acetylcholine loss that paralleled the labelling of the lipids. It is possible that although TNBS rendered the vesicular membrane leaky to acetylcholine, it by itself is not permeable. To examine this possibility we ruptured the vesicles by mechanical means (osmotic lysis and ultrasonic irradiation) either before or during the labelling reaction. These treatments resulted in TNBS labelling identical with that observed with intact synaptic vesicles, thus supporting the conclusion that the synaptic vesicles are permeable to TNBS. The difference between labelling of the detergent-treated and mechanically ruptured vesicles is probably due to the complete dissolution of the membrane by the detergent and the consequent unmasking of amino phospholipids that were not exposed in the intact membrane, similar to what was previously suggested by Vale (1977).

Owing to our finding that TNBS cannot be used reliably to determine membrane asymmetry of *Torpedo* synaptic vesicles we utilized the accessibility of the lipids to phospholipase C (*B. cereus*) to determine their organization in the membrane. As shown in Fig. 4, the hydrolysis of the vesicular phospholipids was biphasic: about 45% of the vesicular phospholipids were hydrolysed in less than

Fig. 3. TNBS labelling of *Torpedo* synaptic vesicles and its effect on their integrity

The relative content of amino lipids exposed to TNBS labelling in intact vesicles is denoted by ○. Synaptic vesicles that were disrupted by Triton X-100 (0.4%) and then labelled by TNBS (●) were used as a reference for total labelling of amino lipids. The integrity of the vesicles was assessed by measuring the release of vesicular acetylcholine at 23°C in the presence (■) and absence (□) of TNBS, and at 4°C with (▲) and without (△) TNBS.

Fig. 4. The effect of incubation of *Torpedo* synaptic vesicles with phospholipase C (*Bacillus cereus*) on their phospholipid (▲) and acetylcholine (●) contents. The acetylcholine content of a control in which the vesicles were incubated without phospholipase C denoted by ○.

Fig. 5. Distribution of glycerophospholipids between the two faces of the synaptic vesicles membrane (derived from the experiments with phospholipase C).

The bars represent $\pm$ s.d. Key: 1, PtdSer; 2, PtdCho; 3, plasmenylethanolamine; 4, PtdEtn; 5, PtdIns; 6, total glycerophospholipids.

1 min, after which the reaction slowed markedly. During the first phase there was virtually no loss of vesicular acetylcholine (<5%), whereas in the second phase there was a gradual loss of acetylcholine, which eventually led to complete depletion. After 3½ h, about 85% of the glycerophospholipids were hydrolysed (about 93% of PtdCho, 87% of PtdEtn, all the PtdIns and 30% of PtdSer). As expected, SPM was not hydrolysed at all by this enzyme. The biphasic characteristic of the phospholipid hydrolysis, the intactness of the vesicles during the first phase (Fig. 4) and the interpretation of similar data from other systems (Patzner *et al.*, 1978; Etemadi, 1980b) suggest that the lipids hydrolysed first are localized in the outer leaflet and that those composing the inner leaflet are gradually being exposed to the enzyme owing to the consequent vesicle disintegration. Accordingly, we analysed the lipid composition after a 1 min exposure to phospholipase C (Fig. 4) and compared it with that of the untreated vesicles (Table 1). These data were utilized to calculate the 'outside/inside' distribution ratio of each phospholipid (eqn. 1), where

$$\frac{PX_{\text{out}}}{PX_{\text{in}}} = \frac{PX_{\text{total}} - PX_{\text{in}}}{PX_{\text{in}}} \quad (1)$$

PX_{total} = proportion of each phospholipid from the total untreated phospholipids (%) and PX_{in} = proportion of each phospholipid (from the total phospholipids) remaining after 1 min exposure to phospholipase C (%). The putative distribution of glycerophospholipids thus calculated is depicted in Fig. 5. As shown, the vesicular membrane is asymmetric. All the PtdIns, 77% of the PtdEtn ester, 47% of the plasmenylethanolamine and 58% of

PtdCho are in the outer leaflet. Also there are indications that the content of PtdSer in this leaflet is small. It should be noted that PtdSer is a poor substrate of phospholipase C (Moore *et al.*, 1977). Therefore the finding that PtdSer remained unhydrolysed at the end of the first phase (Fig. 4) and was only poorly hydrolysed after a 3½ h exposure to the enzyme (less than 30% hydrolysis of PtdSer compared with over 90% hydrolysis of PtdCho) may be due to the poor activity of the enzyme and not only due to the unavailability of PtdSer. This possibility was confirmed by control experiments utilizing either PtdCho/PtdSer (1:1) liposomes or a diethyl ether/buffer biphasic assay system. In these experiments the rate of hydrolysis of PtdCho was found to be respectively 2-fold and 5-fold higher than that of PtdSer. Thus the data obtained with phospholipase C are only suggestive for PtdSer and cannot be employed reliably to determine its exact inside/outside ratio of distribution.

Discussion

In the present paper we report the lipid composition of purified cholinergic synaptic vesicles and their distribution between the inner and outer leaflets of the vesicular membrane. The major lipids of the vesicles are cholesterol (38.5 mol%), PtdCho (25.2 mol%), total PtdEtn (22.2 mol%), SPM (7.4 mol%) and PtdIns (2.3 mol%). These findings, obtained with *Torpedo ocellata* cholinergic vesicles, are similar to, but not identical with, those recently reported by Deutsch & Kelly (1981) with *Narcine*

brasiliensis cholinergic vesicles. The ethanolamine phospholipids in the *Torpedo* vesicles consist of PtdEtn and plasmenylethanolamine at a molar ratio of 2.1. The cholesterol content of the synaptic vesicle membrane is much higher than its content in most intracellular membranes, though it is lower than in most normal plasma membranes (for review, see Rouser *et al.*, 1972; Barenholz & Thompson, 1980; Van Blitterswijk *et al.*, 1981). The origin of the high cholesterol content and its effect on the properties of the synaptic vesicles is still unclear.

The *Torpedo* synaptic vesicle membranes exhibit higher fluidity than expected on the basis of their cholesterol and SPM content (Shinitzky & Barenholz, 1978; Barenholz & Thompson, 1980; Van Blitterswijk *et al.*, 1981). The concepts of fluidity and microviscosity are controversial (Shinitzky & Yuli, 1982). In the present paper, 'fluidity' is used not as a measure of lipid dynamics but related to the membrane structural parameter (Lakowicz *et al.*, 1979). It is obtained from r_{∞} (limiting fluorescence anisotropy of DPH (Lakowicz *et al.*, 1979; Van Blitterswijk *et al.*, 1981). This may be explained by the high content of docosahexaenoic acid ($C_{22:6}$) in the phospholipids (Deutsch & Kelly, 1981). Our preliminary results on rat synaptic vesicle membranes (Y. Barenholz & T. E. Thompson, unpublished work) support this hypothesis.

The latter membranes, which are less fluid than the *Torpedo* synaptic vesicle membranes, have similar cholesterol and SPM content but only about 50% of the docosahexaenoic acid of the *Torpedo* synaptic vesicle membranes (Y. Barenholz & T. E. Thompson, unpublished work). The difference in fluidity between the *Torpedo* and rat synaptic vesicle membrane may be related to differences in ambient temperatures (approx. 20°C for the *Torpedo* and 37°C for the rat). Similar effects were found for the synaptosomal membranes of the goldfish (Cossins, 1977).

No major phase separation was detected for *Torpedo* synaptic vesicle membranes between 10 and 45°C as a result of the high cholesterol content together with the high degree of unsaturation (for review, see Shinitzky & Barenholz, 1978; Jain & Wagner, 1980; Barenholz & Thompson, 1980).

The distribution of lipid components between the two leaflets of a membrane can be assessed by using various approaches. These include chemical labelling, enzymic degradation of membrane lipids, phospholipid-exchange proteins and physical methods (for review, see Etemadi, 1980b). The basic assumptions underlying the validity of any approach are as follows. (i) Under the conditions of the experiment, the reacting agent does not penetrate the membrane and the membrane remains intact. (ii) The changes occurring in the membrane during the

procedure do not affect the lipid distribution. (iii) Flip-flop of lipid components is on a slower scale than the assay itself.

It seems that physical methods, and especially n.m.r., are theoretically the most promising since they do not induce major chemical changes in the membrane. Unfortunately the n.m.r. technique is not applicable to synaptic vesicles, since the signal of the lipids cannot be observed (Y. Barenholz & T. E. Thompson, unpublished work).

We investigated the asymmetry of the vesicular membrane by two well established methods: chemical modification with TNBS and enzymic digestion by phospholipase C (Etemadi, 1980b). Our finding with TNBS demonstrates that the first of the above three assumptions is not met. On the contrary, it seems that under all conditions (lower temperatures and lower pH values) TNBS permeates the vesicles and induces the release of the vesicular contents (Fig. 3). This assertion is further supported by the fact that the same degree of labelling by TNBS was obtained with intact and with disrupted vesicles. Similar findings have been reported with human erythrocytes (Heast *et al.*, 1981). Thus the increased labelling of *Torpedo* amino lipids by TNBS caused by the detergent is not due to exposure of the membrane inner leaflet to TNBS. It is probably due to the increased exposure of amino groups, which are hindered by interaction with proteins (Vale, 1977), or due to physical limitation on the extent of the labelling reaction in the intact membrane (Bishop *et al.*, 1979) or both.

Our present findings are similar to those previously reported by Deutsch & Kelly (1981; Fig. 1). However, in view of the novel finding that TNBS disrupts synaptic vesicle membrane intactness, the use of TNBS labelling for the study of this membrane topology is questionable. In contrast with TNBS labelling our finding with phospholipase C demonstrates that conditions can be met under which the intactness of the vesicles is unaffected by lipid hydrolysis.

It is accepted that the susceptible lipids represent the lipid components in the outer leaflet of the membrane (Etemadi, 1980b). It is noteworthy that independently of the experimental approach the second and third requirements have to be checked. The isolated synaptic vesicles do not contain any enzyme involved in lipid metabolism (Whittaker, 1973; Mahadik *et al.*, 1978) and in this respect are non-active membranes. Most of the non-active membranes, such as red-blood-cell and viral membranes, have relatively low rates of flip-flop (Thompson, 1978). Thus, because the reaction time with phospholipase C is short (only 1 min), the effect of lipid flip-flop should be minimal.

On the basis of the above assumption the analysis of the phospholipids hydrolysed by phospholipase C

indicates that the synaptic vesicle membrane is asymmetric. The results show that this method is applicable to analysis of PtdCho, PtdEtn, plasmenyl-ethanolamine and PtdIns. The outside/inside membrane ratio is 1.4 for PtdCho, 0.9 for plasmenyl-ethanolamine and 3.4 for PtdEtn. All the PtdIns is found in the external leaflet. The physiological significance of lipid asymmetry in membranes is still unclear. There are many speculations, but none proven (Etemadi, 1980b). This is also true for the asymmetry of the synaptic vesicles, although a few speculations on the role of lipid asymmetry in the fusion process were made by Deutsch & Kelly (1981); as yet there is no evidence in support of any of them.

In this regard it is noteworthy that in some preparations hormone-induced PtdIns breakdown leads to the subsequent liberation of arachidonate and to enhanced prostaglandin synthesis (Bell *et al.*, 1979; Bell & Majerus, 1980; Banschbach & Hokin-Neaverson, 1980; Marshall *et al.*, 1981). We have shown that acetylcholine release from *Torpedo* nerve terminals is regulated by presynaptic muscarinic acetylcholine receptors (Michaelson *et al.*, 1979) and that this effect is mediated by prostaglandins (I. Pinchasi, M. Schwartz, A. Raz & D. M. Michaelson, unpublished work). It is therefore tempting to speculate that the vesicular PtdIns, which *in vivo* faces the synaptic cytoplasm, may be implicated in the muscarinic regulation of acetylcholine release.

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