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PHOSPHOLIPID AND FATTY ACID COMPOSITION OF TETRODOTOXIN RECEPTOR-RICH MEMBRANE FRAGMENTS FROM *ELECTROPHORUS ELECTRICUS*

HANS H. GRÜNHAGEN [a,*], HANSJÖRG EIBL [b], GERHARD KREBS [c] and PETER REITER [a]

[a] *Institut für Physiologische Chemie der Universität, D-6650 Homburg/Saar,* [b] *Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen and* [c] *A. Nattermann & Cie, D-5000 Köln 30 (F.R.G.)*

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To study the interaction of voltage-sensitive Na^+-channels with membrane lipids, the phospholipid and fatty acid composition of highly purified membrane fragments from the remarkably differentiated plasma membrane of *Electrophorus electricus* has been analyzed. After density gradient fractionation and carrier free electrophoresis, fractions with up to 30 pmol tetrodotoxin binding/mg protein can be obtained, which may correspond to a 50% pure preparation of the extrasynaptic part of the excitable face. Phospholipid classes and cholesterol are separated by one-dimensional thin-layer chromatography in acidic and alkaline solvent systems. The following mean molar contents are found: 40% phosphatidylcholine, 23% phosphatidylserine, 30% phosphatidylethanolamine and 7% sphingomyelin. In a series of 11 animals, significant deviations from these mean values have been observed. The fatty acid composition of the phospholipids has been determined by gas chromatography. Phosphatidylcholine contains more than 50% 16:0, and about 20% unsaturated fatty acids in the C-18 group. Compared to other plasma membrane fractions, this phospholipid is the least differentiated. By contrast, phosphatidylethanolamine and phosphatidylserine show many characteristics in different membrane fractions, especially in their unsaturated components representing more than 50%. 22:6, as the major constituent in these fractions, accounts for a quarter to a third of all fatty acids in these fractions. 18:0 is the main saturated component in these two phospholipids with abundances of typically a quarter or less of all fatty acids. Knowledge of the lipid composition of these excitable membranes may help to conserve binding and structural properties when analyzing lipid-sensitive Na^+-channels in vitro. It is also useful as a guideline for systematic reconstitution studies.

Introduction

The function of excitable membranes depends on specific channels and on the properties of the membrane phase as a whole. The membrane phase is known to contribute to the control of electric phenomena via general electric properties such as charge density or dielectric constant. Little is known, however, about more specific requirements of channel proteins for special components or a particular thermodynamic state of the membrane phase. Voltage-sensitive Na^+-channels are essential constituents of nerve, muscle and electroplaque cells propagating an action potential. Their specific interaction with tetrodotoxin is used to identify and to characterize the corresponding re-

* Present address: c/o BASF AG, Hauptlabor B9, D-6700 Ludwigshafen, F.R.G.

ceptor in vitro (for reviews, see Refs. 1–3). In contrast to other membrane proteins, e.g., the acetylcholine receptor [4], the tetrodotoxin receptor has turned out to be very sensitive to changes in its hydrophobic environment. After solubilization, loss of binding capacity can be slowed down only if the detergent solution is supplemented with lipids [5,6]. Loss of functional properties is expected to be even more sensitive to changes of the lipid environment.

For systematic studies of these effects and to provide data for reconstitution experiments, a detailed lipid analysis of electrically excitable membranes appears important. Progress in this field depends on the availability of data from highly purified membrane fragments. Corresponding results have been published for nerve tissue from lobster leg nerve, squid stellar nerve, garfish olfactory nerve and squid retinal nerve [7,8], for skeletal muscle [9,10] and for *Electrophorus electricus* electroplax [11,12]. Recently, we have developed a procedure to prepare tetrodotoxin receptor-rich membrane fragments from *E. electricus* electroplax [13]. With centrifugation techniques, specific [^{3}H]tetrodotoxin binding [14] of up to 5 pmol/mg protein is reached, and further purification of these membrane fractions by carrier-free column electrophoresis yields specific [^{3}H]tetrodotoxin binding up to 30 pmol/mg. Only in such preparations does the excitable membrane constitute a substantial part and not a minor fraction of the whole membrane material subjected to lipid extraction and analysis.

This paper describes the phospholipid – and fatty acid – composition of a series of highly purified membrane fragment preparations. In individual animals, significant deviations from the typical lipid pattern have been observed. The results of the analysis are of importance for a functional transfer of ion channels from vesicular to planar host membranes (Schindler, H. and Grünhagen, H.H., unpublished data).

Methods

Preparation of [^{3}H]tetrodotoxin receptor-rich membrane fragments. E. electricus, approx. 1 m long, were purchased from World Wide Scientific Animals, Ardsley, NY 10502, U.S.A. They were kept in fresh water at 25–28°C for 2 weeks to 6 months before excision of the electric organ. During this period the animals were fed with live fresh-water fish and/or pieces of herring meat (approx. 30–60 g/animal per week). Purification of membrane fractions by sucrose density gradient centrifugation and by carrier-free column electrophoresis was carried out as described [13]. Specific tetrodotoxin binding was determined with chemically tritiated tetrodotoxin [14]. It ranged from 3 to 5 pmol/mg protein for density gradient fractions (F3 with under 1.10 g/ml and F5 with over 1.10 g/ml sucrose density) and from 20 to 30 pmol/mg for the tetrodotoxin receptor-rich subfraction after electrophoresis (III). Preparations were used for lipid analysis fresh or after storage in liquid nitrogen, no difference was observed between the results from these conditions. Membrane protein was determined with bovine serum albumin as a standard [15].

Isolation of membrane lipids [16]. 5 ml of membrane suspension with up to 2 mg protein/ml were mixed with 6 ml methanol and supplemented with EDTA to yield at least a 5-fold molar excess over phospholipids and calcium. After 10 min, 5 ml chloroform were added, and after vortexing and phase separation the lower phase was collected. The upper phase was extracted twice more with 2.5 ml of chloroform. The combined lower phases were evaporated (Rotavapor) and kept under N_2.

Separation of major lipid classes. Two alkaline systems and one acid system were used for separation of phospholipids by one-dimensional thin-layer chromatography on 20 cm silicagel plates (Merck Silicagel 60, No. 5721). The alkaline systems (a) and (b) ($CHCl_3$/ CH_3OH/ 25% NH_4OH/ H_2O; (a) 100 : 50 : 1.5 : 2 and (b) 120 : 50 : 5.5 : 3) were chosen for optimal isolation of cholesterol, whereas the acid system (c) ($CHCl_3$/ CH_3OH/ CH_3COOH/ H_2O; 100 : 60 : 20 : 5) yields an excellent separation of the phospholipids (cf. R_F values in Table I). Different commercial phospholipid preparations (Sigma, Calbiochem) with minor variations of R_F values were used as a reference. The lipid fractions were visualized on the plate with iodine vapor, scraped off, eluted with $CHCl_3$ and evaporated with N_2. Phosphorus in phospholipids was

TABLE I

R_F VALUES OF MAJOR LIPID CLASSES IN THE THREE SOLVENT SYSTEMS USED FOR ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY (SILICAGEL MERCK 60 PLATES)

	$CHCl_3/CH_3OH/25\%\ NH_4OH/H_2O$		$CHCl_3/CH_3OH/CH_3COOH/H_2O$
	a 100:50:1.5:2	b 120:50:5.5:3	c 100:60:20:5
Sphingomyelin	0.05	0.23	0.19
Phosphatidylserine	0.09	0.25	0.59
Phosphatidylcholine	0.16	0.40	0.34
Phosphatidylethanolamine	0.44	0.47	0.81
Cholesterol	0.84	0.92	1.0

analyzed according to the turbidimetric assay of Eibl and Lands [17] (reagent kit from Serva), cholesterol was determined according to Clark et al. [18] with cholesterol from Serva as a standard.

Fatty acid analysis. For fatty acid analysis, the purification of membrane fragments, the chromatographic separation of lipids and all further steps were carried out under argon. No visualization with iodine vapor was used; instead, the phospholipid fractions were detected on moistened plates after spraying with distilled water. Fatty acids were analyzed by quantitative gas chromatography. 0.1–1 μmol chromatographed phospholipid was treated for 5 min with 2 ml of 0.5 N sodium methoxide in methanol to form the methyl esters [19]. 5 ml *n*-hexane were added and the system vortexed. After 10 min, the upper layer was taken and dried over solid sodium sulfate/sodium bicarbonate (2:1, w/w). The solution was decanted, evaporated in a vacuum centrifuge, and dissolved in 0.1 ml undecane for gas chromatograph injection. In a Hewlett-Packard gas chromatograph (5880) volumes from 1 to 2.5 μl were injected for analysis. A 50 m × 0.25 mm (i.d.) glass capillary with Silar 10 C (Gerstel) was used as the column. Injector and detector temperature was 250°C. The split was set to approx. 40 ml/min, the carrier gas was H_2 with a 1.0 bar prepressure. A temperature program with 1 min at 150°C, followed by heating to 230°C with 3 K/min and 10 min at 230°C was chosen. A mixture of 40 reference fatty acids (Sigma) covering the range from 8:0 to 22:6 was used for comparison. Standard computer programs were applied for qualitative identification of fatty acids included in the reference mixture and for quantitative evaluation of peak areas.

Results

Table II summarizes the results of phospholipid class analyses carried out during a period of 13 months with 11 individual *E. electricus* animals. When aliquots of the same samples were independently analyzed, the results were within an experimental error range of 1–2 mol%. The wider range of results observed in the series of analyses clearly demonstrates significant differences among animals. Typical phosphatidylcholine contents are around 40%, being lower in density gradient fractions and slightly higher after purification of membrane fragments by electrophoresis. High phosphatidylserine levels are found in all fractions. Phosphatidylethanolamine content is around 30–35%, being lower after electrophoresis. Sphingomyelin is present at 5–7%. No other component (lysophosphatidylcholine, phosphatidic acid, etc.) was detected above 1%. Compared to earlier analyses of membrane fragments from the same source [11,12], the chosen purification procedure yields preparations with lower phosphatidylcholine and higher phosphatidylserine content. The percentages in Ref. 12 are close to extreme cases in our population of animals but differ significantly from our mean values. Remarkable differences exist between these data and those in Ref. 11, where 62% phosphatidylcholine, 20% phosphatidylethanolamine, 11% sphingomyelin, and 7%

TABLE II

MOLAR PHOSPHOLIPID CONTENT AND PHOSPHOLIPID TO CHOLESTEROL RATIO OF DENSITY GRADIENT FRACTIONS AND ELECTROPHORESIS SUBFRACTIONS

F3, F5: density fractions with lower, respectively higher sucrose density than 1.10 g/ml; F5 II, F5 III: subfractions from F5 after carrier free electrophoresis (cf. Ref. 13); data represent mean values and S.D. of results from 11 individual animals (see text).

	Molar phospholipid content (%)				Molar phospholipid/ cholesterol ratio
	Phosphatidyl-choline	Phosphatidyl-serine	Phosphatidyl-ethanolamine	Sphingo-myelin	
F3	33 ±3	22±2	38 ±2	7±1	4.0±0.8
F5	34.5±4	24±3	35.5±3	6±1	3.2±0.7
F5 II	41.5±4	23±3	29.5±2	6±1	3.5±1.0
F5 III	40 ±3	23±2	30 ±3	7±1	3.9±0.9

phosphatidylserine have been found in sucrose density fractions.

The molar phospholipid-to-cholesterol ratio is also shown in Table II. The values are obtained from the same series of preparations as used for phospholipid content analyses. Mean molar ratios are around 3.5 (estimated experimental error ±0.5). Again, individual animals show distinct deviations. The value in Ref. 12 (1.67) is slightly lower than the lower extreme from the comparable fraction F5 in our study. For sarcolemma preparations from skeletal muscle, being closely related to *E. electricus* electric organs, molar ratios of approx. 5 [10] and approx. 9 [20] have been published. In our study, the mean ratios are slightly lower in heavier than in lighter density gradient fractions, whereas the two electrophoresis subfractions have very similar ratios.

The analysis of fatty acids was carried out with preparations from two different animals with phospholipid and cholesterol contents close to the mean values (cf. Table II). Table III shows the data derived from these two sets of analyses. The following conclusions may be drawn for different phospholipid fractions:

(1) In the phosphatidylcholine fractions, 16:0 is the major component. It constitutes about 50% (48 ± 6%) of all fatty acids and close to 90% of the saturated fraction in this phospholipid. Unsaturated components are found mainly in the 18 : *n* group, in which identified peaks account for about 20–25% of the fatty acids. Within this group, 18:1 − 9, 18:1 − 7 and 18:3 − 6 are dominating. 22:6 constitutes about 5–10%.

(2) In phosphatidylserine and phosphatidylethanolamine fractions, high amounts of 22:6 (up to 37%) are most obvious. Together with other components in the range from 22:1 to 22:6, this group may represent more than 50% of all fatty acids and about two-thirds of the unsaturated fraction. In contrast to phosphatidylcholine fractions, these phospholipids have higher 18:0 levels, typically more 18:0 than 16:0. The sum of both saturated fatty acids amounts to only one-third or less of the material.

(3) All phospholipids contain detectable amounts of uneven fatty acids, e.g., 13:0, 15:0 and 17:0.

In comparison to an earlier analysis [12], our preparations contain slightly higher levels of 16:0 in phosphatidylcholine fractions, although both analyses reveal this fatty acid being the major component in this phospholipid. If phosphatidylserine and -ethanolamine data from our density gradient fractions F3 and F5 are taken together, they compare reasonably well with data from Ref. 12: 22:6 in abundance, and 18:0 as the major saturated fatty acid.

Comparing the four different membrane preparations in our analysis, the following characteristics result:

(a) The lighter F3 and the heavier F5 density gradient fractions, which are both enriched plasma membrane preparations [13], show significant differences in their unsaturated fatty acid contents: F3 contains higher amounts of 20:4 in all phos-

TABLE III

FATTY ACID COMPOSITION IN DIFFERENT PHOSPHOLIPID- AND MEMBRANE FRACTIONS

Pooled fractions F3, F5 (specific binding 3–5 pmol tetrodotoxin/mg), F5 II (under 2 pmol/mg) and F5 III (10–30 pmol/mg): see legend of Table II. PC, PS, PE: phosphatidylcholine, -serine, -ethanolamine. Percentage composition according to sum of eluted peak areas, approximately representing weight percentage (in a mixture, low molecular weight components will have higher mole percentage figures).

Fatty acid	Relative retention time	F3			F5			F5 II			F5 II		
		PC	PS	PE	PC	PS	PE	PC	PS	PE	PC	PS	PE
08:0	3.95	0.1	0.1	0.2	0.2	1.1	0.3	0.1	0.2	0.2	0.1	0.2	0.1
12:0	6.94			0.5	0.1				0.3	0.6		0.8	
13:0	8.14	0.5	0.5	1.2	0.4	3.6	0.7	0.2	1.0	1.8	0.3	2.5	0.8
14:0	9.51	0.7		1.7	1.1	1.1	0.8	1.4	0.1	2.0	1.3	2.0	0.5
15:0	11.04	1.1	0.1	2.0	0.6	0.8	0.6	0.7	1.2	3.4	0.7	3.4	0.9
	12.45			2.4	0.8				1.5	4.7			1.2
16:0	12.68	42.7	2.6	5.9	46.3	6.8	4.7	51.2	4.5	6.9	53.9	7.3	4.1
16:1–7*trans*	13.68	0.4			0.2	0.7	0.5	0.4			0.4		
16:1–7	14.13	0.9	0.6	2.8	1.6	1.7	0.4	0.6	1.9	5.9	0.5		1.4
17:0	14.42	1.7	0.7	0.7	0.8	1.6	0.6	0.9	0.5	0.3	0.9	5.6	0.4
	15.76			2.9	1.3	0.2	0.3		1.5	6.1			1.1
18:0	16.20	6.0	23.4	11.6	2.8	4.7	19.5	3.1	23.7	7.3	3.1	8.1	27.9
18:1–7*trans*	17.27		2.0	2.9	1.6		0.5	0.4	1.5	5.8	0.4		1.2
18:1–9	17.59	8.4	4.8	2.6	5.5	0.4	4.8	5.3	3.7	1.9	3.6		4.4
18:1–7	17.76	12.0	0.9	0.9	13.1	1.3	1.0	12.6	1.5	1.9	13.7	4.8	1.5
18:2–6*trans, trans*	18.67		3.4	2.6	1.2		1.5		1.1	5.0			0.7
18:2–6	19.58	1.9	0.8	0.7	0.3	0.5		0.2			0.4	5.0	0.4
18:3–6	21.06	1.2	0.5	0.7	3.4	2.3	1.7	3.5	0.2	2.1	3.9		0.7
18:3–3	21.84		4.2	0.3	1.0	2.2							
	20.85		4.0	2.0	1.1	0.3	0.4			3.4		4.1	
	21.22						1.6		1.7				2.4
	22.55			1.6						2.2			
20:2–6	23.24	1.3			0.8	0.2		0.8			0.9	3.3	
20:3–3	25.50	0.4			0.2			0.2			0.3		
20:4–6	25.86	7.1	6.9	19.8	0.5	2.9	1.3	0.9	2.8	6.8	0.8	5.1	1.9
21:0	21.69			0.4	0.2							1.4	
22:1–9	24.78	0.5	3.4	0.2	1.6	0.3	2.0	1.7	1.1	0.6	1.9	2.4	1.1
	29.03		1.7		0.4								
	29.65	0.8	5.2	1.8	0.1		2.5	0.5	2.2	0.8	0.3		2.4
	30.74	1.2	4.7	2.4	0.4	0.4	2.6	0.9	2.7	0.9	0.4	1.3	2.7
	31.71	1.6	1.5	0.4	0.6	10.0	3.9	0.8	1.7	1.5	0.5	4.0	1.8
	32.30	0.4	3.2	1.0			4.8	0.6	2.6	0.6	0.3		3.0
22:6–3	33.73	5.6	18.0	21.4	6.2	35.0	37.3	10.8	35.8	18.2	5.7	21.0	33.0
24:0	26.75		0.2	0.1	0.1					0.6		0.5	
	27.38		2.2	0.5	0.4								
24:1–9	28.16	1.7	0.2	1.2	1.3	2.7	0.7	0.9	1.2	3.4	0.9	0.9	0.9
	34.34					5.2						6.8	
Other components		1.8	4.2	4.6	3.8	14.0[a]	5.0	1.3	3.8	5.1	4.8	9.5[a]	2.8

[a] Mainly components with retention times between those of 8:0 and 10:0, e.g., 4.40 and 4.47.

pholipids, whereas F5 has significantly higher contents of 22:*n* fatty acids, especially of 22:6 in phosphatidylserine and -ethanolamine. Less obvious are differences in the population of saturated acids: 16:0 appears more frequent in F5 than in F3, and 18:0 shows a different phospholipid dis-

tribution in both fractions.

(b) Electrophoresis subfraction F5 III, displaying highest levels of α-neurotoxin [13] and [^{3}H]tetrodotoxin binding, is not much different from F5 II, as far as the fatty acid composition of phosphatidylcholine is concerned. F5 III has significantly more than 50% saturated fatty acids in this phospholipid, indicating the existence of lipid molecules with saturated hydrocarbon chains only. There are, however, differences between subfractions II and III, if one compares the phosphatidylserine or the phosphatidylethanolamine components, respectively. The differences are marked in the cases of 18 : 0, 20 : 4 and 22 : 6. For some fatty acids, enriched occurrence in phosphatidylserine appears to be compensated by lower levels in phosphatidylethanolamine, and vice-versa.

Discussion

E. electricus electroplax are a rich source of plasma membranes, but the asymmetric morphology of these cells requires additional efforts to purify functionally uniform fractions. Carrier-free electrophoresis of density gradient fractions allows preparation of highly purified Na^+-channel-rich membrane fragments. Subfraction III contains up to 30 pmol/mg specific binding sites for α-neurotoxin and tetrodotoxin and may represent an approx. 50% pure preparation of the extrasynaptic excitable face [13]. In this preparation, lipids from Na^+-channel-bearing membrane regions predominate over lipids from other specializations of the electroplax. In contrast, density gradient fractions contain significant amounts of lipids from various parts of the highly differentiated plasma membrane.

Comparing the mean phospholipid content of subfraction III with the other membrane fractions (cf. Table II) shows that the crude plasma membrane preparations F3 and F5 have slightly lower phosphatidylcholine and slightly higher phosphatidylethanolamine levels, whereas the second electrophoresis subfraction II has almost the same phospholipid content as subfraction III. In view of the lateral mobility of lipid molecules in the membrane plane, a completely different lipid pattern in different specializations of the plasma membrane would be surprising. The existence of minor differences shows, however, that selected lipids may be enriched or depleted locally and that electrophoresis is able to subfractionate density gradient material into more homogeneous fractions, as demonstrated earlier by specific ligand binding and marker enzyme activities [13]. Furthermore, the observed differences preclude a far-reaching mixing of membrane lipids, which might occur after homogenization by membrane fusion processes or by intermembrane exchange of single phospholipid molecules.

We cannot explain as yet the individual variations of phospholipid levels among animals; a correlation between phospholipid content and a single parameter does not appear obvious. Interestingly, in different animals, significant variations of the apparent K_d for the blocking effect of tetrodotoxin have also been observed (Bartels-Bernal, E., Hof, D. and Grünhagen, H.H., unpublished data). The reconstitution experiments with defined phospholipid mixtures may allow us to study the dependence of Na^+-channel properties on the lipid composition.

The relative abundance of cholesterol in cellular membranes appears to differ in different organelles and has been found highest in plasma membranes [21]. Since all membrane preparations analyzed in this study represent purified plasma membranes, no major differences were expected. In addition, differences originally existing in plasma membrane fragments may diminish in vitro because of exchange processes, which are much faster with neutral lipids than with polar lipids [22–24].

The phosphatidylserine component appears most interesting among the phospholipids. Earlier preparations of excitable membranes from the same source [12] or from other animals and tissues [7,9,25] had already revealed high levels of this class. The preparation procedures chosen for this study yield values around 20% (cf. Table II). A comparison of data from different tissues and different cellular membranes [21,22] suggests the occurrence of higher phosphatidylserine levels, (1) in excitable tissues as compared to nonexcitable tissues and (2) in plasma membranes as compared to intracellular organelles. In addition to the abundance of phosphatidylserine, its fatty acid composition qualifies this lipid as a modulator of excitable membrane properties. Whereas phos-

phatidylcholine has a fatty acid composition similar to that of many saturated acids in all membrane fractions, phosphatidylserine has high levels of unsaturated fatty acids, and their distribution differs markedly in different fractions. The highly purified fraction F5 III with Na^+-channel-rich extrasynaptic fragments has 15 fatty acids above 2% in the serine component, but only six above 2% in phosphatidylcholine. Only phosphatidylethanolamine shows a degree of differentiation comparable to phosphatidylserine. An interesting phenomenon can be noted when comparing F5 II and F5 III with regard to several fatty acid levels in serine and ethanolamine phospholipids: If F5 III has, for example, a higher level of 22 : 6 in the ethanolamine class than F5 II, F5 III has a lower level of this fatty acid in the serine class than F5 II, and vice-versa for other fatty acids.

Although fish oils are known to contain high amounts of unsaturated fatty acids [26], there is evidence for a special enrichment of these compounds in excitable tissue [27]. In particular, the docosahexaenoic acid, 22 : 6, has been found enriched in other neuronal tissue such as brain and rods of the retina [30–32]. High amounts of unsaturated fatty acids increase membrane fluidity as a macroscopic parameter [28]. In addition, complex mixtures of fatty acids may serve for domains of microheterogeneity in the membrane plane. The existence of lateral specializations is obvious on electroplax with its extra- and subsynaptic regions, its transverse tubuli and its overall asymmetry [13]. The diversity of fatty acids may also reflect the selective association of special proteins with specific lipids. In view of the low flip-flop exchange rates, an uneven distribution of phospholipids in the two monolayers of the membrane may be of importance for the control of functional proteins, too. Such asymmetric planar membranes are available now for reconstitution experiments [29].

Knowledge of the lipid composition may help to preserve functional properties of the Na^+-channels, when this protein is analyzed in vitro. It can contribute to an understanding of physiological and experimental control of channel function and it provides guidelines for systematic reconstitution experiments.

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