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Phospholipid asymmetry in cardiac sarcolemma. Analysis of intact cells and 'gas-dissected' membranes *

Jan A. Post [a], Glenn A. Langer [b,**], Jos A.F. Op den Kamp [c] and Arie J. Verkleij [d]

[a] *Institute of Molecular Biology and Medical Biotechnology,* [c] *Department of Biochemistry,* [d] *Department of Molecular Cell Biology, State University of Utrecht, Utrecht (The Netherlands) and* [b] *Departments of Medicine and Physiology, Cardiovascular Research Laboratory, UCLA School of Medicine, Los Angeles CA (U.S.A.)*

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The investigation focuses on the phospholipid composition of the sarcolemma of cultured neonatal rat heart cells and on the distribution of the phospholipid classes between the two monolayers of the sarcolemma. The plasma membranes are isolated by 'gas-dissection' technique and 38% of total cellular phospholipid is present in the sarcolemma with the composition: phosphatidylethanolamine (PE) 24.9%, phosphatidylcholine (PC) 52.0%, phosphatidylserine/phosphatidylinositol (PS/PI) 7.2%, sphingomyelin 13.5%. The cholesterol/phospholipid ratio of the sarcolemma is 0.5. The distribution of the phospholipids between inner and outer monolayer is defined with the use of two phospholipases A_2, sphingomyelinase C or trinitrobenzene sulfonic acid as lipid membrane probes in whole cells. The probes have access to the entire sarcolemmal surface and do not produce detectable cell lysis. The phospholipid classes are asymmetrically distributed: (1) the negatively charged phospholipids, PS/PI are located exclusively in the inner or cytoplasmic leaflet; (2) 75% of PE is in the inner leaflet; (3) 93% of sphingomyelin is in the outer leaflet; (4) 43% of PC is in the outer leaflet. The predominance of PS/PI and PE at the cytoplasmic sarcolemmal surface is discussed with respect to phospholipid-ionic binding relations between phospholipids and exchange and transport of ions, and the response of the cardiac cell on ischemia-reperfusion.

Introduction

Phospholipids of the plasma membrane of myocardial cells are thought to play an important role in the physiology as well as the pathology of the heart. The myocardial response to ischemia followed by reperfusion is attended by dramatic changes in the sarcolemma. We recently proposed a model for the disruption of the sarcolemma associated with ischemia-reperfusion, which is based on the physicochemical properties of the sarcolemmal phospholipids and the assumption of the presence of negatively charged phospholipids in the inner leaflet [1,2]. In addition, the impor-

* This paper is dedicated to Arthur Kornberg on the occasion of his 70th birthday.

** Reprint requests should be addressed to: G.A. Langer, Depts of Medicine and Physiology, Cardiovascular Research Laboratory, UCLA School of Medicine, Los Angeles, CA 90024, U.S.A.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; TNBS, trinitrobenzene sulfonic acid; HPTLC, high performance thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Correspondence: J.A. Post, Institute of Molecular Biology and Medical Biotechnology, State University of Utrecht, P.O. Box 80054, 3508 TB Utrecht, The Netherlands.

tance of sarcolemmal phospholipids in Ca^{2+} binding and possibly in the control of contraction is indicated by a series of studies [3–6]. These studies showed a strong correlation between sarcolemmal bound Ca^{2+} and contractile force. At least 80% of the sarcolemmal bound Ca^{2+} can be attributed to binding to the phospholipids [7]. In order that sarcolemmal Ca^{2+} relations be further defined, it is necessary to know the phospholipid composition and its distribution within the sarcolemma. Several studies have reported on the phospholipid composition of the sarcolemma [8–10], but nothing is known of the phospholipid distribution within the lipid bilayer. The distribution will have important implications for sarcolemmal Ca^{2+}–phospholipid interactions and would, therefore, have an impact on models for ischemia-reperfusion damage and for cardiac excitation-contraction coupling.

Phospholipid distribution in the sarcolemma of any myocardial tissue has, until now, not been reported. In erythrocytes and platelets it has been well established that the phospholipid classes are asymmetrically distributed over the two monolayers of the plasma membrane [11,12]. The negatively charged phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI) are almost exclusively present in the inner monolayer. The zwitterionic phospholipid phosphatidylethanolamine (PE) is preferentially present in the inner monolayer and the choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin are preferentially present in the outer monolayer.

There are a number of prerequisites for the study of phospholipid distribution in the plasma membrane of living cells: (1) a technique for preparation of relatively pure sarcolemmal membrane should be available; (2) the cells should not be contaminated with other cell types, e.g. endothelial cells and fibroblasts; (3) the various membrane probes used need to have complete access to the cells and their action should be nonlytic. The cultured neonatal rat heart preparation, with a unique method of membrane isolation, meets these requirements. Furthermore, the preparation has the advantage that detailed information is available on the Ca^{2+}-binding characteristics of the cells and their sarcolemma and of the physiology relevant to this binding. Thus, the findings might be related to function.

Therefore, the major goal of this study is the definition of phospholipid distribution in the sarcolemma of a myocardial cell. To define this distribution the cells were treated with two phospholipases A_2, sphingomyelinase C and trinitrobenzene sulfonic acid in order to hydrolyse or label phospholipids of the outer monolayer of the sarcolemma. To interpret these data it is necessary to know the phospholipid composition of the sarcolemma. Therefore, we have used the 'gas-dissection' method for preparation of the sarcolemma [13,14] and evaluated the purity of the isolated membranes.

Materials and Methods

Cell culture

Culturing of the cells was done according to a modification of the method of Harary and Farley [15]. Neonatal rats (1–2 days old) were decapitated, the hearts were excised and minced. The mince was incubated in a spinner flask at 37°C with 0.1–0.05% trypsin (in 137 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose and penicillin (100000 units/l)/streptomycin (100 mg/l). The incubation fluid was decanted and new medium was added. The supernatant from the first three incubations (15 min each) was discarded; during the following 6–8 incubations (10 min each), the mince was almost completely digested. The cell pellets were spun (8 min, $430 \times g$) and resuspended in growth medium (Gibco, Paisley, U.K.: Ham F10, supplemented with 10% fetal calf serum, 10% horse serum, penicillin (100000 units/l)/streptomycin (100 mg/l), arabinose C (10 μM, to inhibit fibroblast growth) and $CaCl_2$ (final concentration 1 mM)). The cells were plated on Falcon 3000 dishes for 2–3 h, during which time fibroblasts adhere and myocytes remain freely suspended [16]. Finally, the myocytes were plated on Primaria-treated culture disks (Falcon Plastics, Sumter, SC, U.S.A.) and within 3 days a confluent monolayer of spontaneously beating cells was formed. The disks used were cut from the bottom of 50 mm standard Primaria dishes and resterilized by exposure to ultraviolet light for 45 min on each surface. The disks were then placed in another culture dish and the cells allowed to settle and attach to the disk surface. Before use the disks

with cells attached were extensively washed with buffer W (133 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl, 5 mM glucose (pH 7.2)).

Isolation of the sarcolemma

The gas dissection technique has been previously described [13,14]. Briefly, the disk with cell monolayer attached is placed at the center of a platform in a stainless steel chamber. The chamber is closed. The platform, with disk, is then elevated so that a valve which extends into the chamber makes firm contact with the center of the disk. This valve is in series with a inlet valve outside the chamber that controls the entry of N_2 gas at 2000–2100 psi. The distal valve is conical such that its flat lower surface (8 mm diameter) sits flush on the center of the monolayer on the disk. The dimensions and configuration of the conical valve are of critical importance to the membrane isolation. It is designed so that upon rapid (< 1 s) opening of the inlet valve N_2 exits in a high velocity stream parallel to the surface of the monolayer. The circumferential opening of the valve is also critical. This is set at 0.3 mm. With an input pressure of 2000–2100 psi this opening gives a gas velocity that is optimal for membrane dissection. As the N_2 stream travels radially over the surface of the monolayer the upper surface of the cells is sheared open, the cellular material blown out and the sarcolemma left in a fenestrated layer and, in some areas, in a wrinkled or rolled form attached to the disks. The electron micrographs shown in Fig. 1 indicate the membrane configuration. Sections perpendicular to the plane of membranes showed a variety of configurations of the gas-dissected membranes (Fig. 1A). Planar sheets of membrane as well as rolled vesicular forms are found. Sections more oblique to the plane of membranes showed at some places, presumably the rolled parts, a configuration which very much resembles a vesicular sucrose gradient preparation (Fig. 1B).

Marker enzymes

The following marker enzymes were used to characterize the membrane preparation: Na^+/K^+-ATPase [17], vanadate insensitive Ca^{2+}-ATPase for the sarcoplasmic reticulum [18], succinate dehydrogenase for mitochondria [18]. To estimate the recovery of succinate dehydrogenase activity, cells were scraped from 50% of a disk, homogenized and enzyme activity was measured. The other half of the disk was gas-dissected an the activity of the enzyme in the membranes was measured. Protein analysis was carried out according to Lowry et al. [20].

Lipid extraction

Extraction was accomplished by immersion of the disk with attached cells or membranes in 4 ml isopropanol (Merck, analytical grade). After 15 min incubation no phospholipids could be detected in the material which remained on the disks. Thus, the standard immersion of 60 min assured complete extraction. A small amount of plastic was extracted during this procedure. This could be removed by evaporation of the isopropanol, redis-

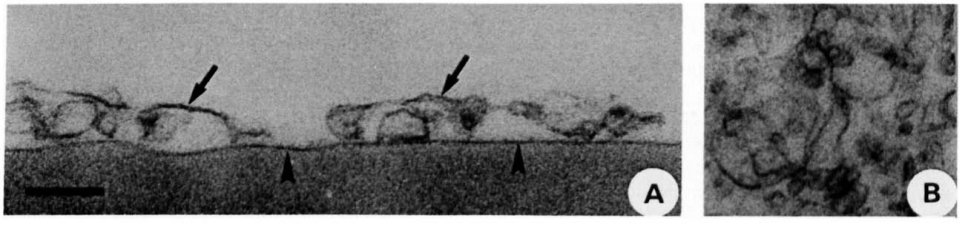

Fig. 1. Electron micrograph A shows the configuration of the gas-dissected membranes sectioned perpendicular to the disk surface. The dark line (arrowheads) running the length of the section represents the surface of the disk. The membranes are indicated by arrows. Fig. B shows a section oblique to the disk surface and a configuration which very much resembles a sucrose gradient preparation (Magnification: 64400×, bar represents 0.2 μm).

solving in chloroform/methanol (2:1, v/v) in which the plastic is not soluble and spinning down the plastic residue (5 min, 2000 × g). This leaves the lipids in the chloroform/methanol phase.

Lipid analysis

Total phospholipid content was determined by drying the samples, destruction of the phospholipids by 70% perchloric acid (30 min, 180°C) and measuring inorganic phosphorus [21]. Cholesterol content was determined with the use of an enzymatic kit (Boehringer) on a total lipid sample [22]. The phospholipid composition of each lipid extract was determined by two-dimensional thin-layer chromatography on HPTLC plates (Merck), which were first developed in chloroform/methanol/water/ammonia (90:54:5.5:5.5, v/v) followed by chloroform/methanol/water/acetic acid (90:40:12:2, v/v) according to Broekhuyse [23]. The individual phospholipid spots were detected by iodine, scraped, destructed and inorganic phosphate was determined as described above.

Phospholipase treatment

Incubation of the cells or membranes on disks with phospholipase A_2 was done with a mixture of bee venom phospholipase A_2 (Sigma) and *Naja naja* phospholipase A_2 (Sigma) at 37°C with gentle shaking. 5 I.U. of both enzymes were added per disk in 4 ml buffer W in which $CaCl_2$ was supplemented to 10 mM.

Incubation with a modified porcine pancreatic phospholipase A_2 (pal-AMPA) [24] was done under the same experimental conditions, only less phospholipase A_2 was used (2 I.U./disk of cells).

Sphingomyelinase C from *Staphylococcus aureus* was purified as described by Zwaal et al. [25]. Incubations were performed in buffer W, 37°C with gentle shaking. 5 I.U. of the enzyme in 4 ml buffer W was used to treat a disk of cells.

At the end of the incubations the disks were first extensively rinsed in buffer W and subsequently in this buffer with 50 mM ethylenediamine tetra acetic acid (EDTA) (Merck) to stop the phospholipase activity.

Labelling with trinitrobenzene sulfonic acid (TNBS)

Cells were incubated with 1 and 2 mM TNBS (Sigma) in buffer W (pH 8.0) at 4°C for 30 min. The incubation was stopped by removal of TNBS and replacement with buffer W containing 6 mM glycylglycine (Merck) at room temperature in order to remove unreacted TNBS (twice 30 min). After removal of the unreacted TNBS the lipids were extracted and analysed.

In order to estimate whether TNBS penetrated intracellularly labelling of whole cells was carried out for up to 60 min with samples taken at 10 min intervals. Further more, gas-dissected membranes were labelled with TNBS to estimate whether the phospholipids in the inner monolayer are able to react with TNBS. Under these conditions of pH TNBS does not react with the amino group of Tris.

Control of cell lysis

The extent of cell lysis during the treatments was determined by assay of the release of lactate dehydrogenase (LDH) from the cells [26]. The total amount of activity present in the incubation fluid was compared to the total activity present in the homogenate of a disk of cells.

Electron microscopy

The gas-dissected membranes were fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4), post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide, dehydrated with the use of methanol and finally a capsule containing Epon was placed on the disk with the attached membranes. After polymerization the capsules were snapped from the disks and the adherent membranes were sectioned parallel or perpendicular to the plane of the disk, using a diamond knife. The sections were mounted on copper grids, stained with uranyl acetate and examined in a Philips 301 electron microscope.

Results

Characterization of the gas-dissected membranes

The activity of typical marker enzymes present in the gas-dissected membranes and other characteristics of the membranes as compared with a homogenate of cultured cells are shown in Table I. The sarcolemmal marker, Na^+/K^+-ATPase was, due to the culturing of a pure population of myocardial cells, already enriched by a factor of

TABLE I

CHARACTERISTICS OF THE GAS-DISSECTED MEMBRANES, COMPARED TO HOMOGENATE OF CULTURED CELLS

Data are given as mean ± S.E., number of determinations is given in parenthesis.

	Cells	Membranes
Phospholipid (nmol/disk)	171.8 ± 6.9 (10)	32.8 ± 1.2 (36)
Phospholipid/protein (μmol/mg)	0.24 ± 0.01 (10)	1.40 ± 0.14 (19)
Cholesterol/phospholipid (nmol/nmol)	0.35 ± 0.01 (38)	0.49 ± 0.02 (18)
ATPase (0 mM K^+) [a]	4.2 ± 0.04 (8)	20.3 ± 1.4 (8)
ATPase (20 mM K^+) [a]	8.0 ± 0.04 (8)	59.4 ± 3.0 (8)
Na^+/K^+-ATPase [a]	3.8	39.1
Ca^{2+}-dependent vanadate-insensitive ATPase [a]	6.5 ± 0.2 (4)	non detectable (4)
Succinate dehydrogenase [b]	0.18 ± 0.02 (5)	0.48 ± 0.05 (5)

[a] μmol P_i/mg protein per h.
[b] μmol $DCIPH_2$/mg protein per h.

4.8 as compared to whole tissue homogenate of adult heart, which had a specific activity of 0.77 μmol/h per mg. Isolation of the sarcolemma produced a further 10-fold enrichment of this marker and thus a total specific activity increase of 50 was achieved. 46% of total cellular Na^+/K^+-ATPase activity was recovered. The marker enzyme of the sarcoplasmic reticulum (vanadate-insensitive Ca^{2+}-dependent ATPase) was not detectable in the gas-dissected membranes. The mitochondrial marker succinate dehydrogenase was present with even a small mitochondrial 'enrichment' (see Discussion). 13% of total cellular succinate dehydrogenase activity was retained. The cholesterol/phospholipid and phospholipid/protein ratios are increased (in the gas-dissected membranes as compared to whole cells) by 1.4 and 5.8, respectively.

The phospholipid composition of the gas-dissected membranes and of the cells is shown in Table II. Of interest is the fact that phosphatidic acid (PA) is present in the whole cells whereas it is non-detectable in the gas-dissected membranes. Since PA is an intermediate in phospholipid synthesis, most of which takes place in the sarcoplasmic reticulum, this observation is another indication of the absence of this intracellular organelle in the sarcolemmal preparation. Note that the membranes are somewhat enriched in sphingomyelin and consistent with the presence of succinate dehydrogenase activity (see Table I), the mitochondrial marker enzyme, cardiolipin is present. We detected 172 nmol (±6.9, $n = 10$) of phospholipid on a disk with cells prior to gas-dissection and 33 (±1.2, $n = 36$) nmol of phospholipid on a disk after membrane preparation.

Treatment with sphingomyelinase C

Incubation of the whole cells with sphingomyelinase C for up to 2 h gave no significant lysis of the cells. Lactate dehydrogenase activity released in the incubation fluid was less than 2% of total activity, which was not significantly greater than that seen without the enzyme present. After at least 60 min of treatment sphingomyelin degradation reached a plateau at a level of 55% of total cellular sphingomyelin hydrolyzed (Table III). When sphingomyelinase C incubation was stopped and cells were subsequently gas-dissected, lipid

TABLE II

PHOSPHOLIPID COMPOSITION OF CULTURED NEONATAL HEART CELLS AND THEIR GAS-DISSECTED MEMBRANES

Sph, sphingomyelin.

	Phospholipid composition (%)	
	Cells ($n = 7$)	Membranes ($n = 4$)
PE	26.2 ± 0.8	28.0 ± 0.6
PC	49.6 ± 1.5	49.6 ± 0.7
PS/PI	9.0 ± 1.6	6.5 ± 1.0
Sph	8.9 ± 1.1	10.9 ± 0.8
CL	3.5 ± 0.3	2.5 ± 0.2
PA	1.5 ± 0.4	non detectable
?	1.2 ± 0.3	2.4 ± 0.5

TABLE III

ACCESSIBILITY OF PHOSPHOLIPIDS TO REAGENTS IN INTACT NEONATAL MYOCARDIAL CELLS

The accessibility of PC and PE have been determined by measuring the decrease in PC or PE and the increase of the lyso compounds or labelled compound. In the case of sphingomyelin the decrease of sphingomyelin was used to determine the accessibility. Data are expressed as mean percent of each phospholipid class ± S.E. (n).

Probe	PC	PE	PS/PI	Sphingomyelin
Phospholipase A_2	17.6 ± 0.6 (7)	7.7 ± 1.0 (8)	0.0 (8)	-
Pal-AMPA	16.7 ± 0.3 (11)	10.9 ± 0.4 (3)	0.0 (11)	
Sphingomyelinase C	-	-	-	55.3 ± 1.0 (4)
Trinitrobenzene-sulfonic acid	-	9.5 ± 0.3 (22)	0.0 (22)	-

analysis showed that up to 90% of the sphingomyelin present in the membranes was degraded. This clearly shows that sphingomyelinase C has access to the attached surface of the cell and thus interacts with the entire sarcolemmal surface. With the use of these data the percentage of cellular phospholipids present in the sarcolemma can be obtained using the method of Chap et al. [27] (see Discussion).

Incubation with phospholipase A_2

Since phospholipase A_2 is a smaller molecule than sphingomyelinase C (M_r 13600 and 38000, respectively) and sphingomyelinase C has complete access to the entire sarcolemmal surface it is likely that phospholipase A_2 also interacts with the entire sarcolemmal surface. Phospholipase A_2 treatment of the cultured neonatal cells did not cause a significant lysis of the cells (less then 2.0%). A plateau was reached after 60 min of incubation at which time 17.6% of the total cellular PC was degraded (Table III). A subsequent incubation of 60 min, with the addition of sphingomyelinase did not increase this hydrolysis percentage. In whole cells 7.7% of total PE was hydrolysed by phospholipase A_2 treatment. No degradation of the negatively charged phospholipids PS and PI could be detected (Table III). Incubation of gas-dissected membranes with phospholipase A_2 resulted in a complete degradation of the glycerophospholipids indicating full activity of the enzyme preparation and complete accessibility of the phospholipids after gas-dissection.

Incubations with pal-AMPA

Treatment of the cells with this phospholipase A_2 did not cause any significant lysis of the cells either. A plateau of phospholipid hydrolysis was reached after 15 min of incubation, at which time 16.7% of total cellular PC was degraded. Of the total cellular PE 10.9% could be degraded. No degradation of the negatively charged phospholipids PS and PI could be detected (Table III).

Labelling with TNBS

At low temperature (4°C) and at concentrations of 1 and 2 mM the amine-probe TNBS did not penetrate the sarcolemma, since a plateau of PE labelling is reached after 20 min of incubation with an excess of TNBS present. Labelling of the gas-dissected membranes resulted in labelling of 88% of the PE present. It could, therefore, be used to localize PE and PS in the intact myocardial cells. In the lipid extracts of previously labelled cells 9.5% of the total PE was labelled. No labelling of PS could be detected (Table III).

Discussion

Purity of the sarcolemmal preparation

Isolation of the sarcolemma of cultured myocardial cells has the advantage that the isolated plasmamembrane fraction will not be contaminated by plasma membrane fractions of endothelial cells or fibroblasts. Comparison of the data summarized in Table I with other reported pre-

parations [8,9,10] shows that our sarcolemmal preparation is highly purified. The 50-fold enrichment of the Na^+/K^+-ATPase is one of the higher ones reported. The cholesterol/phospholipid and phospholipid/protein ratios are increased compared to whole cells and are comparable to other reported values.

Contamination of the gas-dissected membranes with sarcoplasmic reticulum could not be detected (no vanadate insensitive Ca^{2+}-ATPase activity) and the absence of PA in the gas-dissected membranes indicates little retention of this intracellular organelle. The recovery of cellular Na^+/K^+-ATPase activity in membranes is 46%. This is extremely high compared to other reports (20%, [10]; 4.3%, [9]; 26%, [8]), and is in agreement with the high protein yield of 4.6% in the gas-dissected preparation.

The mitochondrial marker succinate dehydrogenase was somewhat enriched (2.7-fold, Table I) in the gas-dissected membranes, indicating a small 'co-purification' of mitochondria. However, it is important to note that the specific activity found in the membrane is comparable to other reported values [8]. Because of the presence of succinate dehydrogenase-activity in the membranes it is not surprising that we find cardiolipin in the gas-dissected membranes (Table II), since cardiolipin is a phospholipid which is characteristic of the mitochondria [28]. Others have reported the presence of cardiolipin in sarcolemma and the question arises whether all the cardiolipin found in the gas-dissected membranes is of mitochondrial origin or whether there is, indeed, cardiolipin present in the sarcolemma. Therefore, we compared the recovered succinate dehydrogenase-activity in the gas-dissected membranes with the cardiolipin retention. 14% of total cellular cardiolipin was present in the membranes as compared to 13% of total succinate dehydrogenase activity. These almost identical values and the fact that both are present in the inner mitochondrial membranes indicate very strongly that all the CL present in the gas-dissected membranes is of mitochondrial origin. The specific activity (μmol/h per mg protein) of succinate dehydrogenase in terms of protein content is indeed increased (Table I), but if one expresses the specific activity based on phospholipid content (μmol/h per μmol P_i) a decrease is found. This indicates that the mitochondria that are retained have been broken up and soluble mitochondrial protein as well as other soluble cellular protein has been washed away.

TABLE IV

'PURIFICATION' OF THE GAS-DISSECTED MEMBRANES

Sph, sphingomyelin.

	Gas-dissected membranes		Mitochondria (Daum [29])		'Purified' sarcolemma	
	% PL (*a*)	nmol (*b*)	% PL (*c*)	nmol (*d*)	nmol (*b* − *d*)	% PL
CL	2.5	2.5	12.0	2.5	0.0	0.0
PE	28.0	28.0	40.0	8.3	19.7	24.9
PC	49.6	49.6	41.0	8.5	41.1	52.0
PS/PI	6.5	6.5	4.0	0.8	5.7	7.2
Sph	10.9	10.9	1.0	0.2	10.7	13.5
?	2.4	2.4	2.0	0.5	1.9	2.4
Total	100.0	100.0	100.0	20.8	79.1	100.0

We undertook several approaches to remove the mitochondrial contamination, but were not successful. Therefore we 'purified' the gas-dissected membranes in an artificial manner: purified rat heart mitochondria contain about 12% cardiolipin [29] and our gas-dissected membranes 2.5%. This implies that the mitochondrial membranes have been 'diluted' 4.8-fold and thus that 20.8% of the phospholipid of the gas-dissected membranes is of mitochondrial origin. Table IV shows the sequence used for 'purification', which is possible because the mitochondrial phospholipid composition [29] as well as the percentage of mitochondrial contamination of the gas-dissected preparation is known. The 20.8% of phospholipids present in the gas-dissected membranes is distributed over the phospholipid classes according to the mitochondrial phospholipid composition (columns c and d). Substraction of column d from column b gives the phospholipid content of the 'purified' sarcolemma.

Mitochondrial phospholipids are not hydrolysed during the phospholipase A_2 and sphingomyelinase C treatment of the whole cells since the phospholipases have access only to the outer monolayer of the sarcolemma. Thus, the percentage of degraded phospholipids in the sarco-

lemma is underestimated because of the mitochondrial contamination. For example, correction for this contamination shows that in the 'purified membranes', obtained after treatment with sphingomyelinase C, 12.4% (instead of 9.9%) of the total phospholipids is degraded.

Percent of cellular phospholipids present in the sarcolemma

With the use of the data obtained after the sphingomyelinase C treatment one can estimate the percentage of cellular phospholipid present in the sarcolemma. This is necessary in order to calculate the lipid distribution of the sarcolemma on the basis of the probes used on whole cells, since these cells possess a considerable amount of intracellular membrane. The percentage of cellular phospholipid present in the sarcolemma can be calculated using the method of Chap et al. [27].

Sphingomyelin hydrolysis occurred under nonlytic conditions and the sphingomyelinase C activity was blocked at the end of the incubation. This means that the degraded sphingomyelin is derived solely from the phospholipid pool of the plasma membrane. If one expresses the hydrolysed sphingomyelin as percentage of total phospholipid of whole cells (4.7%) and of 'purified sarcolemma' (12.4%), one can deduce the amount of phospholipid present in the sarcolemma, relative to the whole cells. Thus, the percentage of sphingomyelin degraded in whole cells divided by the percentage of sphingomyelin degraded in pure sarcolemma = 4.7/12.4 = 0.38. Thus, 38% of total cellular phospholipid is located in the sarcolemma. With this value one can estimate the amount of each phospholipid class in the sarcolemma.

Percent of each sarcolemmal phospholipid class in the cell

The percent of each of the sarcolemmal phospholipids of total cellular phospholipid is derived from: (percent of class in 'purified' sarcolemma (last column Table IV)) × (percent of total cellular phospholipid in sarcolemma (38%)). The values appear in the first column of Table V. The percent of each class of the total of that particular class in the cell is then derived from: (value in first column of Table) : (percent of class in the whole cell (first column, Table II). These are listed in the second column of Table V.

TABLE V

PERCENT OF EACH SARCOLEMMAL PHOSPHOLIPID CLASS IN THE CELL

Sph, sphingomyelin.

	% of total cellular phospholipid	% of each phospholipid class
PE	9.5	36.1
PC	19.8	39.8
PS/PI	2.7	30.0
Sph	5.1	57.3
?	0.9	75.3

Membrane recovery

We calculate that about 57% of cellular sphingomyelin is present in the sarcolemma, which is 5.1% of total cellular phospholipid (Table V). The average amount of phospholipid on a disk of cultured cells is 172 nmol (Table I). Thus, there are 8.7 nmol sphingomyelin in the sarcolemma of these cells. In the 'purified' gas-dissected sarcolemma 3.5 nmol sphingomyelin is present. This gives a 'purified' membrane retention of 40.3% (3.5 : 8.7). This is in agreement with the high plasma membrane marker enzyme Na^+/K^+-ATPase recovery of 46% in the preparation.

Transbilayer distribution of phospholipids in the sarcolemma

Tables III and V summarize the data from which the transbilayer distribution of the sarcolemmal phospholipids can be obtained. From these data we derive the fraction of sarcolemmal phospholipid present in the outer monolayer from:

TABLE VI

RELATIVE AMOUNTS (%) OF SARCOLEMMAL PHOSPHOLIPIDS PRESENT IN THE OUTER MONOLAYER

Sph, sphingomyelin.

Probe	PC	PE	PS/PI	Sph
Phospholipase A_2	44.2	21.3	0.0	–
Pal-AMPA	42.0	30.0	0.0	–
Trinitrobenzene-sulfonic acid	–	26.3	0.0	–
Sphingomyelinase C	–	–	–	93.0

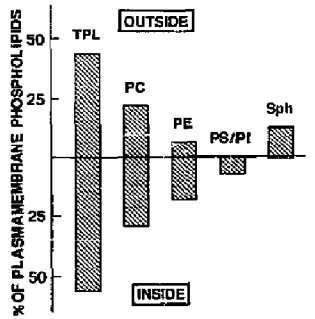

Fig. 2. Phospholipid composition and transbilayer distribution in the sarcolemma of cultured rat heart cells. (TPL, total phospholipids; Sph, sphingomyelin).

(fraction of particular phospholipid class accessible to probe in the whole cell (Table III) : (fraction of its class present in the sarcolemma (Table V)). The values found with the various probes are summarized in Table VI and outside/inside ratios presented in Fig. 2. It can be seen that PS and PI are exclusively located in the inner monolayer of the sarcolemma and sphingomyelin is almost exclusively located in the outer monolayer. PE is preferentially present in the inner monolayer, whereas about 43% PC is in the outer monolayer.

Comparison of the phospholipid asymmetry of the sarcolemma from cultured rat heart cells with the phospholipid distribution in plasma membranes of other cell types shows the same tendency. The negatively charged phospholipids (PS and PI) and PE are preferentially present in the inner monolayer [11,12] and the choline-containing phospholipids (PC and sphingomyelin) have a preference for the outer monolayer. Despite differences among several cell types this general distribution seems to be present in all.

Implications of the data

Since we now know the phospholipid composition of the sarcolemma of the cultured neonatal myocardial cells and we know the amount of Ca^{2+} bound to these membranes, one can estimate the stoichiometry of the Ca^{2+} phospholipid binding. Previous results [14] showed that the gas-dissected membranes contained 19.1 μg protein/mg dry cell weight per disk, which means that the average disk (32.4 μg protein) contained 1.7 mg dry weight of whole cells. Lanthanum (La^{3+}) has been used to displace Ca^{2+} bound at the surface of intact cells, which was shown to be the equivalent of Ca^{2+} displaced from the outer as well as the inner surface of gas-dissected membranes [14]. This probe displaces 3.3 mmol/kg dry weight cells ($[Ca^{2+}]_o = 1$ mM) or 5.6 nmol Ca^{2+} per disk of cells. One disk of whole cells contains 65 nmol of sarcolemmal phospholipids (0.38×172 nmol) of which 20 nmol is PI, PS and PE, the possible candidates for Ca^{2+} binding. This gives a phospholipid/Ca^{2+} stoichiometry (at $[Ca^{2+}]_o = 1$ mM) of 3.6 (20/5.6), a value which is not unreasonable.

The presence of all the negatively charged phospholipids and most of the PE in the inner monolayer of the sarcolemma has serious implications for the Ca^{2+} binding to these phospholipids and for their possible role in excitation-contraction coupling. It has been shown that the K_d of this sarcolemmal bound calcium is about 1 mM [30]. Intracellular Ca^{2+} concentration in the myocardium varies between $5 \cdot 10^{-6}$ and 10^{-7} M and therein lies a problem. At these Ca^{2+} concentrations only a small percentage of the putative inner monolayer Ca^{2+} binding sites would be occupied. It might be speculated that close to the inner monolayer a micro-environment exists in which there is a much higher Ca^{2+} concentration. A candidate for the micro-environment region is the space between the junctional sarcotubular system and the inner sarcolemmal leaflet, the space in which the 'foot-like' processes extending from the sarcotubules to the sarcolemma are found [31,32].

With respect to the localization of the anionic phospholipids in the inner monolayer it should be noted that both the Na^+/Ca^{2+} exchanger and the sarcolemmal Ca^{2+} pumps are markedly stimulated by the addition of anionic phospholipid to vesicular preparations in vitro [33,34]. If this interaction between anionic phospholipids and the transport systems is also present the in vivo situation, then the present study would suggest that such interaction is at the inner monolayer.

Under pathological conditions Ca^{2+} has been suggested to induce membrane damage [35]. During ischemia Ca^{2+} is lost from the sarcolemma

[36] and restoration of perfusion with repletion of Ca^{2+} is associated with Ca^{2+} influx into the cells [37]. The changes observed by electron microscopy, aggregation of the intramembranous particles of the sarcolemma and the extrusion of lipids [2,38] can only be interpreted by assuming that the negatively charged phospholipids, i.e. PS and PI, are asymmetrically distributed. The present study clearly confirms this lipid asymmetry. All PS and PI, and most of the non-bilayer preferring phospholipid PE, are present in the inner monolayer. This makes it possible that the increase in intracellular Ca^{2+} upon reperfusion can produce the observed destabilization and disruption of the sarcolemma by interaction with negatively charged phospholipids in the inner monolayer of the sarcolemma.

In summary, this study shows that the phospholipids of the sarcolemma of myocardial cells are asymmetrically distributed. The negatively charged phospholipids are exclusively and the zwitterionic PE is preferentially located in the cytoplasmic leaflet. This lipid asymmetry may have significant implications with respect to the control of Ca^{2+} movements at the sarcolemma.

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