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Plasmalogen content and distribution in the sarcolemma of cultured neonatal rat myocytes

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Phospholipids are believed to play an important role in pathology and physiology of the myocardium. Because of the distinct physico-chemical properties of plasmalogens we studied the plasmalogen content and distribution in the sarcolemma of cultured rat myocytes. Treatment with phospholipase A_2 degraded all glycerophospholipids in the outer monolayer. The hydrolysis products were analyzed for plasmalogen content. It is shown that the inner sarcolemmal leaflet is highly enriched in phosphatidylcholine and ethanolamine plasmalogen. This distribution of the plasmalogens might affect bilayer stability and thereby be involved in the destruction of the sarcolemma upon ischemia and reperfusion.

Phospholipid asymmetry; Plasmalogen; Plasma membrane; Phospholipase; (Cultured myocyte; Neonatal rat heart)

1. INTRODUCTION

Phospholipids play an important role in the physiology and pathology of the myocardium. They are believed to be involved in the destruction of the sarcolemma during the calcium paradox [1] and ischemia reperfusion [2–4], as well as in excitation-contraction coupling in the myocardium [5,6]. Based on our morphological observations [3,4] we proposed that the physico-chemical characteristics of the sarcolemmal phospholipids play a key role in the destabilization and destruction of the sarcolemma during reperfusion after prolonged ischemia [7]. Since, in this hypothesis, the localization of the phospholipids in the sarcolemma is considered to be of particular importance, we recently established the phospholipid distribution over the two halves of the sarcolemmal lipid bilayer [8]. This study showed a highly asymmetric distribution: negatively charged phospholipids were found to be exclusively present in the inner monolayer, this leaflet also containing 75% of the phosphatidylethanolamine (PE) and 57% of the phosphatidylcholine (PC), sphingomyelin being largely (93%) confined to the outer monolayer.

The physico-chemical properties of a phospholipid are determined not only by its polar head group, but also by the hydrophobic part of the molecule [9]. Both the fatty acid composition as well as the nature of the bonds between the aliphatic chains and glycerol backbone should be considered in this respect. The phospholipids of many biological membranes can be subdivided into diacyl and ether glycerophospholipids. In the latter group, the aliphatic chain at the *sn*-1 position is linked to the glycerol moiety by an ether rather than an ester bond. In most biological preparations, two types of ether phospholipids may occur [10]: (i) the 1-(1′-alkenyl)-2-acylglycerophospholipids or plasmalogens, having a *Z* double bond in the 1-alkenyl moiety next to the ether linkage; and (ii) the 1-alkyl-2-acylglycerophospholipids, which have a saturated bond at this position.

Ether phospholipids are less polar than the ester

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analogues because of the absence of the ester carbonyl dipole(s), whereas they also differ in the steric arrangement of their respective 1′-alkenyloxy, alkoxy and acyloxy groups [9]. Because of these differences in chemical structure, the subclasses possess different physico-chemical properties. This has been clearly shown in studying the phase behaviour of multilamellar dispersions of the three PE subclasses, which contained identical aliphatic moieties [11]. It was shown that the phase transition temperature from the gel to the liquid crystalline state for 1-(1′-alkenyl)-2-acylglycerophosphatidylethanolamine (GPE) is about 5–6°C lower than that of the corresponding 1-alkyl-2-acyl-GPE and diacyl-GPE [11,12]. ^{31}P-NMR showed that the liquid to hexagonal II phase transition temperature (T_h) of plasmalogen PE (30°C) is dramatically lowered when compared to 1-alkyl-2-acyl-GPE (53°C) and diacyl-GPE (69°C) [11,13].

As it is obvious that the plasmalogens may contribute to the overall physico-chemical properties of the lipid bilayer of the sarcolemma, we determined the content as well as the transbilayer distribution of these phospholipid species.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Culturing of cells was performed according to a modification of the method of Harary and Farley [14]. Neonatal rat (1–2 days old) hearts were excised, minced and trypsinized as in [8]. Cells were plated on Falcon 3000 dishes for 2–3 h, during which fibroblasts adhere and myocytes remain freely suspended [15]. Finally, myocytes were plated on Primaria-treated culture disks (Falcon Plastics, Sumter, SC) and within 3 days a confluent monolayer of spontaneously beating cells was formed. Before use, on the third day, the disks with attached cells 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).

2.2. *Isolation of the sarcolemma*

The gas dissection technique that we used has been described [8,16]. Briefly, the disk with attached cell monolayer is placed at the centre of a platform in a stainless-steel chamber. The chamber is closed and the platform that supports the disk is then elevated so that a valve, which extends into the chamber, makes firm contact with the centre of the disk. The construction of this valve is such that, upon rapid (< 1 s) opening of the inlet, a burst of highly compressed N_2 produces a stream of high velocity parallel to the surface of the monolayer. This stream of N_2, which travels radially over the surface of the monolayer, tears open the upper surface of the cells, thereby blowing away any intracellular material and leaving the sarcolemma attached to the disk. Previous characterization [7] showed that such membranes are of high purity, although a small degree of mitochondrial contamination might occur.

2.3. *Phospholipase treatment*

Incubation of cells with phospholipase A_2 was carried out with a mixture of bee venom phospholipase A_2 (Sigma) and *Naja naja* phospholipase A_2 (Sigma) at 37°C under gentle shaking for 75 min. 5 IU of both enzymes were added per disk in 4 ml buffer W in which $CaCl_2$ was supplemented to 10 mM. At the end of incubations, disks were first extensively rinsed in buffer W and subsequently in this buffer containing 10 mM EDTA (Merck) to arrest phospholipase activity. Lysis of the cells was determined by measuring the release of lactate dehydrogenase [17].

2.4. *Lipid extraction and isolation of different phospholipid classes*

Extraction was accomplished by immersion of the disk with the attached cells or membranes in 4 ml isopropanol (Merck, analytical grade) for 60 min, which has been shown to extract all the lipids [8]. The phospholipids of gas-dissected membranes and of untreated cells were separated by two-dimensional thin-layer chromatography on HPLC plates (Merck). Phospholipids of phospholipase-treated cells were separated by two-dimensional thin-layer chromatography on TLC plates (Merck). Plates were first developed in chloroform/methanol/water/ammonia (90:54:5.5:5.5, v/v) followed, after drying, by chloroform/methanol/water/acetic acid (90:40:12:2, v/v) according to Broekhuyse [18]. Individual phospholipid spots were detected by mild iodine exposure and identified by using pure standards. After removal of iodine, the silica spots containing PC, PE, lyso-PC and lyso-PE were scraped off and phospholipids extracted as described by Arvidson [19].

2.5. *Hydrolysis of bovine brain PE and pig brain PC to LPE and LPC*

Bovine brain PE (Sigma) or isolated pig brain PC were dissolved in ether/methanol (98:2, v/v). Phospholipase A_2 (bee venom and *N. naja*, 1:1, U/U) was dissolved in 10 mM Tris-HCl (pH 7.4) containing 10 mM Ca^{2+} and added to the lipids. The reaction was performed by continuous vigorous stirring at room temperature. At the end of incubation, the mixture was centrifuged briefly to achieve good phase separation, the ether phase collected and the aqueous phase washed twice with ether. The reaction was monitored by using one-dimensional TLC on HPLC plates using the above acidic solvent system.

2.6. *Determination of plasmalogen content*

Lipid extracts were dried under a stream of N_2, dissolved in 100 μl chloroform/methanol (1:2, v/v) and applied to an HPLC plate. In order to cleave the acid-labile 1′-alkenyl ether bond of the plasmalogens, the HPLC plates were placed in the fumes of concentrated HCl [20] for 11 min. After drying, plates were developed using the above basic solvent system. The various products were visualised by iodine vapour and phosphorus reagent, scraped off and analyzed for phosphorus content [21] after destruction with 70% perchloric acid (30 min, 180°C). It should be noted that the products of acid hydrolysis

of the 1'-alkenyl ether bond in lyso-PC and lyso-PE, i.e. glycero-3-phosphocholine and -ethanolamine, remain at the origin of the TLC plate and do not react with the phosphorus reagent.

3. RESULTS

Determination of the plasmalogen content of PC and PE in whole cells and of isolated sarcolemma was carried out directly on total lipid extracts as described in section 2. In order to assess the distribution of PC and PE plasmalogens over the two monolayers of the sarcolemma, the following procedure was used: cells were treated with exogenous phospholipase A_2 in order to degrade all glycerophospholipids present in the outer monolayer of the sarcolemma. This can be achieved without cell lysis [8]. Lipids were extracted from the phospholipase-treated samples and degraded phospholipids isolated and analyzed with respect to plasmalogen content.

Table 1 shows the plasmalogen content of PC and PE isolated from cultured neonatal cells as well as gas-dissected membranes. The plasmalogen content of PE is markedly higher than that of PC. Furthermore, the plasmalogen content of either phospholipid in whole cells is slightly higher than that in the gas-dissected membranes.

Treatment of cells with phospholipase A_2 did not cause any significant lysis of myocardial cells, as assessed from the absence of any significant release of lactate dehydrogenase by cells during incubation. Incubation with phospholipase A_2 resulted in hydrolysis of about 10% of total cellular PE and 19% of total cellular PC. These degraded fractions of PE (10%) and PC (19%) account for the hydrolysis of all PE and PC present in the outer monolayer of the sarcolemma [8]. The plasmalogen content of each of these two lysophospholipids was significantly lower than that of the corresponding native phospholipids in the gas-dissected membranes (table 1).

In order to ascertain that the determination of the plasmalogen content of lysophospholipids can indeed be carried out by using the same technique as is normally used in the case of intact phospholipids, bovine brain PE and pig brain PC were completely hydrolysed by phospholipase A_2. Thereafter, the above procedure was applied to determine the plasmalogen content of both the original phospholipids and the two lysophospholipids derived from them. The plasmalogen content of lyso-PE, derived by complete hydrolysis of bovine brain PE, is essentially identical to that of the original PE (table 1). The same is observed for PC.

Table 1

Plasmalogen content of phospholipids of myocardial cells, their isolated sarcolemma, and of purified phospholipids and their completely hydrolyzed lysophospholipids

	% plasmalogen	
Cell PE	43.3 ± 5.3 (8)	
Cell PC	18.8 ± 2.7 (6)	
Gas dissected membrane PE	39.6 ± 2.5 (7)	
Gas dissected membrane PC	15.6 ± 1.4 (7)	
LPE	24.2 ± 2.4 (8)	$p < 0.001$
LPC	11.3 ± 0.8 (4)	$p < 0.005$
Bovine brain PE	61.8 ± 1.7 (3)	
LPE (derived from B.b.PE)	59.3 ± 0.7 (3)	
Pig brain PC	6.2 ± 1.3 (3)	
LPC (derived from P.b.PC)	8.6 ± 2.7 (3)	

Means ± s (n); p: values of unpaired, two-tailed t-test compared to values of gas-dissected membranes

4. DISCUSSION

The unsaturated ether bond found in plasmalogens is cleaved by HCl vapour [20], resulting in a long-chain aldehyde and a 2-acyl-*sn*-glycero-3-phospholipid, which can be easily separated from other phospholipids. Using pig brain PC and bovine brain PE, as well as the corresponding lyso derivatives that had been derived from them by phospholipase A_2 treatment, it could be demonstrated that cleavage of the vinyl ether linkage in a 1-alkenyl lyso derivative is as effective as in the case of the original 1-alkenyl-2-acyl-glycerophospholipids. This proves that the plasmalogen content of lysophospholipids, obtained after phospholipase A_2 treatment of intact cells, can be determined by using exposure to HCl vapour and subsequent one-dimensional TLC and phosphorus analysis. Furthermore, it appeared that incubation of bovine brain PE and pig brain PC with phospholipase A_2 from bee venom and *N. naja* venom resulted in the complete hydrolysis of these phospholipids. This is in agreement with the observation that phospholipase A_2 from *N. naja*, unlike most other phospholipases [22], shows high

activity toward all three subclasses of PC [23] and may therefore be used for deacylation of the three subclasses [22]. We therefore used this enzyme, in combination with bee venom phospholipase A_2, to treat cells and the results indeed indicate complete hydrolysis of all glycerophospholipids in the outer monolayer of the sarcolemma.

The data presented in table 1 can be used to calculate the distribution of plasmalogens over the two monolayers of the sarcolemma. We have previously shown that 43% of the total sarcolemmal PC is present in the outer monolayer [8]. It now appears that 11.3% of this PC fraction, determined as lyso-PC, is present as plasmalogen (table 1). This implies that $(43 \times 0.0113 =)$ 4.9% of the sarcolemmal PC is present in the outer monolayer in the form of plasmalogen and that consequently $(43 - 4.9 =)$ 38.1% is present as diacyl- and alkylacyl-GPC. The inner monolayer of the sarcolemma, containing 57% of the sarcolemmal PC, then contains $(15.6 - 4.9 =)$ 10.7% of the sarcolemmal PC as plasmalogen and 46.3% as diacyl- and alkylacyl-GPC.

A similar calculation shows that of the 25% of the sarcolemmal PE we have shown to be present in the outer monolayer [8] 24.2% consists of the plasmalogen form (table 1), representing 6.1% of total sarcolemmal PE. The inner monolayer, which contains 75% of the sarcolemmal PE, then contains 33.5% of the sarcolemmal PE in the plasmalogen form.

These results, summarized in fig.1, clearly show that the plasmalogen forms of PC and PE are both asymmetrically distributed in the sarcolemma. Not less than 84% of plasmalogen PE is present in the inner monolayer, whereas this layer accommodates 74% of total sarcolemmal PE. This implies that the inner monolayer is highly enriched in plasmalogen PE, compared to the outer monolayer. Plasmalogen PE accounts for 44.7% of PE in the inner monolayer and for only 24.2% of that in the outer sarcolemmal leaflet. Similar calculations show that, of the total PC in the inner monolayer, 18.8% is plasmalogen, this value being 11.3% for the outer monolayer pool.

It should be noted that the sarcolemmal preparation we used has some mitochondrial contamination [8] which, by itself and in the case where mitochondria might have a very high plasmalogen content, could already partly explain the above

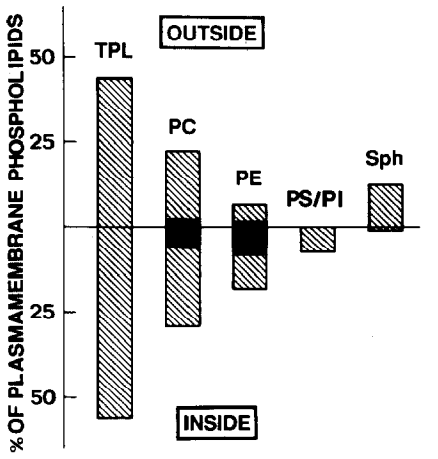

Fig.1. Phospholipid composition and transbilayer distribution in the sarcolemma of cultured rat heart cells. PC and PE plasmalogens are indicated by the black portion of the bars (TPL, total phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS/PI, phosphatidylserine/-inositol; Sph, sphingomyelin).

results. Gross [24], however, showed that cardiac mitochondria contain little plasmalogen PE compared to sarcolemma. The plasmalogen content we determined for the PC and PE from the gas-dissected membranes is lower than that reported for canine sarcolemma [25]. However, it has been shown that the plasmalogen content may vary between myocardial tissue of different species [26].

The transbilayer distribution of plasmalogens reported here is at variance with that reported for the red cell membrane. By using trinitrobenzenesulfonic acid no significant differences in the molecular species composition of PE localized in the inner and outer monolayer were observed [27]. It was observed recently that the relative amounts of plasmalogen PE in both outer and inner monolayer pools of PE in the human red cell membrane are essentially identical (Middelkoop, E., personal communication).

One interesting aspect of the relatively high plasmalogen content of the inner monolayer of the sarcolemma might be a role in the control of cellular calcium. High plasmalogen contents seem to be a characteristic of electrically active tissue which suggests some involvement in the control of excitation. It has been shown that choline plasmalogens appear to differ in their molecular dipole as compared with diacyl-GPC [9,28] and ex-

hibit a decreased surface potential vs diacyl-GPC [28]. Seelig et al. [29] have demonstrated that the binding/adsorption of metal ions modifies the membrane surface potential. Conversely, metal ions may be attracted to, or released from a membrane surface by changes in its surface potential. It might very well be that this mechanism, together with specific properties of the plasmalogens, is involved in excitation/contraction coupling, in which sarcolemmal bound calcium is believed to play a central role [5,6].

Another important aspect of the relative high plasmalogen content, especially of PE plasmalogen, of the sarcolemmal inner leaflet is the rather low lamellar to hexagonal II transition temperature (T_h) of the PE plasmalogen as compared with diacyl- and alkylacyl-GPE [11,13]. We recently proposed [7] that an increase in intracellular Ca^{2+} during reperfusion after prolonged ischemia, causes a lateral phase separation of the phospholipids in the inner monolayer of the sarcolemma. Due to this separation, the lipid bilayer destabilizes because of the 'no longer controlled' non-bilayer behaviour of PE. The present data show that plasmalogen PE accounts for 45% of the PE present in the inner monolayer of the sarcolemma, having a relatively low T_h. This implies that the inner monolayer of the sarcolemma is in a rather unstable bilayer configuration, which might be easily disturbed by the occurrence of lateral phase separations, for instance those caused by an increase of cytosolic Ca^{2+} upon prolonged ischemia and reperfusion.

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