Loss of Asymmetric Distribution of Sarcolemmal Phosphatidylethanolamine During Simulated Ischemia in the Isolated Neonatal Rat Cardiomyocyte


In the present study we have investigated the reorganization of the sarcolemmal phospholipids during the first 60 minutes of simulated ischemia ("ischemia") as induced by anoxia, volume restriction, and nutrient deprivation. Experiments were carried out on [3H]acetate-labeled neonatal rat cardiomyocytes and isolated (nonradiolabeled) sarcolemmal membranes obtained from the same culture system. After 60 minutes of "ischemia," cellular high-energy phosphate (ATP) levels had decreased to approximately 40% of the control values, but no significant phospholipid hydrolysis was detected. Labeling experiments using the nonpermeant (primary amine-containing phospholipid) probe trinitrobenzenesulfonic acid and nonlytic treatment with (different) exogenous phospholipases A2 were both indicative of a shifted transbilayer distribution of the hexagonal H phase-prefering and fusion-promoting sarcolemmal phosphatidylethanolamine in favor of the outer membrane leaflet. This specific change in sarcolemmal phospholipid asymmetry preceded the loss of integrity of the sarcolemma, monitored by the release of lactate dehydrogenase as well as by scanning electron microscopy. It is proposed that, in addition to the previously reported lateral phospholipid reorganization, uncontrolled transbilayer movement of the non-bilayer-prefering phosphatidylethanolamine from the inner to the outer leaflet of the sarcolemma is an additional factor in destabilizing the lipid bilayer, eventually leading to the irreversible membrane damage seen after a prolonged period of ischemia. (Circulation Research 1993;73:514-523)

KEY WORDS • neonatal rat cardiomyocytes • simulated ischemia • sarcolemma • phospholipid asymmetry • phosphatidylethanolamine • hexagonal H phase • irreversible membrane damage

A n increasing body of evidence has shown that the development of myocardial membrane dysfunction plays an important role in the pathogenesis of ischemic myocardial cell injury. It is known that ischemia induces a complex series of noxious alterations such as energy depletion, cellular acidosis, redistribution of cellular calcium ions, and accumulation of metabolic intermediates (eg, acyl-CoA esters, long-chain acyl carnitines, and lysophospholipids), which may all contribute to sarcolemmal dysfunction. However, the exact mechanism(s) underlying irreversible ischemic membrane damage remains to be established.

To study molecular changes occurring in the sarcolemma, the probes to be used need to have free access to this membrane. This is not possible to achieve in the intact heart because of the endothelial barrier between the vascular space and the interstitial space. Therefore, one has to use isolated cell models, which have previously been successfully applied to studies on anoxia- and/or ischemia-like conditions. Since the sarcolemma is the major focus of this study, the prerequisites are as follows: (1) The architecture of this membrane under control conditions should be known. (2) One should be able to isolate the sarcolemma after different experimental interventions. Cultured neonatal rat heart cells fulfill these requirements. They are the only myocyte system in which the sarcolemmal phospholipid topology is established and, in combination with the use of the so-called "gas-dissection" technique, it is possible to isolate the sarcolemma (in high purity and yield) in just a few seconds after ending the experimental protocol.

Caution must be exercised when extrapolating results from an in vitro situation to in vivo ischemia. In addition, developmental changes that occur in the sarcolemma when neonatal myocytes mature into adult cardiomyocytes could have important implications for mechanisms of membrane injury. Nevertheless, the system used in this study, ie, oxygen and volume restriction on isolated neonatal rat cardiomyocytes, gave rise to changes in various morphological, functional, and biochemical parameters that parallel those in Langendorf-perfused hearts and in vivo situations. The following observations are relevant: (1) Membrane vesicles are formed in the mitochondria after 15 to 30 minutes of simulated ischemia. This "blebbing" (vesiculation) of

Received December 4, 1992; accepted May 20, 1993.
From the Institute of Biomembranes, CBLE, Department of Lipid Biochemistry, Utrecht University (The Netherlands).
Reprint requests to Institute of Biomembranes, CBLE, Department of Lipid Biochemistry, Utrecht University, P.O. Box 80.084, 3508 TB Utrecht, The Netherlands (Dr Musters).
mitochondrial membranes eventually results in deformation and destruction of the mitochondria.2,3 There is a gradual reduction of high-energy phosphate (ATP) levels4 (down to approximately one third of control values after 60 minutes of “ischemia,” in the present study). (3) A change takes place in the interaction between the sarcolemma and the underlying cytoskeleton.3 Electron microscopic evidence has been presented showing that, after 60 minutes of simulated ischemia, local uncoupling between structures occurs.5 (4) Freeze-fracture electron microscopy reveals rearrangements of lipids and proteins in the sarcolemma. After 60 minutes of “ischemia,” there is a slight aggregation of the sarcolemmal intrinsic membrane proteins, indicating that phase separation occurs in the phospholipid matrix, which is accompanied by the extrusion of intrinsic membrane protein-free multilamellar membrane structures.3 (5) Redistribution of calcium within the myocardial cell occurs after 60 minutes of simulated ischemia.6 (6) Heat shock proteins are expressed after 30 minutes of “ischemia.”7

Simulating ischemia at the level of the individual cell offers useful opportunities for studying ischemia-induced progressive alterations in the organization of the sarcolemmal phospholipids. These specific modifications, as summarized above, have led to the proposal of a model that may explain the sequence of events eventually leading to membrane disruption.11,12 This model describes the previously observed lateral membrane reorganization in terms of a change in its physicochemical characteristics, and in this context, it has been postulated that acidification is the basis for such a membrane reorganization. Apart from this lateral membrane reorganization, it is possible that the specific transbilayer distribution of the phospholipids, which has been reported before,3 gradually changes after a prolonged period of ischemia because of both the ATP depletion and a disturbed interaction between the sarcolemma and the underlying cytoskeleton.3,12 A first indication for such a rearrangement has already been reported.13

In the present article, we report on a biochemical and structural study of the fate of the phospholipids in both intact neonatal rat cardiomyocytes and their gas-dissected sarcolemmal membranes during simulated ischemia, examined in relation to the integrity of the myocytes. The results of labeling experiments with the nonpermeant (primary amine–containing phospholipid) probe trinitrobenzenesulfonic acid (TNBS) indicate a specific change in transbilayer distribution of sarcolemmal phosphatidylethanolamine (PE). In addition, similar alterations in lipid topology of the sarcolemma are demonstrated by experiments in which (different) phospholipases A2 (PLA2s) are used. Because of the physicochemical properties of the hexagonalII phase–preferring PE, it is suggested that such a drastic phospholipid reorganization could play an important role in the loss of stability and integrity of the sarcolemma after a prolonged period of ischemia, eventually leading to a transition from reversible to irreversible membrane damage.

Materials and Methods

Cell Culture

Culturing of the cells was done according to a modification of the method of Harary and Farley14 and has been described previously.3 Briefly, hearts of 1- to 2-day-old neonatal rats were excised, minced, and digested by trypsin. Myocytes were purified by successive fractionating steps and plated on culture dishes or dishes at a seeding density of 4×10⁶ to 5×10⁶ cells per Petri dish (60 mm in diameter, Falcon 3802 Primaria). Within 3 days, a confluent monolayer of spontaneously beating cells was formed. The growth medium was changed every other day, and the dishes or dishes with attached cells were used on their fourth, fifth, or sixth day in culture.

Labeling With [1H]Acetate

Cells were labeled with [1H]acetate for 6 days (medium change every other day, 1 μCi per dish, [1H]acetate acid sodium salt, New England Nuclear, Boston, Mass) and used on their sixth day in culture. Before use, the dishes with attached cells were washed extensively in buffer W containing (mM) NaCl, 133; KCl, 5; MgCl₂, 1; CaCl₂, 1; Tris-HCl, 10; and glucose, 5 (pH 7.35).

Simulation of Ischemia

Ischemia was simulated by using a slightly modified in vitro model of Vemuri et al12 in which normal air can be easily replaced by an atmosphere of water-saturated argon (Ar 99.99 vol%). Control cells were incubated under normoxic conditions in buffer W (4 mL per dish). During the “ischemic” incubations, the extracellular medium of the cells was both nutrient deprived and volume restricted (2 mL buffer W without glucose per dish), as has been described previously.3

Cell Integrity

The integrity of the myocytes was checked both biochemically, by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH), and morphologically, by using scanning electron microscopy. The LDH activity of the supernatants was assayed as described by van der Schaft et al.15 All measurements were done in duplicate, and the relative LDH release was determined by comparing the activities of the different supernatants with the activity of total cell homogenates. Cells cultured on collodion-coated Thermacon disks (12 mm in diameter) were used for scanning electron microscopy. The myocytes were fixed with 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2) and postfixed with 1% OsO₄ in the same buffer. The cells were subsequently dehydrated by ethanol treatment, freeze-dried, and, finally, sputtered with gold. The samples were examined on a CAM Scan S-2 scanning electron microscope.

ATP Levels

Cellular high-energy phosphate (ATP) levels were assayed by using a bioluminescent assay kit (Boehringer). The cellular ATP was extracted in nucleotide releasing agent for somatic cells (NRS) buffer (pH 6.9) for 2 minutes under gentle shaking. Subsequently, the extracts were rapidly frozen and stored in liquid nitrogen until analysis was performed. All measurements were made in duplicate on a Lumac-2000 bioluminescence meter. The relative ATP levels were determined by comparing the absolute bioluminescence values of the different samples with those of control cells.
Labeling With TNBS

Cells were incubated with 2 mM TNBS (Sigma Chemical Co, St. Louis, Mo) in buffer W (pH 8.0) at 4°C for 30 minutes in the dark. It has previously been shown that under these conditions TNBS does not penetrate the sarcolemma and labels all the primary amine–containing phospholipids present in the outer leaflet of the sarcolemma. The kinetics of this labeling was such that a plateau is reached after 20 minutes of labeling; thus, during the standard incubation of 30 minutes, steady-state labeling is reached. The incubation was terminated by removal of the TNBS, and cells were washed three times at room temperature with buffer W (pH 8.0), containing 6 mM glycylglycine (Merck) in order to remove the unreacted TNBS. In addition, cell integrity during TNBS labeling was examined by measuring LDH release (TNBS does not interfere with the LDH assay).

Phospholipase Treatment

Cells were exposed to a mixture of bee venom PLA$_2$ (Sigma) and Naja naja PLA$_2$ (Sigma) for 15 minutes at 37°C. Per dish, 5 IU of each enzyme was added in 4 mL buffer W, in which the CaCl$_2$ concentration was increased to 10 mM. At the end of the incubations, the dishes were first rinsed in buffer W and, subsequently, in this buffer with 50 mM EDTA (Merck) to arrest phospholipase activity. In addition, all supernatants were checked for LDH release.

Isolation of the Sarcolemma

The sarcolemma was isolated using the gas-dissection technique, which has previously been described by Langer et al. Briefly, the disk with attached cell monolayer was placed at the center of a mobile platform inside the dissection chamber. The platform, with disk, was then elevated to make firm contact with the protruding inner horizontal gas outlet. Subsequently, the inlet valve was opened rapidly (<1 second) to allow the entry of N$_2$ gas at a pressure of 1900 to 2000 psi. As the N$_2$ stream bursts radially over the surface of the monolayer, the upper surface of the cells is sheared open, the cellular material is blown out, and the sarcolemma is left in a fenestrated layer and, in some areas, in a wrinkled or rolled form attached to the disks. The characterization of these membrane preparations by marker enzymes and transmission electron microscopy has been described by Post et al.

Lipid Extraction

Extraction of the phospholipids was accomplished by immersion of the dishes with cells in 3 mL isopropanol (analytical grade, Merck). After 45 minutes, no residual phospholipids could be detected in the material that was left behind on the dishes. Thus, a standard immersion of 60 minutes ensured complete extraction. A small amount of plastic was extracted during this procedure. The isopropanol extracts were dried under N$_2$ and purified according to Bligh and Dyer.

Lipid Analysis

The total phospholipid content was determined after drying the different fractions under N$_2$, destruction of the phospholipids by 70% perchloric acid (30 minutes at 180°C), and measuring inorganic phosphorus. Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on TLC plates (DC-Fertigplatten Kieselgel 60, 200×200 mm, Merck) or on high-performance TLC plates (HPTLC-Fertigplatten Kieselgel 60, 100×100 mm, Merck), which were first developed in chloroform:methanol:ammonia (25%): water (90:54:5.5:5.5 [vol/vol/vol/vol]), followed by drying and subsequent development in the second direction in chloroform:methanol:acetic acid:water (90:40:12:2 [vol/vol/vol/vol]) according to Broekhuysen. TLC plates for separating the neutral lipids were developed in ether:petroleum ether:formic acid (40:60:1.5 [vol/vol/vol]) in one dimension only. All lipids were identified by the use of standards. The trinitrophenyl (TNP)–labeled derivatives of PE and phosphatidylserine (PS) were clearly separated from the other lipids. The individual lipid spots were detected by iodine, scraped, and quantified either spectrophotometrically as inorganic phosphorus or by using a liquid scintillation counter (Packard TriCarb-1500) for the $^3$H-labeled fractions.

Experimental Design

After simulating ischemia, both dishes and disks with attached myocytes were either labeled with TNBS or incubated with the PLA$_2$s. In part of the experiments, gas dissection was performed to isolate the sarcolemma after labeling with TNBS. Subsequently, lipid extracts were made from the dishes with whole cells, as well as from the disks with the isolated gas-dissected sarcolemmal membranes. Finally, lipid extracts were pooled to obtain sufficient material to perform lipid analysis. All data are presented as mean±SD, and statistical significance was determined by using the (bidirectional) Student’s t test.

Results

LDH Release and ATP and Total Phospholipid Content

The LDH release of the cardiomyocytes after 30, 60, and 120 minutes of control (normoxic) incubation was 2.1±1.1%, 3.2±1.3%, and 3.5±1.5%, respectively (n=4). The data on both LDH release and total phospholipid content of the myocytes after different periods of simulated ischemia are shown in Table 1. During the first 60 minutes of “ischemia,” there was neither a significant LDH release (P>.2) nor a change in total phospholipid content, as compared with the control incubations with the same period of time. Fig 1 shows the morphology of the control cardiomyocytes as revealed by scanning electron microscopy. After 60 minutes of simulated ischemia, the morphology of the myocytes was comparable to that of the control cells, which is shown in Fig 2. In addition, the myocytes resumed spontaneous beating after reoxygenation. However, beyond 60 minutes of simulated ischemia, the cardiomyocytes started to lose LDH (up to 18.5% after 120 minutes of “ischemia”), indicating loss of cell integrity, an event that was confirmed by scanning electron microscopic images (Fig 3). The latter figure also shows that there was morphological heterogeneity after 120 minutes of simulated ischemia. Some of the cardiomyocytes had completely lost cell integrity; other myocytes appeared to be intact. However, this impression matched the LDH release of 18.5%. At the same
time, there was a decrease in the total phospholipid content of the myocytes (up to approximately 10% after 120 minutes of "ischemia"). Furthermore, these cardiomyocytes did not resume spontaneous beating after subsequent reoxygenation.

The high-energy phosphate (ATP) content of control cells was approximately 18 nmol/mg protein, which is in agreement with previously reported values. The relative cellular ATP levels after different periods of simulated ischemia are shown in Table 1. During the first 60 minutes of "ischemia," ATP levels had decreased to 40.1% of the control values, whereas after 120 minutes of simulated ischemia, ATP levels were as low as 16.0% of the control values.

The distribution of \(^3\text{H}\) label in the individual phospholipids of the cardiomyocytes after control and "ischemic" incubations is shown in Table 2. During the first 60 minutes of simulated ischemia, neither a significant extent of phospholipid hydrolysis nor a change in phospholipid class distribution could be detected. However, after 120 minutes of "ischemia," phospholipid degradation did occur and was accompanied by the release of free fatty acids in which 15.3% of the \(^3\text{H}\) label was found, without causing any specific change in phospholipid class distribution. In addition, no increase in \(^3\text{H}\)-labeled lysophospholipids was observed.

**TABLE 1.** Total Phospholipid Content, Lactate Dehydrogenase Release, and ATP Content of the Cardiomyocytes After Control and "Ischemic" Incubations

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Total phospholipid (nmol Pi/dish) (n=4)</th>
<th>LDH release (%) (n=4)</th>
<th>ATP content (%) (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia 120 min</td>
<td>113.7±3.2</td>
<td>3.5±1.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Ischemia 30 min</td>
<td>115.4±1.2</td>
<td>2.4±1.3</td>
<td>72.2±14.9</td>
</tr>
<tr>
<td>60 min</td>
<td>111.8±3.6</td>
<td>5.4±2.5</td>
<td>40.1±9.5</td>
</tr>
<tr>
<td>120 min</td>
<td>101.4±2.0</td>
<td>18.5±4.5</td>
<td>16.0±6.3</td>
</tr>
</tbody>
</table>

Pi, inorganic phosphate; LDH, lactate dehydrogenase. Values are mean±SD.

**FIG 1.** Scanning electron microscopic image of the cardiomyocytes after 60 minutes of control incubation (normoxia). Bar=7 μm.
Labeling With TNBS

The distribution of TNP label in the HPTLC-separated phospholipids of both whole myocytes and their isolated gas-dissected sarcolemmal membranes after control and "ischemic" incubations is shown in Table 3.

In control cells, 7.7±0.7% of the total cellular PE was labeled by TNBS. No increase in this labeling was observed after 30 minutes of "ischemia" (not shown). However, after 60 minutes of simulated ischemia, the amount of PE labeled by TNBS increased significantly to 13.5±1.6% (P<.01). In addition, no labeling of PS was observed in either control cells or in cells subjected to 60 minutes of "ischemia." Furthermore, as shown in Tables 1 and 4, no significant lysis was detectable during the 60 minutes of simulated ischemia or subsequent labeling with TNBS, as compared with the control incubations (P>.2). Since the percentage of cellular PE present in the sarcolemma of the neonatal rat cardiomyocyte is known, whereas only the PE present in the outer monolayer of the sarcolemma is labeled, the distribution of PE across the sarcolemmal bilayer can be calculated, as has been described in Post et al.5 Fig 4 shows the percentages of TNP-labeled sarcolemmal PE representing the PE in the outer membrane leaflet, calculated from the cellular data in Table 3. In control cells, 21.4±2.0% of the sarcolemmal PE was labeled by TNBS, whereas after 60 minutes of simulated ischemia, this percentage was significantly increased to 37.3±4.3% (P<.01).

The percentages of sarcolemmal TNP-PE in the "purified," isolated gas-dissected membranes are also shown in Fig 4. The data in Table 3 on the sarcolemmal membranes, isolated after TNBS labeling, have been used to calculate these percentages according to Post et al.5 After gas dissection of the control cells, 19.8±5.5% of the sarcolemmal PE was found to be labeled by TNBS. This percentage was significantly increased to 40.6±7.8% after 60 minutes of "ischemia" (P<.03). These data match those calculated from the cellular data and confirm that there is a real increase in the quantity of PE that can be labeled in the outer sarcolemmal phospholipid monolayer after 60 minutes of simulated ischemia.

Beyond 60 minutes of "ischemia," when significant release of LDH had occurred, we found that more of the PE was labeled than is present in the sarcolemma. Furthermore, labeling of PS was observed (not shown). This is in agreement with the loss of the sarcolemmal integrity, upon which TNBS has gained access to the intracellular milieu and will label cytosolic exposed PE and PS.

Phospholipase Treatment

As shown in Table 4, during the subsequent 15-minute incubation of the "ischemic" cells with the PLA2
cocktail (from bee and Naja naja venom), no significant additional LDH release was found as compared with the incubation of the control cells ($P>0.2$). This indicated that the action of the phospholipases remained restricted to the outer sarcolemmal membrane leaflet. Furthermore, both PLA$_2$s used have previously been shown to have free access to the entire sarcolemmal surface. The results of the treatment with the PLA$_2$ cocktail both in control cells and in cells after 60 minutes of simulated ischemia are shown in Table 5. In addition, Fig 5 shows the relative amounts of hydrolyzed sarcolemmal PE in control cells (27.3±1.8%, calculated

**FIG 3.** Scanning electron microscopic image of the cardiomyocytes after 120 minutes of simulated ischemia. Note that one of the myocytes has completely lost its sarcolemmal integrity (see arrow), whereas the other two are still intact. Bar=14 μm.

**TABLE 2. Distribution of $^3$H Label in the Cardiomyocytes After Control and “Ischemic” Incubations**

<table>
<thead>
<tr>
<th>$^3$H distribution (%)</th>
<th>Normoxia (n=4)</th>
<th>60 min (n=3)</th>
<th>120 min (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>43.4±1.1</td>
<td>43.2±0.8</td>
<td>36.4±5.5</td>
</tr>
<tr>
<td>PE</td>
<td>21.8±6.2</td>
<td>21.9±4.5</td>
<td>21.1±6.1</td>
</tr>
<tr>
<td>Sph</td>
<td>19.3±3.0</td>
<td>18.8±3.8</td>
<td>16.2±2.8</td>
</tr>
<tr>
<td>PS/PI</td>
<td>8.0±0.7</td>
<td>7.4±0.4</td>
<td>5.9±0.9</td>
</tr>
<tr>
<td>CL</td>
<td>1.8±0.4</td>
<td>1.9±0.3</td>
<td>2.3±0.6</td>
</tr>
<tr>
<td>LPC</td>
<td>1.6±0.8</td>
<td>1.5±0.9</td>
<td>1.4±0.7</td>
</tr>
<tr>
<td>LPE</td>
<td>0.9±0.4</td>
<td>1.0±0.5</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>FFAs</td>
<td>2.5±0.9</td>
<td>3.8±1.1</td>
<td>15.3±5.0</td>
</tr>
<tr>
<td>ORI</td>
<td>0.9±0.4</td>
<td>0.7±0.4</td>
<td>0.6±0.4</td>
</tr>
</tbody>
</table>

PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; PS/PI, phosphatidylserine/phosphatidylinositol; CL, cardiolipin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; FFAs, free fatty acids; ORI, origin. Values are mean±SD.

**TABLE 3. Phospholipid Composition of Both Intact Cardiomyocytes and Isolated “Gas-Dissected” Sarcolemmal Membranes After Control and “Ischemic” Incubations and Subsequent Exposure to Trinitrobenzenesulfonic Acid**

<table>
<thead>
<tr>
<th>Total cellular PL (mol%)</th>
<th>PL in gas-dissected SL (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (60 min)</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
</tr>
<tr>
<td>PC</td>
<td>47.1±2.5</td>
</tr>
<tr>
<td>PE</td>
<td>27.4±1.0</td>
</tr>
<tr>
<td>TNP-PE</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>PE+TNP-PE</td>
<td>29.7±1.0</td>
</tr>
<tr>
<td>PS/PI</td>
<td>11.0±0.9</td>
</tr>
<tr>
<td>Sph</td>
<td>8.5±1.1</td>
</tr>
<tr>
<td>CL</td>
<td>3.7±0.2</td>
</tr>
</tbody>
</table>

PL, phospholipid; SL, sarcolemma; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TNP-PE, trinitrophenyl-PE (labeled PE); PS/PI, phosphatidylserine/phosphatidylinositol; Sph, sphingomyelin; CL, cardiolipin. Values are mean±SD.
According to Post et al. as well as in the cardiomyocytes after 60 minutes of simulated ischemia, when this percentage was significantly increased to 36.5±1.9% (P<.01). The hydrolysis of PE was quantitative, since during the 15-minute PLA₂ incubation of control cells the same amount of PE was hydrolyzed as in our previous study in which we incubated the cells for 60 minutes and steady-state hydrolysis was reached. In addition, no hydrolysis of PS or phosphatidylinositol was observed. These data correspond well with the data from the labeling experiments with TNBS (shown in Fig 3). Hence, both types of experiments demonstrate that, after 60 minutes of "ischemia," there is a real increase in the amount of PE exposed in the outer sarcolemmal membrane leaflet.

**Discussion**

In the sarcolemma of the myocardial cell, the phospholipids are asymmetrically distributed over the two

![Figure 4](http://circres.ahajournals.org/doi/fig/10.1161/01.RES.73.3.520)

**TABLE 4.** Lactate Dehydrogenase Release During Trinitrobenzenesulfonic Acid Labeling and Phospholipase A₂ Incubation

<table>
<thead>
<tr>
<th>LDH release during lipid topology studies (%)</th>
<th>Normoxia (60 min)</th>
<th>Ischemia (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia (60 min)</td>
<td>1.1±0.6</td>
<td>1.6±0.8</td>
</tr>
<tr>
<td>PLA₂ cocktail (15 min)</td>
<td>2.7±2.3</td>
<td>4.9±2.6</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase; TNBS, 2 mM trinitrobenzenesulfonic acid; PLA₂, 5 IU phospholipase A₂. Values are mean±SD.

**TABLE 5.** Phospholipid Class Distribution of the Cardiomyocytes After Nonlytic Treatment With the Phospholipase A₂ “Cocktail” (From Bee and Naja naja Venom) Following Control and “Ischemic” Incubations

<table>
<thead>
<tr>
<th>Total cellular PL after PLA₂ cocktail (mol%)</th>
<th>N (60 min)</th>
<th>I (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>44.3±0.4</td>
<td>41.2±1.8</td>
</tr>
<tr>
<td>LPC</td>
<td>6.2±0.1</td>
<td>8.8±0.1</td>
</tr>
<tr>
<td>PC+LPC</td>
<td>50.5±0.4</td>
<td>50.0±1.8</td>
</tr>
<tr>
<td>PE</td>
<td>25.5±0.1</td>
<td>23.4±1.3</td>
</tr>
<tr>
<td>LPE</td>
<td>2.8±0.1</td>
<td>4.4±1.0</td>
</tr>
<tr>
<td>PE+LPE</td>
<td>28.3±0.1</td>
<td>27.8±1.6</td>
</tr>
<tr>
<td>PS/PI</td>
<td>9.5±0.5</td>
<td>10.2±1.6</td>
</tr>
<tr>
<td>Sph</td>
<td>8.0±0.5</td>
<td>8.4±0.3</td>
</tr>
<tr>
<td>CL</td>
<td>3.7±0.4</td>
<td>3.6±0.3</td>
</tr>
</tbody>
</table>

PL, phospholipid; PLA₂, phospholipase A₂; PC, phosphatidylcholine; LPC, lyso-PC; PE, phosphatidylethanolamine; LPE, lyso-PE; PS/PI, phosphatidylserine/phosphatidylinositol; Sph, sphingomyelin; CL, cardiolipin. Values are mean±SD.

The negatively charged phospholipids PS and phosphatidylinositol are exclusively present in the cytoplasmic leaflet of the sarcolemma, which also contains 75% of the sarcolemmal PE. At present, the exact physiological role of this asymmetric distribution is not known. However, it has been proposed that the asymmetric distribution, via its effect on calcium binding, dramatically affects the Ca²⁺ concentration in the subsarcolemmal region. Consequently, this would be an

![Figure 5](http://circres.ahajournals.org/doi/fig/10.1161/01.RES.73.3.520)
important factor with respect to excitation-contraction coupling in the myocardial cell. Furthermore, the activity of many plasma membrane transport proteins, such as the Ca\(^{2+}\)-ATP\(\sigma\),\(^{21,16,22}\) the Na\(^+-\)Ca\(^{2+}\) exchanger,\(^{23}\) and the Na\(^+-\)K\(^+-\)ATP\(\sigma\),\(^{23}\) have been shown to be dependent on the phospholipid environment in which they are embedded. In this way, the asymmetric distribution might play an additional role in the physiology of the cardiomyocyte.

So far, the maintenance of this asymmetric distribution under normal conditions has not been studied in the cardiomyocyte. It has, however, been extensively studied in the red blood cell and in the platelet,\(^{24}\) which both exhibit a transverse phospholipid distribution in their plasma membranes that is very similar to that in the myocyte sarcolemma.\(^{25,26}\) It has been shown that two mechanisms are most likely responsible for the maintenance of the asymmetrical distribution: (1) an interaction of the primary amine–containing phospholipids with the underlying cytoskeleton and (2) an ATP-dependent process that translocates the primary amine–containing phospholipids from the outer to the inner lipid monolayer, as mediated by the so-called amino-phospholipid translocase.\(^{27,28}\)

In the present study it is shown that during the first 60 minutes of simulated ischemia, there is neither a significant accumulation of lysophospholipids and free fatty acids nor a change in phospholipid class distribution. After 120 minutes of “ischemia,” when cell lysis has started, phospholipid degradation does occur and results in a gradual reduction of all phospholipids with a concomitant increase in free fatty acids. However, still no increase in lysophospholipids could be detected, which indicates that these compounds are subjected to additional degradation processes. These results are in agreement with those recently reported by Chien et al.,\(^{29}\) Hagve et al.,\(^{30}\) and Bersohn et al.\(^{31}\)

Furthermore, the fact that no cell lysis occurred up to 60 minutes of simulated ischemia allowed us to investigate the transbilayer organization of the sarcolemmal phospholipids during the first 60 minutes of “ischemia.” After 60 minutes of simulated ischemia, before any significant LDH release is observed and cellular ATP levels have decreased to approximately 40% of the control values, there is a significant increase in the fraction of sarcolemmal PE that can either be labeled by TNBS or hydrolyzed by (different) PLA\(_2\)s, techniques that are commonly used in phospholipid topology studies.\(^{32}\) In addition, it should be stressed that during the first 60 minutes of “ischemia” neither labeling nor hydrolysis of PS could be detected. In contrast, after a prolonged period of simulated ischemia, once LDH release has started, TNBS has access to the cytoplasm and is indeed capable of labeling PS located in the cytoplasmic exposed membrane layer. Therefore, the increased labeling or hydrolysis of PE after 60 minutes of “ischemia” in intact cells is not due to a change in the sarcolemmal permeability for TNBS or PLA\(_2\), respectively, but reflects migration of PE from the inner toward the outer leaflet of the sarcolemma, thus resulting in a net increase in the amount of PE exposed in the extracellular membrane leaflet.

Another possible explanation for our data can be considered. This is that the increased susceptibility of PE to treatment with TNBS and PLA\(_2\) is a consequence of lateral phase separation of this lipid in the extracellular leaflet of the sarcolemma. Such a possibility implies that part of the PE present in the outer sarcolemmal monolayer is protected against both TNBS labeling and PLA\(_2\) hydrolysis. However, in a previous study in which we elucidated the transbilayer distribution of the sarcolemmal phospholipids in isolated neonatal rat cardiomyocytes, it was shown that in intact cells approximately 25% of the sarcolemmal PE could be hydrolyzed by PLA\(_2\), whereas 100% of the PE is degraded when isolated sarcolemma is incubated under exactly the same conditions.\(^{6}\) This result shows that the 25% sarcolemmal PE hydrolyzed in the intact cell represents all of the sarcolemmal PE exposed to the extracellular milieu. In addition, the observation that both labeling with a small molecule like TNBS and hydrolysis with two types of phospholipases give identical results makes the alternative explanation mentioned above very unlikely. Therefore, the increased labeling or hydrolysis of PE after 60 minutes of simulated ischemia, as observed in the present study, must be explained by an increased presence of PE in the outer sarcolemmal monolayer.

Intriguingly, recent experiments with metabolically inhibited neonatal rat cardiomyocytes, as induced by treatment of the cells with both iodoacetic acid and deoxyglucose, are also indicative of a changed sarcolemmal phospholipid asymmetry for PE.\(^{33}\) After 30 minutes of metabolic inhibition, when cellular ATP levels have decreased to less than 10% of control values, even up to 50% of the sarcolemmal PE can be labeled by TNBS, which suggests a complete loss of the asymmetrical distribution of PE over the two phospholipid monolayers. At the same time, no labeling of PS is observed.

Concerning the possible mechanisms that could be responsible for the loss of the asymmetrical distribution of sarcolemmal PE during simulated ischemia, we can speculate that, in analogy with the erythrocyte system, the decrease in cellular ATP results in a decrease in the activity of the aminophospholipid translocase.\(^{24,27}\) Furthermore, decreased ATP levels, intracellular acidification, and Ca\(^{2+}\) redistribution could all affect the stability of the cytoskeleton, resulting in a loss of interaction between the primary amine–containing phospholipids and this protein network.\(^{3,12,27,34,35}\) This is schematically shown in Fig 6.

**Implications of Loss of Asymmetric Distribution of Sarcolemmal PE**

Bringing the data obtained in this study into the context of what is known about phospholipid involvement in membrane structure and function leads to the following proposal: a shift in the transbilayer distribution of the sarcolemmal PE will eventually contribute to the irreversible damage of the sarcolemma seen after a prolonged period of ischemia. The first interpretation is that alterations in phospholipid distribution are expected to alter, and possibly inhibit, the activity of sarcolemmal transport systems and to interfere with other functions of the sarcolemma. In this way, the change in lipid topology could impair sarcolemmal functioning, which then subsequently leads to a disruption of the sarcolemma by cell swelling\(^{36,37}\) or contraction.\(^{38}\) More importantly, the transbilayer movement of PE is crucial for the sarcolemma, since a net increase in the outer leaflet of this primary amine–containing phos-
phasolipid, which is known to be a nonbilayer, hexagonal II phase–preferring, and fusogenic lipid, may induce destabilization of the phospholipid bilayer itself. The sarcolemma contains two different species of PE, namely diacyl and plasmalogen PE, of which the latter one has even stronger hexagonal II-promoting properties. We have not yet studied the species composition of the increased amount of PE in the outer sarcolemmal monolayer; this would not have a qualitative but could have a quantitative effect on the tendency to promote nonbilayer behavior and thus fusion. Furthermore, a recent physicochemical study in which the fusogenic tendencies of PE-containing large unilamellar vesicles were tested demonstrates that the fusogenicity of the lipid bilayer as a whole is largely determined by the properties of the individual lipid monolayers and clearly supports a regulatory role for phospholipid asymmetry in maintaining overall membrane stability. In addition, both lateral phase separation and the tendency to form nonbilayer hexagonal II-type structures have been shown to affect adversely the barrier function of the lipid bilayer.

It is clear, however, that further studies are necessary to characterize fully the progressive alterations in lipid topology of the sarcolemma during simulated ischemia, as well as the mechanisms by which these alterations are governed. At the same time, this experimental approach might help us to gain a better understanding of the physiologic significance of membrane asymmetry in cardiac myocytes under both normal and pathological conditions.

Acknowledgments

This study was supported by the Netherlands Heart Foundation (Grants 89.076 and 88.257). The research of Dr Post has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. The authors wish to specially thank Dr Ben Roelofsen for his valuable discussions and critical reading of the manuscript.

References

Loss of asymmetric distribution of sarcolemmal phosphatidylethanolamine during simulated ischemia in the isolated neonatal rat cardiomyocyte.


doi: 10.1161/01.RES.73.3.514

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/73/3/514