特 集 (special edition)

Protection of the ischemic heart: a possible role for phospholipase inhibitors?

Ger J. van der Vusse* and Robert S. Reneman*

Introduction

Ischemia- and reperfusion-induced damage of the heart is a multifactorial process[1]. A variety of events, including disturbances in ion-homeostasis, acidification of the cellular milieu, depletion of high-energy phosphate stores, physical and chemical changes in the membranes enclosing the cell and its subcellular organelles, activation of lysosomal enzymes, and decreased capacity to scavenge endogenous oxygen free radials, most likely contribute to the loss of cell viability during the ischemic attack. Reperfusion of the previously ischemic area, although meant to prevent the cardiac cells from an inevitable death, may add to the ischemia-induced injury. Increased production of oxygen free radicals, enhancement of the cellular Ca^{2+}-content resulting in myofibrillar contracture and activation of Ca^{2+}-dependent hydrolases, and osmotic load are thought to be specific mechanisms of reperfusion-induced damage. Despite several decades of scrutinous investigation our knowledge of mechanisms underlying the loss of cell viability due to ischemia and reperfusion is still incomplete. Nevertheless, scientists concerned with the life-threatening features of cardiac ischemia are searching for pharmacological tools to delay or prevent loss of cell viability caused by flow-deprivation and reperfusion of cardiac tissue.

Loss of cell membrane integrity has been generally accepted as the ultimate event in the transition from reversible to irreversible injury of cardiac cells, ultimately leading to loss of cell viability and cell death[1]. Hence enzymatic degradation of phospholipids, being an important building block of cellular membranes, by phospholipases has been suggested to play a crucial role in impairment of membrane function. As a consequence, it has been investigated whether the putative injurious action of cardiac phospholipases could be prevented by chemical means.

In the present overview, the current ideas on the mechanisms responsible for the destabilization of cardiac cell membranes are discussed. Special attention will be paid to phospholipids, endogenous phospholipid hydrolyzing enzymes and drugs with phospholipase blocking properties.

Role of phospholipids in cell viability

Cell viability depends, among others, on proper functioning of the plasmalemma, the membrane enclosing the cellular content. When the plasmalemma loses its ability to main-

*Department of Physiology, Cardiovascular Research Institute Maastricht, University of Limburg
P. O. Box 616, 6200 MD Maastricht,
The Netherlands

tain a barrier between the extra and intracellular compartment, the cell is irreversibly damaged and will die. As a consequence of the loss of the semi-permeable properties of the plasmalemma, constituents of the cytoplasma, such as co-factors, metabolites and proteins, will be released from the cell and extracellular macromolecules and ions, like Ca^{2+}, will freely diffuse into the interior of the cell. The remnants of the cell will be subjected to lytic processes by the action of lysosomal enzymes and invading macrophages.

Like all biological membranes, the plasmalemma and intracellular membranes are composed of phospholipids, cholesterol and proteins[1]. Phospholipids are present in a variety of species, the nature of which is determined by the chemical composition of the headgroup. Phospholipids contain glycerol as backbone (Fig. 1). Fatty acyl chains are connected to the hydroxyl groups at the Sn_1 and Sn_2 positions of

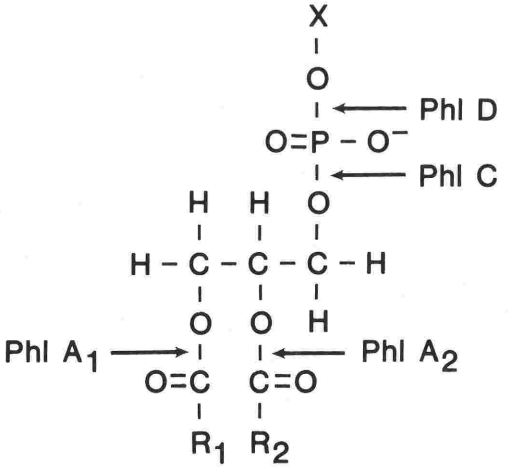

Fig. 1 Chemical structure of a phospholipid and sites of action of phospholipase A_1, A_2, C and D. R_1 and R_2 are aliphatic chains connected to the carbon atom of the glycerol backbone at the Sn_1 and Sn_2 position, respectively. The species of the phospholipid is determined by the nature of the X-group, which is linked via a phosphate ester to the third carbon atom of glycerol. After[76], with permission of the publisher.

glycerol through an esterbond. This part of the molecule determines the hydrophobic nature of phospholipids. The hydrophilic headgroup is a complex alcohol molecule bound to the third (Sn_3) carbon atom of the glycerol backbone via a phosphate group[1]. The most common phospholipid species in cardiac membranes are phosphatidyl choline and phosphatidyl ethanolamine. Phosphatidyl inositol, phosphatidyl serine and cardiolipin are present in smaller amounts. In addition to phospholipids, sphingomyelin, a molecule with sphingosine as backbone, is present in cardiac membranes. The composition of the fatty acyl chains in the hydrophobic part of the phospholipid molecule may vary considerably. In general, the number of carbon atoms per fatty acyl chain varies from 14 to 24. The number of unsaturated bonds can vary from zero to 6. Moreover, part of the phospholipids is present in the plasmalogen form: the first aliphatic chain of the molecule is connected with the glycerol backbone through a vinyl ether linkage instead of an ester bond. In some animal species up to 40% of the cardiac plasmalemmal phospholipids are plasmalogens[1].

All biological membranes consist of two leaflets, the so-called lipid bilayer. In case of the plasmalemma, the hydrophilic headgroups of the outer and inner leaflets of the bilayer point towards the extracellular and intracellular space, respectively. The hydrophobic acyl chains form the core of the lipid bilayer. The distribution of the various phospholipid spcies across the inner and outer leaflet is asymmetric. The outer leaflet mainly consists of sphingomyelin and phosphatidyl choline. The inner leaflet is composed of phosphatidyl serine, phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl choline[2]. The asymmetrical distribution is maintained by an ATP-dependent aminophospholipid translocase[3]. Due to the asymmetric phospholipid distribution the fluidity is different in the inner and outer leaflet of the membrane. To compensate for this dif-

ference, more cholesterol is present in the outer leaflet[1]. Phospholipids in membranes are present in the liquid-crystaline phase rather than in the gel phase.

The ensure signal transduction and transport of hydrophilic substances, such as ions and nutrietns, across the membrane, proteins are present in the lipid bilayer.

The stability of the plasmalemma is maintained by a variety of factors. The fluidity of the membrane, the liquid-crystalline state of the phospholipids, the chemical composition of the hydrophobic aliphatic chains, the asymmetrical distribution of phospholipids over the inner and outer leaflet, and the presence of membrane proteins most likely determine the stability of the biological lipid bilayer. Additional strength to the plasmalemma is provided by anatomical structures present on both the intra and extracellular side of the membrane. The outer leaflet of the plasmalemma is connected to an outer lamina or basement membrane, whereas the inner leaflet is attached through vinculine and other proteins to the part of the cytoskeleton that forms a subcortical lattice near the plasmalemma. To adept to damage of the fatty acyl chains or differences in external circumstances, fatty acyl chains are continuously removed and reincorporated (the so-called deacylation-reacylation cycle) from and into their phospholipids. This process is considered to be a self-regenerative property of the plasmalemma.

Ischemia- and reperfusion-induced damage

Underperfusion of the heart results in loss of cardiac cell viability when timely restoration of flow does not occur. So far the precise nature of the chain of events at the molecular level leading to cell death has not been elucidated in full detail. Since it has been recognized that the integrity of the plasmalemma plays a crucial role in the maintenance of cellular homeostasis, research has been focussed on the specific changes occurring in cardiac membranes during ischemia and reperfusion[1].

Membrane destabilization most likely precedes overt disrupture of the sarcolemma. Both physical and chemical mechanisms underlying seem to be involved in loss of membrane stability.

Rouslin and colleagues[4] have suggested that a shift of cholesterol molecules from the plasmalemma to mitochondrial membranes negatively influences the stability of the cell membrane. Increased acidity of the cytoplasma of ischemic cells may cause solidification of negatively charged phospholipids, such as phosphatidyl serine and phosphatidyl inositol[5]. As a consequence of phase transition of part of the membrane phospholipids solid islands or lipid domains are formed in the bilayer, area's from which membrane proteins are expelled. With freeze fracture electron microscopy aggregation of intramembranous particles, most likely reflecting segregated membranous proteins, can easily be visualized[5]. Since membrane proteins are, to a certain extent, immobilized by attachment to the subcortical lattice of the cytoskeleton clustering of membrane proteins might indicate that the connection between cytoskeletal and membrane proteins is disrupted. In this respect recent findings of Steenbergen and colleagues[6] are worth to mention. In ischemic cardiac tissue they found loss of staining of vinculine, a protein that may serve as the final link between the cytoskeleton and the plasmalemma since it extends into the lipid bilayer of the membrane. Increased Ca^{2+} in the cytoplasma of the oxygen-deprived cells most likely activates calcium-dependent proteases. These proteases are capable of degrading a variety of cytoskeletal proteins including vinculine[6].

The consequences of degradation of the cytoskeletal-plamalemmal complex might be muliple. In addition to segregation of in-

tramembranous particles, the plasmalemma may start to form blebs, filled with intracellular material such as subsarcolemmal mitochondria and cytoplasmic proteins. Moreover, the formation of multi-lamellar vesicles, visible with transmission electron microscopy after proper staining of the lipid material in the plasmalemma[7], readily occurs in the ischemic and reperfused heart. It is uncertain whether the formation of these lipid anomalies is caused by detachment of the cytoskeleton from the plasmalemma or by physical changes in the phospholipid bilayer itself. In the latter case, it has been suggested that the spatial arrangement of phosphatidyl ethanolamine in the lipid bilayer changes into the so-called hexagonal II structure, which may result in the formation of multi-lamellar vesicles and further destabilization of the plasmalemma[5]. Loss of the asymmetric distribution of phospholipids over the inner and outer leaflet of the plamalemma may also add to destabilization of the cell membrane. On the one hand lateral phase transitions in the lipid bilayer may give rise to "scrambling" of phospholipids in the two leaflets of the membrane. On the other hand it can not be excluded that depletion of the cytoplasmic ATP content and increased intracellular Ca^{2+} concentrations impair the ATP dependent Ca^{2+}-sensitive aminophospholipid translocase[3]. Inhibition of this enzym will result in impaired capacity to maintain the phospholipid asymmetry at its physiological level. The consequence of loss of phospholipid asymmetry is severe destabilization of the plasmalemma. Physical forces for example caused by contracture of myofibrils or osmotic load imposed on the energy-depleted cells are thought to play an important role in the overt rupture of the destabilized cell membrane[8].

During reperfusion additional factors are most likely responsible for membrane destabilization and destruction. The formation of oxygen free radicals may result in enhanced peroxidation of the unsaturated fatty acyl chains of membrane phospholipids. The presence of peroxidized phospholipids is thought to negatively influence proper positioning of the phospholipid molecules in the lipid bilayer[9] and, hence, to weaken the plasmalemma. Due to additional influx of Ca^{2+} from the extracellular compartment into the cytoplasmic space during the reperfusion phase a variety of Ca^{2+}-dependent processes will be activated. In addition to the activation of Ca^{2+}-dependent proteases and inhibition of the aminophospholipid translocase, Ca^{2+} may induce lateral phase separation of phosphatidyl serine in the bilayer which may give rise the formation of hexagonal II structures in the plasmalemma[5]. Moreover, the additional mechanical and osmotic load may further pose a burden on the destabilized plasmalemma ultimately resulting in overt disruption of the membrane[8].

During the past two decades several lines of evidence indicate that phospholipase mediated hydrolysis of membrane phospholipids plays a crucial role in ischemia and reperfusion induced damage of cardiac structures[1]. Loss of phospholipid moieties, accumulation of arachidonic acid and lysophospholipids, and the protective action of putative phospholipase inhibitors are suggestive for enzymatic degradation or impaired resynthesis of membrane phospholipids as part of the chain of events leading to irreversible injury of cardiac cells.

Phospholipid turnover and phospholipase activity in the heart

Membrane phospholipids are subjected to a continuous turnover cycle. Degradation of phospholipids by endogenous enzymes keeps pace with the resynthesis of the most important building blocks of cardiac membranes. This turnover cycle allows the cardiac cell to alter the phospholipid composition and the nature of fatty acyl chains incorporated in the phospholipids in the lipid bilayer. By this pro-

cess the cell can, to a certain extent, regulate the chemical and physical properties of its membranes. Several routes of degradation and resynthesis of phospholipids have been indentified in the cardiac cell. Some examples are shown in figure 2. A variety of phospholipases are present to remove the fatty acyl chains or (part of) the hydrophilic headgroup of the phospholipid molecule. Phospholipase A_1 hydrolyzes the Sn_1 glycerol acyl esterbond, phospholipase A_2 cleaves the Sn_2 glycerol acyl ester linkage, phospholipase C breaks the bond between the third carbon atom of the glycerol backbone and the phosphate group of the polar head-group, and phospholipase D hydrolyzes the phosphate-alcohol bond in the polar headgroup (Fig. 1). Lysophospholipase hydrolyses the remaining acyl ester bond in lysophospholipids. Moreover, hydrolytic enzymes specifically attacking plasmalogens (plasmalogenases and plasmalogen specific phospholipase A_2) have been identified in the heart[10-12]. The localization of phospholipid hydrolyzing enzymes appears not to be confined to one particular compartment. Activity of phospholipase A_1 and A_2 has been detected in cytoplasm, mitochondria, sarcoplasmatic reticulum, sarcolemma and lysosomes[10-19]. Phospholipase C has been monitored in cytoplasm and lysosomes[12, 20, 21]. Lysophospholipase activity was found to be present in cytoplasma, mitochondria, sarcoplasmic reticulum and lysosomes[11]. Resynthesis of phospholipids most likely takes place in the sar-

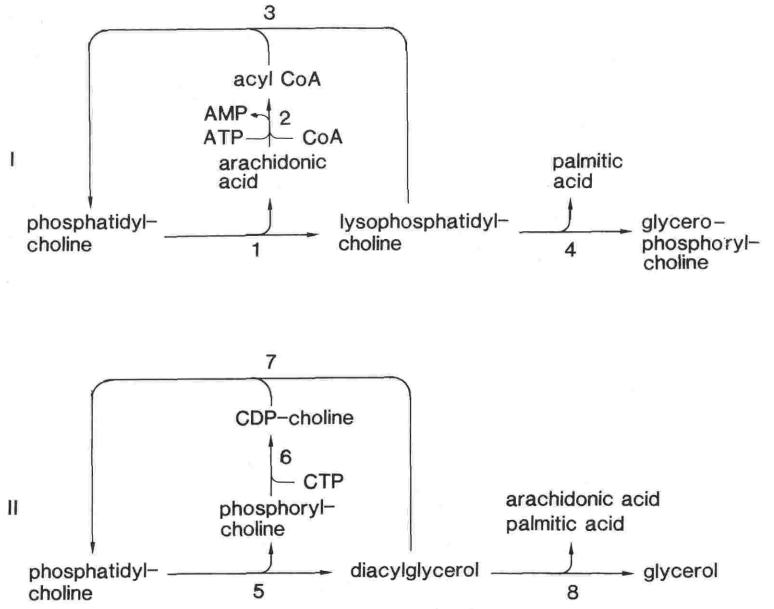

Fig. 2 Schematic representation of the enzymatic turnover and degradation of phosphatidyl choline. Pathway I refers to the deacylation-reacylation cycle. In this example, palmitic and arachidonic acids are connected to the Sn_1 and Sn_2 position of the glycerol backbone, respectively. Situation II describes the turnover of the hydrophilic headgroup of phosphatidyl choline. 1, 2, 3, 4, 5, 6, 7 and 8 refer to phospholipase A_2, acylCoA synthetase, lysophosphatidyl choline acyltransferase, lysophospholipase, phospholipase C, phosphorylcholine cytidylyl transferase, phosphocholine transferase and diacylglycerol +monoacylglycerol lipase, respectively. CTP, cytidine triphosphate. After[76], with permission of the publisher.

coplasmic reticulum. Transport of phospholipids from the site of synthesis to the (intra)cellular membranes is thought to be facilitated by specific phospholipid-binding proteins[22].

Although phospolipid hydrolysis plays an important role in phospholipid turnover detailed information about the regulation and actual rate of this hydrolytic process is lacking. Since most endogenous phospholipases are surrounded by an appreciable amount of potential substrates, the activity of the enzyme is apparently well controlled[23] to prevent loss of membrane phospholipids. A variety of factors and circumstances have been proposed to regulate endogenous cardiac phospholipase activity. These include:

1. Alterations in Ca^{2+}-concentration and acidity of the cytoplasm. Since some phospholipases, like the enzymes located in the mitochondria[19] are Ca^{2+}-dependent, changes in the cellular Ca^{2+}-concentration is thought to be an important regulatory mechanism. However, other endogenous phospholipases are Ca^{2+}-insensitive[11]. Lysosomal phospholipases show an optimum in their hydrolytic activity at lower pH. Hence, increased acidity of the cellular compartment may provoke enhanced phospholipid hydrolysis.
2. Substrate availability. The extent of phospolipid hydrolysis may also be influenced by the amount of phospholipids that become available for the hydrolytic enzymes. Subtle chemical and physical changes in the lipid bilayer of the membrane might render the phospholipid molecules more susceptible to phospholipase activity. These changes might relate to chemical transformation of the phospholipids (e. g. lipid peroxidation), distribution of phospholipids over inner and outer leaflet of the lipid bilayer or phase transitions in the membrane. In this respect, alterations in the interaction between cytoskeleton and lipid bilayer may facilitate phospholipid degradation[1]
3. Intracellular phospholipase inhibition or activation. Recently the presence of proteins displaying either an inhibitory or stimulatory effect on phospholipases has been demonstrated. Inhibitory proteins, commonly indicated by lipocortins or annexins, have been found in a variety of tissues including the heart[24]. Phospholipase activating proteins (PLAP) have been identified by Clark and coworkers[25]. Their significance in cardiac phospholipid degradation remains to be established.
4. Hormonal activation. Some phospholipases, like phospholipase C, appear to be modulated by the activity of G-proteins, which, in turn, are regulated by exogenous hormones[26]. It is generally accepted that phospholipase C mediated phospholipid degradation plays a role in some kinds of signal transduction. It remains to be established whether phospholipase C can provoke mass degradation of phospholipids in the cellular membranes leading to impairment of its physical properties.

Since cardiac phospholipids are subjected to a continuous turnover cycle, net degradation of membrane phospholipids may take place if resynthesis of phospholipid molecules cannot keep pace with degradation. In that case loss of cellular phospholipids will occur without enhancement of the activity of phospholipid hydrolyzing enzymes. As indicated in figure 2 resynthesis of phospholipids can be achieved via various metabolic pathways. Hence, the rate of phospholipid resynthesis can be influenced by a host of intracellular factors and conditions. Among them, cellular ATP, a cofactor required for the formation of acylCoA, and AMP, a well-known inhibitor of acylCoA synthetase, are worth to mention. In addition,

the availability of Coenzyme A, CTP, lysophospholipids and diacylglycerol will support the cell in maintaining its phospholipid content at the required level.

Hydrolysis of phospholipids in the ischemic and reperfused heart

During the past two decades a substantial number of experimentally obtained data favors the notion that hydrolysis of membrane phospholipids plays a crucial role in the onset of irreversible damage of the ischemic and reperfused heart[7,28]. Firstly, ischemia either in association with reperfusion or alone has been reported to decrease the cardiac phospholipid pool and to enhance the content of degradation products, such as lysophospholipids and arachidonic acid, in the affected tissue. Secondly, treatment of isolated cardiac cells or subcellular exogenous organelles with phospholipase A or C provokes changes in the phospholipid composition comparable to the alterations observed in ischemic and reperfused myocardial tissue. Thirdly, compounds with putative phospholipase inhibiting properties, to a certain extent, prevent the heart from the injurious effects of flow-deprivation and reperfusion. Fourthly, alternative models like cultured neonatal cardiomyocytes or isolated adult cardiac muscle cells treated with specific metabolic inhibitors to deplete the intracellular ATP content, indicate a close relationship between phospholipid hydrolysis and loss of cell viability[29].

Following the original studies of Weglicki and coworkers[30], showing the release of arachidonic acid from isolated canine hearts rendered globally ischemia, a substantial number of experiments has indicated that ischemia causes degradation of membrane phospholipids. The most sensitive marker of cardiac phospholipid hydrolysis appears to be accumulation of arachidonic acid, a fatty acid preferentially incorporated at the Sn_2 position of membrane phospholipids. This fatty acid starts to accumulate after 30 minutes of ischemia[27]. In ischemic dog hearts, accumulation predominantly occurred in the subendocardial regions, where flow reduction was most severe[31,32]. In addition to accumulation of lysophospholipids[33-38], net loss of phospholipids in the ischemic heart has been reported by a number of investigators[21,38-40]. This decline seems to be confined to phosphatidyl choline and phosphatidyl ethanolamine since in some studies the content of cardiolipin and phosphatidyl inositol was found to be increased in the ischemic heart[40,41]. Changes in the phospholipid content of subcellular organelles have been reported by Yanagishita et al[42] and Kajiyama and colleagues[43]. A small loss of phosphatidyl choline and cardiolipin was observed in mitochondria isolated from ischemic dog hearts[42]. In contrast, others[44] failed to detect a significant decrease in the phospholipid pool of mitochondria, harvested from ischemic myocardial tissue. In the ischemic sarcoplasmic reticulum the content of both phosphatidyl choline and phosphatidyl ethanolamine was found to be decreased[42]. Interestingly, Suyatna and colleagues[45] observed that the content of phospholipids and lysophospholipids did not change in the sarcolemma isolated from rat hearts, made ischemic for 60 minutes.

A number of studies have indicated that reperfusion following a period of flow-deprivation leads to further hydrolysis of membrane phospholipids. Both substantial reduction in the phospholipid content[46,47] and accumulation of arachidonic acid[27,48] (Fig. 3) have been observed after restoration of flow through previously ischemic cardiac tissue. The decline in phospholipid content of mitochondria isolated from heart subjected to 30 minutes of ischemia and 5 minutes of reperfusion could only be observed in hearts that started to fibrillate after reperfusion[49]. No such decline could be observed in mitochondrial fractions from hearts that

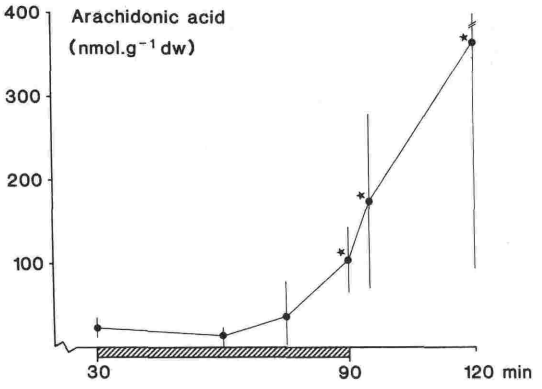

Fig. 3 Accumulation of arachidonic acid in ischemic and reperfused myocardium. Hearts isolated from adult rats were rendered ischemic for 60 minutes. Hearts were frozen at time intervals indicated on the x-axis. Arachidonic acid was monitored with the use of gas chromatography. (Data are obtained from ref 77).

resumed normal electrical activity after restoration of flow.

When intact isolated cardiomyocytes were treated with exogenous phospholipase C, the cells were lysed with loss of lactate dehydrogenase from the intracellular compartments. When intracellular ATP levels were depleted prior to phospholipase C treatment, the sensitivity of the cells towards the hydrolytic enzyme was greatly enhanced[50]. It should be noted that low ATP levels are readily observed in flow-deprived cardiac tissue.

During the past decade, experimental models with isolated cardiomyocytes have been developed to mimic ischemia and reperfusion of intact hearts. Chien and coworkers[29,51,52] used a neonatal heart cell model in which the intracellular ATP was depleted by metabolic inhibition rather than by anoxia or ischeamia. The release of arachidonic acid from membrane phospholipids was found to be closely related to the development of defects in the sarcolemmal membrane, perturbations of electrolyte homeostasis, including Ca^{2+}-accumulation, and loss of cell viability.

Mechanisms underlying ischemia- and reperfusion-induced phospholipid degradation

Despite numerous attempts to clarify the precise nature of the mechanisms leading to the irreversible loss of cell viability during ischemia and reperfusion, and the role of phospholipid hydrolysis therein, no complete picture of all details of the injurious process can be offered. Nevertheless, in recent years a variety of mechanisms explaining net degradation of membrane phospholipids in ischemic and reperfused myocardium have been proposed.

As a consequence of enhanced cellular Ca^{2+}-levels the activity of phospholipase A_2 enzymes that show sensitivity for this cation will be increased. Recent studies of Steenbergen et al[53] indicated that the cytoplasmic Ca^{2+} concentration rapidly elevates during flow-deprivation. Moreover, the production of H^+ during the ischemic episode will increas cellular acidity and, hence, activate phospholipid hydrolyzing enzymes that display their maximal activity at low pH. This is particularly true for lysosomal phospholipases. Weakening of lysosomal membranes with concomitant release of lysosomal proteins into the sarcoplasmic compartment has been described in the ischemic heart[54]. This event might result in increased concentrations of hydrolytic enzymes in the vicinity of cardiac (sub)cellular membranes.

Physico-chemical alterations in the sarcolemma and intracellular membranes might render membrane phospholipids more accessible to the hydrolytic enzymes. Experiments performed on ischemic hearts have shown that clustering of intramembrane particles, most likely associated with lipid domains devoid of proteins in the lipid bilayer, formation of multilamellar vesicles and extrusion of membrane material (membranous "blebs") readily occur in ischemic myocardial tissue[5,7,55]. These alterations,

which are possibly initiated by phase-transition of phospholipids composing the lipid bilayer, might ultimately render the phospholipid constituents of the destabilized membranes more sensitive to endogenous phospholipases.

In in vitro models Weglicki and coworkers[9,56] observed that membrane phospholipids are more suceptible to phospholipase activity after oxidative stress. Oxidative stress is characterized by peroxidation of phospholipids due to enhanced oxygen free radical formation in the affected cells. According to the "free radical-triggered lipolysis by phospholipases theory"[9,56], the lipid bilayer is peroxidized by endogenously generated oxygen free radicals. Subsequently, the peroxidized acyl chains of the phospholipids create alterations in the physico-chemical properties of the membrane, most likely rendering either the unaffected phospholipids or the peroxidized molecules more susceptible for phospholipase A or C activity. Moreover, oxygen free radicals might promote the release of hydrolytic enzymes from the lysosomes into the sarcoplasmic compartment[57]. It should be stressed that oxygen free radical mediated processes are more likely to occur after reinstallation of flow since readmission of oxygen to previously ischemic myocardial tissue has been found to promote the formation of these oxygen species[58] (see below).

Despite experimental findings that phospholipids are degraded during the ischemic attack no direct proof is at hand that the activity of phospholipase A or C is enhanced in the ischemic heart. Although Das and coworkers[47,59] reported an increase in phospholipase A activity in microsomes isolated from ischemic pig hearts, most authors reported a decline rather than an elevation of the activity of phospholipid hydrolyzing enzymes after isolation from the flow-deprived myocardium[21,60,61]. Decreased phospholipase A or C activity in ischemic hearts may be an intrinsic measure of the cardiac cells to protect the phospholipid moieties in their membranes against unwanted activity of phospholipid hydrolyzing enzymes. Inhibition of phospholipase might be achieved by lysophospholipids or acylcarnitine, compounds known to accumulate in oxygen-deprived cardiac tissue, or by structural changes in the phospholipase molecules[1]. It should be kept in mind that impaired resynthesis of phospholipids with a concomitantly unchanged or declined rate of phospholipid hydrolysis will also lead to net loss of phospholipid molecules in the flow-deprived heart. Impairment of reacylation of lysophospholipids (Fig. 2) can be caused by either depletion of ATP, a cofactor required for the formation of acylCoA, or by enhanced cytoplasmic concentration of AMP. AMP is a potent inhibitor of the conversion of fatty acids into their acylCoA esters[62]. Recent studies carried out by Van Bilsen and coworkers[27] strongly suggest that the most likely candidate for acylCoA synthetase inhibition is AMP, since the increase of this nucleotide in ischemic hearts paralleled in time the accumulation of arachidonic acid during the ischemic episode. Moreover, arachidonic acid started to accumulate when the AMP concentration reached the K_i of acyl CoA synthetase for AMP. ATP was found to be a less likely candidate since the residual level of ATP stayed appreciably above the affinity constant of acylCoA synthetase for ATP. The activity of lysophosphatidyl choline acyltransferase in ischemic cardiac tissue was found to be depressed when assayed in vitro[47]. This enzyme is also required for the reincorporation of acyl chains in the phospholipid molecules (Fig. 2).

In addition to oxygen free radical mediated processes (see above), Ca^{2+} may play a substantial role in phospholipid degradation in the reperfused heart. Significant amounts of calcium ions accumulate in the reperfused heart, a condition that might activate Ca^{2+}-sensitive phospholipases[1]. Direct stimulation of

phospholipase A_2 by conformational changes in the enzyme cannot be excluded since its activity was found to be permanently enhanced in microsomal fractions isolated from ischemic and subsequently reperfused pig myocardium[47]. Impairment of the capacity to resynthesize phospholipids in the reperfused heart may add to the net loss of phospholipids after reinstallation of flow. In this respect the experimental findings of Kajiyama and coworders[43] are worth to mention. Their findings indicate that oxidation of glutathione to gluthathione disulfide during ischemia causes loss of activity of the sulfhydryl-sensitive lysophosphatidyl acyltransferase after reinstallation of flow. Decreased lysophosphatidyl acyltransferase activity will impair the capacity to resynthetize phospholipids during reperfusion.

Are phospholipase inhibitors useful to attenuate ischemia- and reperfusion-induced damage?

In spite of uncertainties concerning the role of phospholipid hydrolysis in the chain of events leading to loss of cell viability and the precise nature of the mechanism(s) responsible for enhanced phospolipid degradation in the ischemic and reperfused heart, numerous attempts have been made to block the hydrolytic activity of phospholipid degrading enzymes. Various test models have been used to evaluate the efficacy of inhibiting cardiac phospholipases. The results of a representative number of these studies are summarized in Table 1. Hostetler and Jellison[78] studied the effect of a variety of compounds with established anti-ischemic properties on the Ca^{2+}-insensitive phospholipase A_1 in the sarcoplasmic and cytosolic fraction of rat hearts. Compounds such as chloroquine, chlorpromazine, diltiazem, mepacrine, propranolol, RS 87337 and verapamil showed an inhibitory action on the hydrolysis of phospholipids in this experimental model. The authors[78] concluded that their results provided support for the hypothesis that phospholipid metabolism plays a significant role in myocardial ischemia and suggested that pharmacological interventions to reduce phospholipid catabolism may represent a new way to reduce ischemic injury. Armstrong and Ganote[63] found that mepacrine reduced significantly the rate of cell death in metabolically inhibited or ischemic isolated adult cardiomyocytes. The rate of cell death was related to the inability of the affected cells to exclude exogenous trypan blue. Their findings indicate a close relationship between inhibition of phospholipid degradation and cell death, suggesting that uncontrolled activity of phospholipases may contribute to the destruction of the sarcolemmal membrane[63]. Mepacrine was found to reduce the release of arachidonic acid from ATP depleted cultured neonatal cardiomyocytes by 80%[64]. The steroidal diamine U 26,384 surpassed mepacrine in its phospholipase blocking action. This study further indicates that inhibition of phospholipid degradation is associated with prevention of altered cytoplasmic elemental concentrations, particularly calcium loading, and preservation of morphology. The protective effect of the two phospholipase inhibiting drugs occurred in spite of severe ATP depletion since an 85% or greater ATP reduction was achieved by metabolic inhibition in the presence and absence of drug treatment[64]. Additional support for the notion that inhibition of phospholipid hydrolysis prevents the myocardium from irreversible loss of cell integrity was provided by Van Bilsen and coworkers[65]. They reported that pretreatment of isolated rat hearts with mepacrine partially blocked the accumulation of arachidonic acid, originating from endogenous phospholipids, with a concomitant reduction of lactate dehydrogenase release during reperfusion. Comparable results were obtained by Otani and coworkers[66], who studied the effect of mepacrine and trifluoperazine on

Table 1 Inhibition of cardiac phospholipased by chemical compounds.

compound	model	mode of injury	ref
−choloroquine chlorpromazine diltiazem mepacrine propranolol RS 87337 verapamil	phospholipase A1 isolated from cardiac tissue (cytoplasm and sarcoplasmic reticulum)	none	78
−mepacrine	isolated adult cardiac myocytes	ischemia, metabolic inhibition	63
−U 26,384 mepacrine	cultured neonatal cardiomyocytes	metabolic inibition	64
−U 26,384	cultured neonatal cardiomyocytes	metabolic inhibition	52
−mepacrine	isolated rat heart	ischemia and reperfusion	65
−mepacrine trifluoperazine	isolated rat heart	ischemia and reperfusion	66
−antibodies against phospholipase A_2 and C	isolated rat heart	ischemia and reperfusion	69
−propranolol pindolol	isolated rat heart	ischemia and reperfusion	67
−quinacrine	rat heart in situ	regional ischemia	39
−chlorpromazine	rat heart in situ	regional ischemia	68
−mepacrine	pig heart in situ	ischemia and reperfusion	70
−mepacrine	pig heart in situ	ischemia and reperfusion	47
−benzyl methyl amino[(trifluorotolyl)oxy] propanol	dog heart in situ	regional ischemia	72
−verapamil	mitochodria isolated from dog heart	regional ischemia and reperfusion	43
−chlorpromazine amiodarone	rat heart in situ	isoproterenol treatment	75
−chlorpromazine mepacrine verapamil nifedipine propranolol	rat heart in situ	isoproterenol treatment	74
−chlorpromazine	rat heart in situ	isoproterenol treatment	73

isolated ischemic and reperfused rat hearts. Moreover, β-blockers, such as propranolol and pindolol, were found to reduce the amount of arachidonic acid accumulating in ischemic and reperfused rat hearts. The post-ischemic release of the cytoplasmic enzyme creatine kinase concomitantly declined[67]. The phospholipase A_2 inhibitor quinacrine attenuated the loss of phospholipids in regional ischemic rat hearts in situ and partly prevented the release of creatine kinase from the ischemic area[39]. Chien and coworkers[68] reported the prevention by chlorpromazine of accelerated phospholipid degradation in the sarcoplasmic reticular membranes isolated from regional ischemic rat hearts. Treatment of the heart by this drug also prevented impairment of Ca^{2+}-transport in the sarcoplasmic reticulum[68]. The role of phospholipase A_2 in ischemia- and reperfusion-induced damage has been firmly established by recent studies carried out by Plasad and colleagues[69]. They found that administration of antibodies raised against phospholipase A_2 to isolated rat hearts practically prevented the ischemia- and reperfusion-induced myocardial accumulation of arachidonic acid and the release of lactate dehydrogenase from the cytoplasmic compartment[69]. Inhibition of the depletion of the phospholipid pool of pig hearts in situ, subjected to ischemia and reperfusion, was achieved by pretreatment of the animals with mepacrine[47,70]. Chiariello and associates[71] observed a significant reduction of infarct size by quinacrine when the coronary artery was occluded for 6 hours in dog hearts in situ. Quinacrine also enhanced the phospholipid content in the affected area when compared with untreated hearts[71]. The phospholipase A_2 inhibitor 1-(benzylmethylamine)-3-[α, α, α-trifluoro-m-tolyl)oxy]-2-propanol significantly reduced infarct size in dog hearts, made regionally ischemic by occlusion of the left anterior descending coronary artery[72]. Since the turnover of mitochondrial phospholipids represents one potential area of investigation in which changes in membrane phospholipid content are most likely caused by oxidative stress and Ca^{2+}-sensitive pospholipase A_2, Kajiyama and colleagues[43] studied the effect of verapamil on the function and phospholipid content of mitochondria isolated from dog hearts, rendered regionally ischemic for 60 minutes followed by reperfusion. Treatment of the animals with verapamil caused a significant preservation of the phospholipid composition and respiratory properties of these organelles, as compared to mitochondria isolated from untreated ischemic and reperfused hearts[43]. Interestingly, phospholipase inhibitors also exerted their protective action in in situ hearts damaged by the administration fo relatively high doses of isoproterenol[73,74,75].

Although the above mentioned studies indicate that prevention of phospholipid degradation is closely related to protection of the heart against the deleterious effect of ischemia and reperfusion, no definitive conclusion can be drawn that phospholipase A_2 mediated hydrolysis of membrane phospholipids results in irreversible loss of cardiac function. It cannot be excluded that the drugs used affect other processes in cardiac cells in addition to phospholipid degradation. The specificity of these drugs regarding their inibitory action on phospholipase A_2 is either unknown or poor. For instance, drugs like verapamil have well-established Ca^{2+}-entry blocking and negative inotropic properties. Hence, their action on phospholipase mediated degradation of membrane phospholipids might be the effect of a general protection against ischemia- and reperfusion-induced injury. Moreover, drugs with anti-phospholipase properties might exert their action by stabilization of the membrane instead of direct interaction with the active centre of the enzyme. In view of the fact that phospholipase inhibiting drugs, such as mepacrine and quinacrine, show appreciable

negative inotropic effects, one might argue that reduced oxygen demand will add to the protective action of the drugs. However, it should be kept in mind that, in case of failing hearts, negative inotropic effects might depress the function of the affected heart to a level not compatible with life. In that case, the use of anti-phospholipase drugs should be discouraged. Obviously, detailed pharmacological studies are required to rate the true significance of the above mentioned drugs, showing phospholipid sparing effects in ischemic and reperfused hearts, in the clinical setting.

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