ISSN 1990-7478, Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology, 2020, Vol. 14, No. 4, pp. 267–278. © Pleiades Publishing, Ltd., 2020.
Russian Text © The Author(s), 2020, published in Biologicheskie Membrany, 2020, Vol. 37, No. 5, pp. 323–336.

REVIEWS

Phospholipase A2. Methods for Activity Monitoring

A. S. Alekseeva[a] and I. A. Boldyrev[a], *

[a] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997 Russia*
**e-mail: ivan@lipids.ibch.ru*

Received May 7, 2020; revised May 13, 2020; accepted May 14, 2020

Abstract—Phospholipases A2 (PLA2) are hydrolytic proteins, which cleave fatty acid off the second position (*sn*-2) of the phospholipid. An increased activity of PLA2 correlates with the course of many different inflammatory processes in the body. For the purpose of diagnosing and predicting pathological processes, systems for detecting the PLA2 activity are being developed. The key component of all test systems is a substrate of lipid or non-lipid nature, the breakdown of which by the enzyme leads to the appearance of analytical signal. Lipids as such do not absorb light in the visible region and do not fluoresce. Therefore, to determine the activity of PLA2, substrates with various labels are developed. Test systems for determination of the PLA2 activity can be divided into three groups, depending on the stage of the enzyme action a signal is formed at: (1) systems based on the detection of hydrolysis products; (2) systems based on the cleavage of fluorescently labeled substrates, and (3) systems based on the detection of membrane disintegration. Each of these groups has its own requirements for the structure of the substrate. This review is focused on the structure of PLA2 substrates used in systems to determine the enzyme activity; the proposed classification allows one to identify the strengths and weaknesses of existing detection systems and will be relevant when designing new test systems.

Keywords: phospholipase A2, enzyme activity, lipids, PLA2 substrates, fluorescent probes
DOI: 10.1134/S1990747820040030

INTRODUCTION

Phospholipases A2 (PLA2) is a large superfamily of proteins with a hydrolytic activity against phospholipids, which can selectively cleave fatty acid at the second position (*sn*-2) of phospholipid. As of today, six PLA2 families are typically distinguished: secreted, cytosolic, Ca^{2+}-independent, platelet-activating factor acetylhydrolases (including lipoprotein-associated PLA2), lysosomal PLA2s, and PLA2 in adipose tissue. The family of secreted PLA2s is the most numerous and includes ten groups comprising several subgroups. Secreted (extracellular) PLA2s are found in all mammalian tissues and contained in the venom of snakes and insects. Due to their catalytic activity, PLA2 can release fatty acids (e.g., arachidonic acid) for cyclooxigenases, lipoxygenases, and cytochrome P450 enzymes, which in turn produce different inflammatory mediators including leukotrienes, thromboxanes, and prostaglandins. The most comprehensive description of structural peculiarities, mechanisms of action, localization, and importance for organisms of each type of PLA2 can be found in the reviews [1, 2].

Endogenous PLA2 is of interest for researchers due to the broad range of pathologies involving this enzyme (see reviews [2–5]). Elucidation of the role of PLA2 will enable the development of agents that would have an effect on inflammatory processes in organisms and theoretically control them [6].

The interest in the study of exogenous PLA2s (e.g., from snake venom) is associated with the possibility of finding novel therapeutic agents, similarly to other components of snake venoms [7]. An important feature of PLA2 is the presence of two binding sites in this enzyme: a site recognizing the lipid membrane and another one, responsible for the binding and hydrolysis of a particular lipid molecule (catalytic site) [8]. The structure of the catalytic site is highly conservative and has been rather well studied in different representatives of PLA2s (e.g., for all secreted PLA2, there are the His and Asp residues in the catalytic pocket) [1]. The membrane binding site, on the contrary, is formed by several dozens of amino acids and varies from one enzyme to another [9]. The enzyme activity and selectivity are determined by the function of both sites. PLA2s are the typical representatives of interfacial proteins (i.e., acting on the surface but not in solution), which determines the complex kinetics of the enzymatic process [10].

Like any other protein, PLA2 can be detected using antibodies. IFA techniques have been developed for appreciable set of secreted human [11] and cytosolic PLA2s [12]. The major drawback of IFA techniques for PLA2 is that the presence of the enzyme per se is not yet an indicator of a pathology development—it is important that the enzyme displays its activity (see, e.g., the comparative studies of PLA2 detection with antibodies and assessment of its enzymatic activity

[13, 14]). Biological fluids contain a number of PLA2-binding proteins, which are able to block the membrane binding site and/or cause steric hindrance for lipid substrate binding [15].

The test systems for detecting PLA2 activity have been being developed since the 1970s. However, only recently the first test system has been introduced into clinical practice in the United States to determine the activity of lipoprotein-associated PLA2; it allows prediction of the course of ischemic heart disease [16]. The time it took to move from understanding of the role of PLA2 in the development of ischemic heart disease to the introduction of a test system to clinical practice demonstrates how difficult it was to construct such a test system. The test systems for detecting the activities of other PLA2 types suitable for clinical applications have not yet been developed. However, there are several commercially available test systems for laboratory detection of the activity of secreted PLA2s. A separate trend of research is the development of synthetic low-molecular inhibitors of PLA2 [1, 17]; their efficiency is also assessed by the test systems.

All test systems for detecting PLA2 activity are based on monitoring the hydrolysis of lipids. The latter is a difficult task because lipids do not absorb in the visible spectrum and do not fluoresce. In addition, the enzyme exerts its effect not on individual lipid molecules but on the lipids organized into bilayer membranes. These two factors considerably complicate the detection of lipid hydrolysis. The key components of test systems for detecting PLA2 activity are labeled substrates. The hydrolysis of such substrates either immediately results in emergence of an analytic signal or the products of hydrolysis are compounds reacting with auxiliary substances with the formation of new products that can be monitored analytically. Development of substrates for PLA2 activity detection is a complicated task. This review is devoted to the structure of such substrates and intended for the developers of test systems for detection of the enzymatic activity of PLA2.

EFFECTS OF PLA2 ON LIPID BILAYER AND POSSIBILITIES FOR PLA2 ACTIVITY DETECTION

Enzyme activity is defined as the number of moles of the substrate transformed by the enzyme per unit of time ($\Delta C/\Delta t$). To detect the activity, it is necessary to plot the dependence of substrate concentration on the duration of hydrolysis. One lysolipid molecule and one fatty acid molecule are produced from one phospholipid molecule under the effect of PLA2. PLA2 activity can be monitored both by the decrease in the amount of substrate and by the appearance of one of the products.

PLA2, as a typical representative of interfacial proteins, cannot hydrolyze lipids in solution and functions at the surface of lipid membrane. When developing methods for detection of PLA2 activity, it is convenient to represent the effect of the enzyme on the lipid bilayer as a three-stage process schematically shown in Fig. 1. At the first stage (**i**), the membrane-bound enzyme captures and hydrolyzes a phospholipid with the release of reaction products: fatty acid and lysolipid. At the second stage (**ii**), the products of hydrolysis are distributed over the membrane via diffusion. At the third stage (**iii**), accumulation of hydrolysis products results in membrane disintegration.

According to the scheme in Fig. 1, there are several ways to record enzyme activity, particularly, by monitoring (1) the accumulation of the products of hydrolysis; (2) cleavage of initial phospholipids; and (3) the disintegration of the lipid membrane. Methods utilizing each of the three approaches have been developed.

PLA2 activity can be detected by the appearance of the products of hydrolysis using natural or isotopically labeled lipids (Fig. 2a). The resulting products are separated by chromatography and their amounts are recorded. Sequential detections at different time points of the reaction should be performed to plot the kinetic curve. This is the only measurement scheme that allows the direct calculation of enzyme activity, because the substrate or product concentration is recorded directly. The rate of the change in this concentration is the enzyme activity ($\Delta C/\Delta t$).

If PLA2 activity is detected by the substrates yielding stained products as a result of hydrolysis, the appearance of such products can be monitored by spectrophotometry avoiding chromatography (Fig. 2b). The observed signal is a function of the product concentration and is described by the Beer–Lambert–Bouguer law. However, binding of the labeled substrate by the enzyme is lower than that of natural lipids; therefore, the rate of the hydrolysis observed can be lower.

In addition, labeled substrates are used in mixture with natural lipids, which form the lipid bilayer matrix necessary for normal function of the enzyme. The lipids of the matrix are also hydrolyzed by PLA2. However, according to scheme (b) in Fig. 2, the signal appears only in the case of hydrolysis of the labeled substrates but not the lipids of the matrix.

Thus, a correction should be used in order to assess the actual activity of the enzyme by its observed activity.

Scheme (c) in Fig. 2 shows the detection of PLA2 activity by the cleavage of substrates with a fluorescent label. In this case, fluorescent probes contain a fluorophore attached to one of the hydrophobic chains of the lipid (usually at position *sn*-2) and a specific group, a fluorescence quencher, attached to another hydrophobic chain or to the polar head of the lipid. Fluorescence quenching usually occurs through the mechanism of Förster resonance energy transfer; the intensity of fluorescence quenching is in inverse proportion with the sixth power of the distance between the fluorophore and the quencher. If the fluorophore

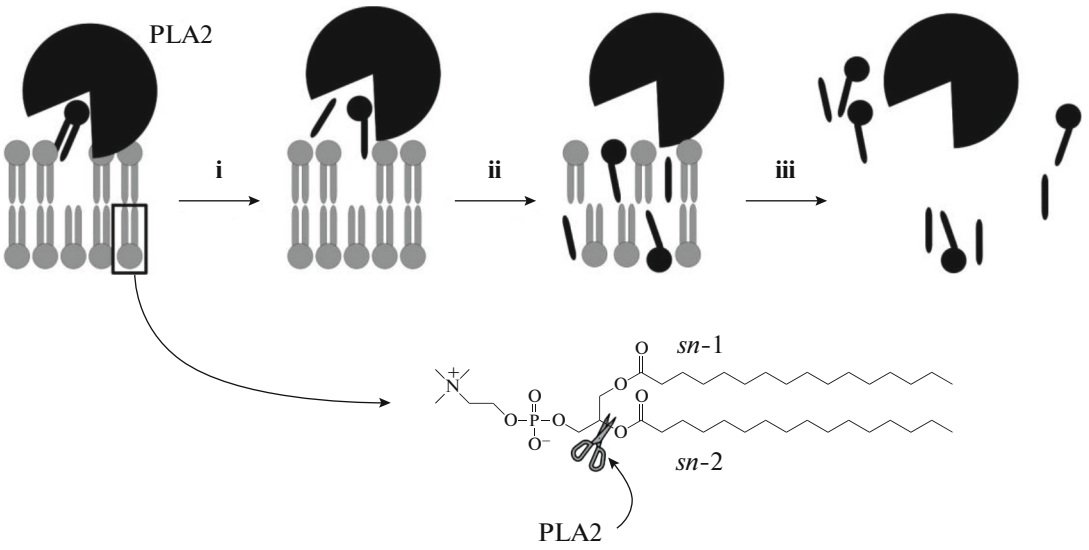

Fig. 1. The schematic representation of the effect of PLA2 on the bilayer: **i**, capture of a phospholipid molecule and hydrolysis; **ii**, distribution of the products of hydrolysis over the membrane; **iii**, membrane disintegration due to accumulation of the products of hydrolysis.

and the quencher are present in the same molecule, the fluorescence intensity is low. During the hydrolysis and subsequent diffusion of the hydrolysis products, the distance between the fluorophore and the quencher increases and the fluorescence signal grows. If the fluorophore and the quencher, despite the hydrolysis, remain in lipid aggregates at a certain distance **r** from each other, residual fluorescence quenching persists given the distance is short.

In addition, the analysis of the observed signal should take into account the influence of the light scattering by liposome dispersions, which in turn depends on the course of the hydrolysis.

The next scheme (Fig. 2d) shows the method for detection of PLA2 activity by disintegration of the bilayer or any other lipid aggregate. In simple cases, liposomes are used, which contain a dye (analyte) in the inner watervolume. Upon liposome destruction by the enzyme, the dye leaks out of the inner water volume of liposomes into the medium and, as a result, the recorded signal changes. In case of the detection according to scheme (d), the analyte is not connected with either to the substrate or the products of hydrolysis. Liposome destruction occurs upon accumulation of the products of hydrolysis to a certain critical level.

Thus, in most cases the observed signal depends in a complex manner on concentration of the products of hydrolysis and thus corrections should be made. The exception is the application of natural lipids or lipids with an isotope label. In the real laboratory and clinical practice, instead of determining all corrections and dependencies, the fluorescent signal is calibrated using standard enzyme solutions with known activity. The latter is determined by the manufacturer of standard solutions according to scheme (a) in Fig. 2. Calibration provides reliable results using systems with colored and fluorescent substrates (schemes (b) and (c) in Fig. 2) and, in many cases, allows correction of the data obtained by destruction of lipid aggregates (scheme (d) in Fig 2). For designing novel PLA2 detection systems, it is necessary to reduce the effects of all extraneous factors (not associated with the enzyme) on the observed signal.

DETECTION OF PLA2 ACTIVITY BY THE ACCUMULATION OF HYDROLYSIS PRODUCTS

In simple systems consisting of the only enzyme and the only type of lipids, thin-layer chromatography with staining according to Vaskovsky [18] proposed as early as in the 1970s is sufficient for detecting the products of hydrolysis . This method of staining is specific to phosphate residues in lipid molecules. The intensity of staining is proportional to the concentration of phosphate groups; therefore, the relative quantity of initial phospholipids and the products of hydrolysis, lysophospholipids, can be easily determined (see the example in [19]). The method does not allow detection of the low amounts of the product and calculation of the minor increases in concentration. However, it is simple and can be used to detect the very fact of hydrolysis.

In complex multicomponent systems such as blood plasma, culture medium, or cell lysates, it is important to know that fatty acids and lysolipids were obtained precisely during the hydrolysis while measuring and had not been present in the system originally. In other words, if PLA2 is present in the system and active, the

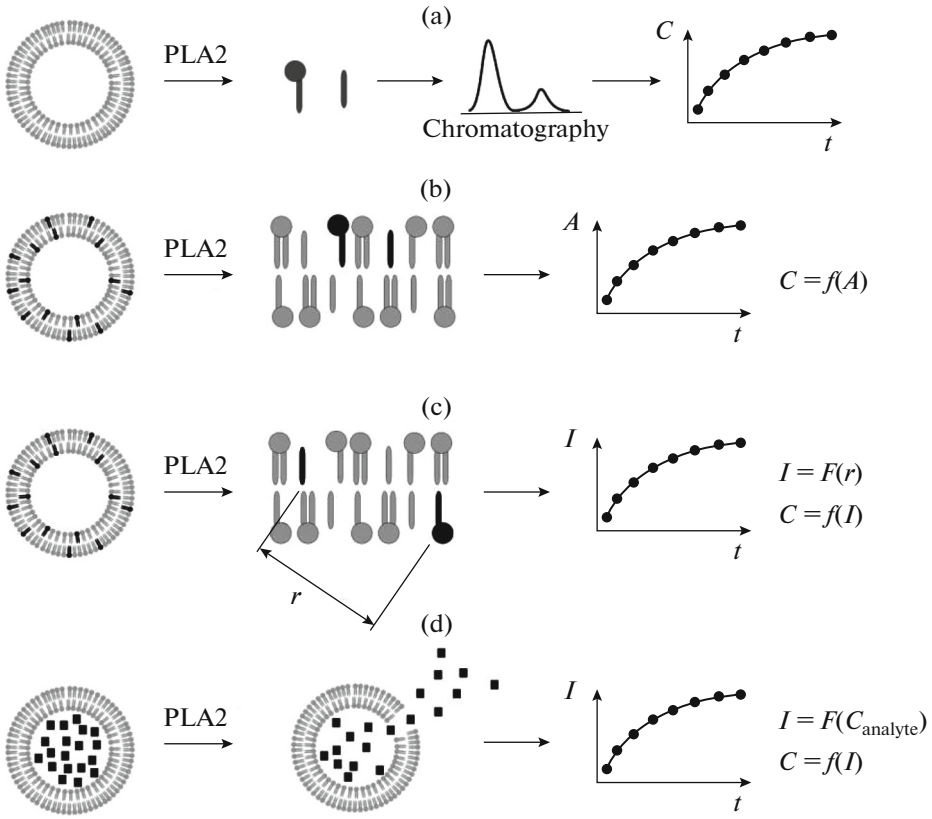

Fig. 2. The schemes of detection of PLA2 activity. (a) Detection of the products of hydrolysis using natural lipids or lipids with an isotope label; (b) detection of the enzyme activity using substrates, which form stained products during the hydrolysis; (c) detection of the enzyme activity using substrates with a fluorescent label; (d) detection of the enzyme activity by destruction of lipid aggregates.

system accumulates the products of hydrolysis. While determining the enzyme activity, one should distinguish them from the products of hydrolysis formed during the analysis. It requires either simultaneous quantitative monitoring of both substrates and products or using labeled substrates. In the former case, it is possible to calculate the increase in the content of the hydrolysis products. In the latter case, labeled products can be easily distinguished from the lipid matrix, as well as fatty acids and lysolipids already present in the system, by a specific signal.

The simultaneous monitoring of the amounts of phospholipids and products of their hydrolysis makes it possible to calculate the quantity of phospholipids hydrolyzed per unit of time, i.e., to immediately determine enzyme activity ($\Delta C/\Delta t$). The approach has been difficult to methodically implement for a rather long time. However, at present, due to the progress in mass spectrometry and the improvement of high-performance liquid chromatography columns, such monitoring is already not an unmanageable issue. For example, there is a method employing high performance liquid chromatography with electrospray ionization mass spectrometry detection [20]. It allows simultaneous detection of phosphatidylcholine as a substrate for PLA2 and its products, fatty acids and lysophosphatidylcholine [20]. The method uses a silica gel column with a short-chain (C5) grafted phase and detects phosphatidylcholine and lysophosphatidylcholine as acetate adducts. Following Schebb [20], other methods of high-performance liquid chromatography combined with mass spectrometry have been developed; they allow simultaneous detection of phosphatidylcholine and the products of its hydrolysis by PLA2 [21]. The analysis requires only a few minutes, which evidences its high efficiency, with simultaneous monitoring of the products of hydrolysis and original lipids.

The method of high-performance liquid chromatography combined with mass spectrometry allows monitoring of lipid hydrolysis by PLA2; however, this monitoring is actually not continuous but discrete—the time between two measurements is equal to the time of analysis. This is why it is so important to reduce the time of analysis, which is the objective of the researchers' work.

The inverse problem (to use the enzyme for lipid detection) can also be solved. Tan et al. successfully developed the method for determining the structure of lipids, with identification of fatty acid binding sites

(*sn*-1 or *sn*-2) using two-dimensional thin layer chromatography of phospholipases A1 and A2 [22].

Application of labeled substrates of PLA2 started with using radioactively labeled phospholipids as early as in the 70–80s. This method is well documented (see, e.g., [23]). Its popularity was due to the accuracy of radioactive methods and the comparative availability of instrumental equipment. The data obtained by the method are much more precise than the data of phosphate group staining according to Vaskovsky.

Radioactive substrates are structurally identical to original lipids. The substitution of one of the ^{12}C carbon atoms with the ^{14}C isotope in the *sn*-2 chain has no effect on the ability of the enzyme to bind and hydrolyze the substrate. In this case, the labeled substrate and the lipid matrix are represented by a single type of molecules. It imparts radioactive substrates with a substantial advantage—they allow for direct measurement of the enzyme activity. The drawbacks of this method are associated with difficulties to obtain permission for the work with radioactive substances. The development of other, more readily available methods for determining PLA2 activity was accompanied by decrease of the popularity of radioactivity-based methods.

Continuous detection (without intermediate chromatography) is possible with spectrometry equipment, e.g., a spectrophotometer. However, the molecules of phospholipids do not absorb light in the visible spectrum. As a result, chemical modification of lipids with the purpose to obtain PLA2 substrates that would carry reporter groups, chromophores, which can be detected with a spectrophotometer, has become a trend.

Since the total concentration of reporter groups in the system cannot change during hydrolysis, the enzyme should cause a change that would lead to the changes in the recorded signal. The enzyme-induced cleavage of the ester group should lead to a change in the color of sample solution under study. Attempts have been made [24, 25] to find substances of non-lipid nature that would be hydrolyzed by PLA2 and the resultant fragments would have the light absorption spectrum different from that of the initial substance.

This is true, for example, for 4-nitro-3-octanoyloxy-benzoic acid **4** (Fig. 3) [24]. This substance absorbs in the UV spectrum but releases nitrophenol derivative **5** during hydrolysis (Fig. 3) with the absorption maximum in the visible spectrum (425 nm). As hydrolysis proceeds, yellow color appears and becomes more intense.

1-Octanoyloxynaphthalene-3-sulfonic acid **6** is a similar substrate [25]; it is well hydrolyzed by PLA2, as has been shown by the authors. The product of its hydrolysis is 1-naphtol-3-sulfonic acid **7**, which reacts with diazonium salt **8** present in the solution (azo coupling reaction) with the formation of a reddish violet azo dye **9** (Fig. 3). The absorption spectrum of the tested solution changes in the course of an additional reaction.

Thio derivatives **10** and **15** are highly similar to natural phospholipids (Fig. 3). In fact, they differ from natural phospholipids by a single substitution, i.e. the oxygen atom in the ester bond is replaced by a sulfur atom. Phospholipase A2 can hydrolyze such bond, and the released thio derivatives react with DTNB (5,5'-dithio-bis-(2-nitro-benzoic acid)) **12** with the formation of stained thionitro derivative **14**. Compounds **10** and **15** are the substrates for secreted and cytosolic PLA2, respectively. A thio derivative **15** carries an arachidonic acid residue, which is necessary for successful detection of the activity of cytosolic PLA2, because cytosolic PLA2s are the only representatives of the PLA2 superfamily exhibiting specificity for the arachidonic acid residue.

One more lipid-like substrate is 1-myristoyl-2-(4-nitrophenylsuccinyl)-phosphatidylcholine **17**. This substrate is a phosphatidylcholine with a derivative of succinic acid and nitrophenol instead of the fatty acid in the second position (Fig. 3). Enzymatic hydrolysis in the *sn*-2 position leads to the formation of a monosubstituted derivative of succinic acid (*n*-nitrophenyl succinate, **18**), which in turn is spontaneously hydrolyzed with the formation of *n*-nitrophenol **19**. The latter gives bright yellow color (the absorption peak at 405 nm). The enzyme activity is determined spectrophotometrically by the color of the solution. The system has been developed for quantification of lipoprotein-associated PLA2 in blood plasma and serum, which makes it possible to predict the course of ischemic heart disease. Reproducibility of the test system was verified both by the developer [26] and by research institutions [16]. At the moment, this is the only test system used in clinical practice.

The above-mentioned PLA2 substrates are not natural phospholipids. Thio derivatives **10** and **15** are chemically modified at a sensitive site (i.e. at bond cleaved by the enzyme), while substrate **17** contains a chromophore group rather close to the active site of the enzyme. As a consequence, the enzyme activity against these substrates differs from the activity against phospholipids. Therefore, in laboratory and clinical practice it is necessary to use reference samples—samples of pure enzyme with the known activity. At the same time, calibration curves should be reproduced for each new sample, which is undoubtedly a limitation. The advantage is that the measured signal—the intensity of light absorption at a certain wavelength—is directly proportional to product concentration, which simplifies the detection of PLA2 activity. The alternative to the PLA2 substrates with chromophore reporter groups changing color are the substrates with fluorescent labels. However, the principle of their action is different.

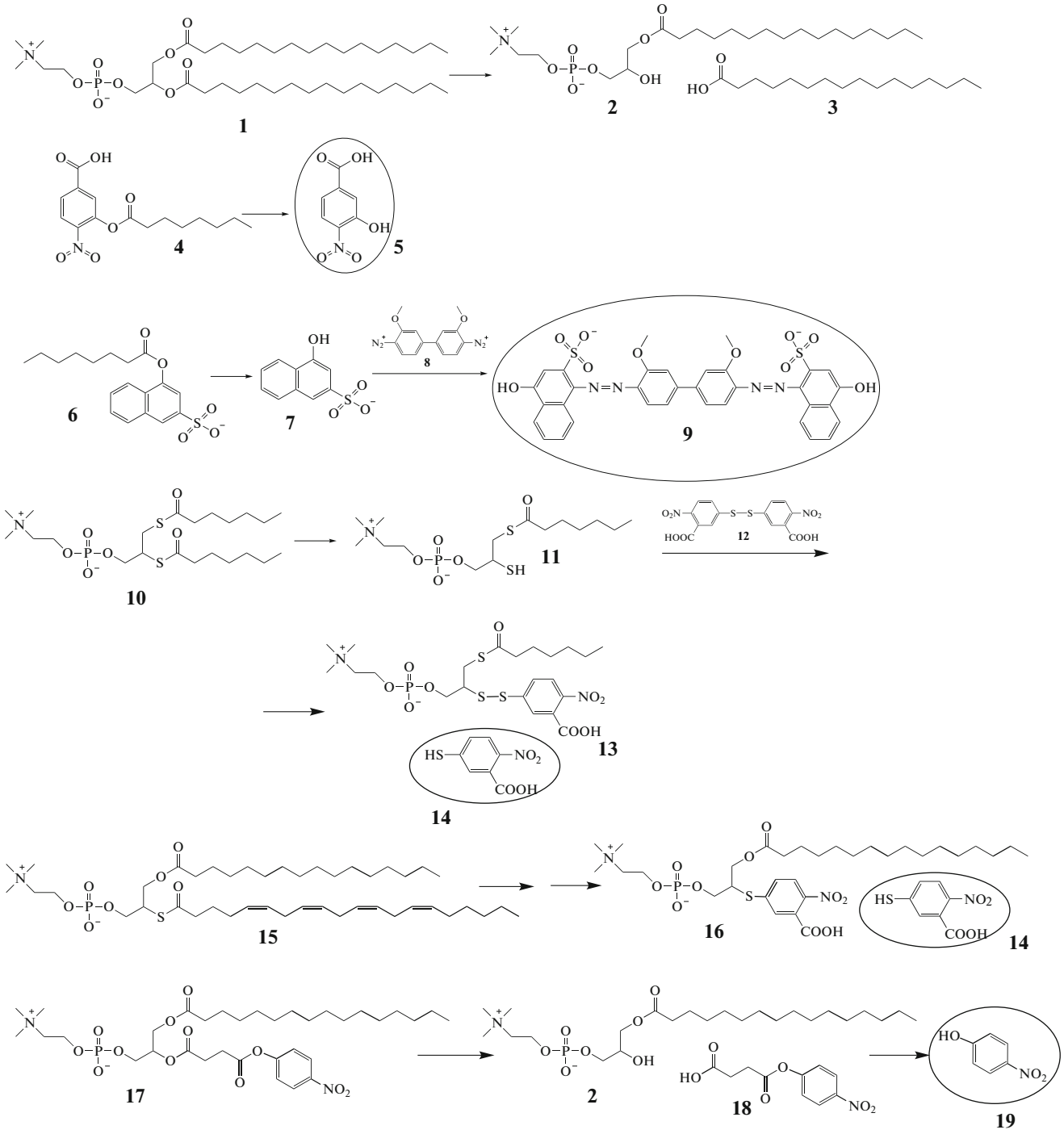

Fig. 3. Detection of PLA2 activity by accumulation of the products of hydrolysis. Phosphatidylcholine (**1**) and the products of its hydrolysis by the enzyme: lysophosphatidylcholine (**2**) and fatty acid (**3**). Colored (detected) products of hydrolysis are encircled. Other explanations are given in the text.

DETECTION OF PLA2 ACTIVITY BY SUBSTRATE CLEAVAGE

The method for detecting PLA2 activity by substrate cleavage is based on the fact that the products of hydrolysis, which are no more bound into a single molecule, diffuse in the lipid matrix, moving away from each other. It leads to changes in the signal. Fluorescent probes contain a fluorophore attached to one of the lipid hydrophobic chains and a fluorescence quencher attached to the other hydrophobic chain or to the polar head of the lipid. As long as the fluorophore and the quencher are within a single molecule,

fluorescence is low. During hydrolysis and subsequent diffusion of the products of hydrolysis, the distance between the fluorophore and the quencher increases and the fluorescence signal grows. In fact, this method registers not the products but the very fact of hydrolysis, that the fact of fatty acid residues not comprising a single molecule but being spatially separated. If the products of hydrolysis do not diverge in space and remain rather close to each other, fluorescent signal will be low.

The important role of diffusion of the products of hydrolysis in the signal formation makes it necessary to choose carefully a lipid matrix. It forms the environment where the products of hydrolysis diffuse. While developing substrates with a fluorescent label, one should take into consideration that fluorophores are rather bulky groups. They can prevent binding by the enzyme, and the hydrolysis of such molecules can be too slow or even not occur at all. The structure of the catalytic site of the enzyme suggests that substrate molecule should contain a fluorophore and a quencher at the end of the hydrophobic chain no closer than 10 carbon atoms from the glycerol residue of the lipid [27], i.e., far from the catalytic site of the enzyme.

Several examples of the substrates for PLA2 for determination of its activity by the changes in fluorescence signal are given below (Fig. 4). With rather great diversity of fluorophores and quenchers, the lipid part of these molecules is unvaried.

The agents that are rather extensively used in laboratory practice are commercial lipid derivatives with BODIPY fluorophores at the end of the fatty acid chain and a dinitrophenyl quencher attached to the lipid polar head (**20**) (Fig. 4) (for an example of application, see [28]), as well as a Red/Green BODIPY PC-A2 fluorescently labeled probe (**21**). The latter is intended for the work with cells and cell lysates. The matrix is composed by phospholipids, a mixture of dioleoyl phosphatidylcholine and dioleoyl phosphatidylglycerol (for examples of applications, see [29, 30]).

Abe et al. proposed phosphatidyl glycerol with fluorescein as a fluorophore and dabcyl as a quencher (**22**) [31]; the hydrophobic chains were 12 carbon atoms in length. At the same time, the probes with fluorescein and dabcyl proved to be the best at the *sn*-1 and *sn*-2 position, respectively (this structure is shown in Fig. 3). Wichmann et al. proposed a series of fluorescent lipid probes with a fluorophore at the end of a long chain, with the chain resembling arachidonic acid (**23**) [32]. Popov et al. [33] proposed the probes with a pheophorbide derivative as a label, which emitted light in the near-infrared spectrum and was attached to the end of the hydrophobic chain of 6 or 12 carbon atoms in length (**24**). The fluorescence quencher was a diazo derivative,– the so-called black quencher. They found out that the short-chain derivative (6 atoms) was not hydrolyzed by PLA2, while the long-chain derivative was hydrolyzed well (Fig. 4).

The variant of Alekseeva et al. [27] is phosphatidylcholine with a BODIPY fluorophore and a specific quencher of its fluorescence, Sudan III dye. Both of them are attached at the ends of long fatty acid chains (**25**). It can be asserted that researchers have succeeded in designing PLA2 substrates that are well hydrolyzed by the enzyme while carrying bulky fluorophores and quenchers. Together with the correctly selected matrices and standard enzyme samples, they give good results.

The main drawback of the systems with fluorophores and quenchers is that the observed signal (fluorescence intensity) depends non-linearly on the concentration of the products of hydrolysis. The effects of side processes are most marked in biological samples, such as blood plasma and cell lysates. The latter have numerous components capable of affecting the lipid matrix and, as a consequence, distribution of labels in the lipid bilayer. It is necessary to use additional methods of correction. The example of accounting side processes can be the work by Alekseeva et al. [27]. For describing the kinetics of the observed fluorescence signal, they have proposed a three-stage model involving the initial substrate in the matrix, the non-hydrolyzed substrate outside the matrix, and the products of hydrolysis. Processing of experimental data according to the model makes it possible to calculate enzyme activity in blood plasma. This achievement, however, is difficult to introduce into wide practice due to the relatively complex mathematical apparatus compared to direct detection of the products of hydrolysis.

DETECTION OF PLA2 ACTIVITY BY LIPID BILAYER DAMAGE

The PLA2-induced lipid hydrolysis and accumulation of the products of hydrolysis—lysolipids and fatty acids—in the lipid bilayer cause membrane packing distortion and then complete destruction of the membrane. These changes can be recorded and used to assess the activity of PLA2.

Monitoring of changes in light scattering of the initial dispersion is one of the variants of this approach. The intensity of the scattered light depends on particle size. Stable liposomes are no less than 50 nm in size and can be prepared by the method of extrusion with a comparatively narrow particle-size distribution. The effect of PLA2 on liposomes leads to the formation of lysolipids, which cannot form a bilayer and, consequently, liposomes. Lysolipids per se form micelles of only several nanometers in size. The changes in average lipid particle sizes lead to the changes in light scattering intensity, which can be monitored and used to assess the activity of PLA2 [34]. However, this technique requires considerable amounts of lipids and the enzyme.

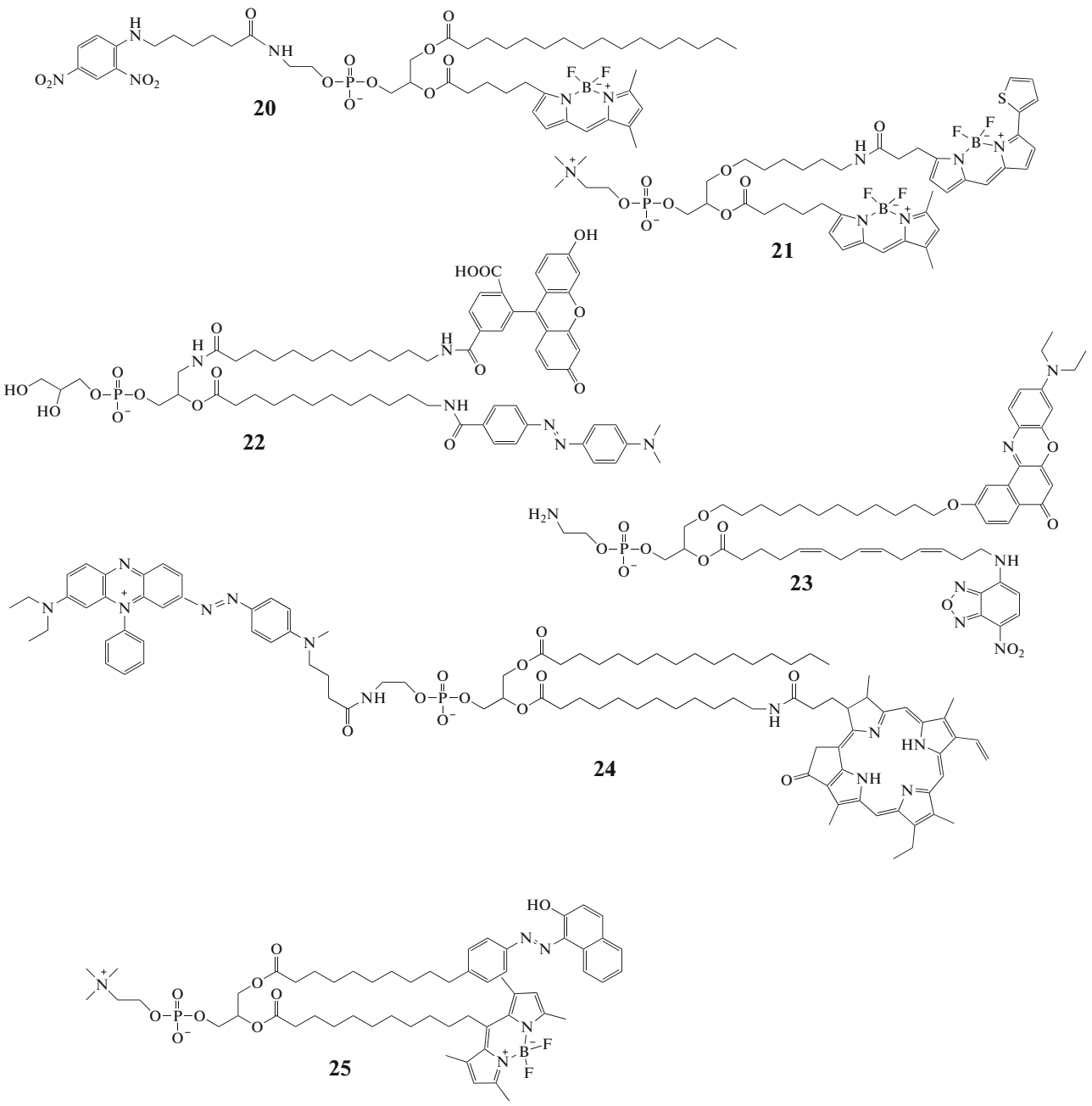

Fig. 4. The fluorescent substrates for detection of PLA2 activity carrying a fluorophore and a specific fluorescence quencher.

Another variant is to use liquid crystal sensors, where the products of hydrolysis released during the function of PLA2 distort the packing of liquid crystal molecules. Such sensors are arranged as follows: a liquid-crystal layer attached to a microgrid is placed above the layer of liposomes. If the enzyme is present in the layer of liposomes (the sensor can be adjusted also for the in-flow operation) and the hydrolysis of lipids takes place, the released fatty acids and lysolipids migrate from the lipid phase and are incorporated into the liquid crystal layer. This changes the polarization pattern of the light that passes through the liquid crystal layer [35]. Alternatively, lipids can initially be inside the liquid crystal layer and their hydrolysis by PLA2 to lysolipids changes the packing of liquid crystals. Detection is also performed by the changes in light polarization [36].

The methods based on fluorescent dyes are simpler and simultaneously more sensitive to destruction of the bilayer. They use the dyes incorporated in the lipid membrane or the dyes present in the inner water volume. The systems are designed so that the membrane damage would cause a change in fluorescence signal.

Huang et al. [37] used the ANS (8-anilino-1-naphthalenesulfonic acid) fluorescent dye incorporated into the lipid bilayer. The fluorescence of this dye depends on the polarity of the medium: it falls upon transition from non-polar to polar environment. Until ANS is incorporated into the membrane, fluorescence signal remains high. Upon membrane destruction, the local microenvironment of the label becomes more polarized and fluorescence decreases. The fluorescence of ANS changes upon its sorption on albumin and many other proteins. Therefore, this method is inapplicable for determining the PLA2 activity in biological samples.

The test system with phospholipids carrying a 10-pyrenyldecanoyl residue in the *sn*-2 position was developed to measure the activity of phospholipase A2 in the presence of albumin [38]. In an aqueous medium, such phospholipids form vesicles with the minimum fluorescence of the monomer (due to excimerization of pyrenyl). 10-(1-Pyrenyl) decanoic acid released as a result of enzymatic cleavage is "dissolved" in the reaction medium only in the presence of albumin, which binds fatty acids with high affinity. At the same time, there is an increase in fluorescence of the pyrenyl monomer. In this system, the analyte (pyrenyldecanoic acid) is a product of hydrolysis. The substrate (pyrenyl-labeled phosphatidylcholine) is different from the substrates with a fluorophore and a quencher: fluorescence quenching occurs during intermolecular interaction but not within a single molecule. Two pyrene residues from different molecules interact with the formation of an excimer (fluorescence of the monomer is quenched). Hydrolysis results in destruction of the aggregates of pyrenyl-labeled phosphatidylcholine; simultaneously, the medium where excimers could be formed disappears. Due to its simplicity, this test system is extensively used in laboratory practice to determine the activity of enzyme samples isolated from various organisms. The examples of application are given in [39, 40].

Tabaei et al. [41] used fluorescently labeled liposomes immobilized on a support. In the field of view of a confocal microscope, these liposomes are bright spots. Under the influence of PLA2, liposomes are destroyed and the products of hydrolysis migrate from the immobilized liposome into the medium. Fluorescence intensity (the brightness of a spot representing a liposome) decreases. The decrease in brightness can be used to calculate the enzyme activity. The authors suppose that under conditions of the assay, one enzyme molecule interacts with a liposome [41].

More frequent is the situation when fluorescence of a dye incorporated into the membrane is intentionally quenched (a fluorescence quencher is located nearby). Upon membrane destruction, the fluorophore and the quencher diverge in space and fluorescence increases. This is the principle of operation of the pair of lipid probes TMB-PC/BCHB-PC [42, 43]. It is important that the fluorophore and the quencher are in different molecules. This system makes it possible to estimate the extent of lipid membrane damage. It was used to find out the non-hydrolytic effect of heterodimeric phospholipases found in the venom of Nikolsky's adder [19].

The impairment of liposome integrity can be monitored by dye efflux from the inner water volume of liposomes. The classical example is calcein-loaded liposomes. This fluorescent dye is present in the liposomes at a high concentration. Due to the effect of internal filter, the fluorescence of concentrated solutions is "quenched." Upon liposome destruction, the dye flows out to the external aqueous phase and is diluted; the level of fluorescence signal increases. This technique has been used in numerous studies of lipid bilayer stability (see, e.g., [44]). In case of PLA2, it was implemented as a method for high performance liquid chromatography [45]. In the method, calcein-loaded liposomes are immobilized on the surface of carrier particles for chromatography. During hydrolysis, the dye is released and its fluorescence is recorded in the system for high performance liquid chromatography. This system was updated to detect polychlorinated biphenols. In the latter case, PLA2 acts as a signal amplifier [46, 47].

Chen et al. loaded liposomes with trinitrophenol and then the emulsion of liposomes was applied to a graphene support. Under the conditions of exposure to PLA2, liposomes were destroyed with the release of trinitrophenol, which was adsorbed on graphene and changed its conductivity [48].

The composite particles of lipids and some nanoparticles are often used instead of liposomes. For example, Li et al. used composite nanoparticles of quantum dots, NBD-labeled phosphatidylcholine, and hydrogenated soybean phosphatidylcholine [49]. The NBD fluorescence is initially quenched by quantum dots. After the enzyme has hydrolyzed the lipids, the particles are destroyed, NBD is released into solution, and fluorescence signal rises. Chen et al. used gold nanoparticles coated with dipalmitoyl phosphatidylcholine. During their hydrolysis, the envelope was destroyed and the particles aggregated, thereby changing the absorption spectrum [50]. Guo et al. [51] created nanoparticles on the basis of gadolinium with perfluoro-15-crown-5 ether and an envelope of phospholipids. The signal (time T2 ^{19}F MRI) was quenched in the presence of gadolinium. Upon destruction of the lipid envelope by the enzyme, the nanoparticles dissociated and the signal appeared. The method of detection is magnetic resonance [51].

All systems of PLA2 detection by destruction of the lipid bilayer have the same drawback. The observed signal depends on hydrolysis not directly but via the stages of accumulation of its products and destruction of the lipid layer. Both of these stages can depend on extraneous processes such as, e.g., the sorption of albumin or other proteins, if the matter in question is

the work with multicomponent media. In other words, extraneous processes may have too much influence on the signal.

CONCLUSIONS

Investigation of the involvement of phospholipase A2 in numerous pathological processes (first of all, inflammatory ones) attracts more and more attention of researchers. It is particularly significant to detect the activity of PLA2 in biological media: culture liquid, cell lysates, blood plasma, and serum. Phospholipases A2 are the components of venoms of various organisms, and the study of the activity of phospholipases A2 from new sources is a pressing and complicated problem.

Starting from the 70s and up to now, the development of a system for determation of the activity of PLA2 has been on-going. However, the creation of such systems for biological samples proved to be a major challenge. At present, there is only one test system for detecting lipoprotein-associated PLA2 in clinical diagnostics, which works with blood plasma and serum. The methods based on detection of the products of hydrolysis and on the cleavage of fluorescently labeled lipids are more adapted to monitor the activity of PLA2 in biological samples. The methods based on detection of the products of hydrolysis are simpler for application. The methods based on detection of the cleavage of fluorescently labeled lipids require the complex mathematical algorithm of experimental data processing. The methods based on detection of membrane destruction are even more difficult to use, because the analyte is not associated with the fact of cleavage of lipid molecules.

The methods of detecting PLA2 activity for clinical applications require further development with due consideration of the difference between the structure of labeled substrate and natural lipids so that the rate of hydrolysis of new substrates will be close to the rate of hydrolysis of natural lipids. For this purpose, the substrates must be structurally similar to the lipids, and the labels should be localized as far as possible from the catalytic enzyme binding site. The future PLA2 test systems should also take into account the extraneous processes influencing the signal, such as destruction of the lipid matrix induced by blood plasma proteins. The current knowledge of substrate structures and the work of test systems based on these substrates already allows the development of systems that would satisfy all these requirements.

ACKNOWLEDGMENTS

The work was supported by the Russian Science Foundation (project no. 19-75-00101).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interests.

This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Dennis E.A., Cao J., Hsu Y.-H., Magrioti V., Kokotos G. 2011. Phospholipase A2 enzymes: Physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* **111** (10), 6130–6185. https://doi.org/10.1021/cr200085w
2. Vasquez A. M., Mouchlis V.D., Dennis E.A. 2018. Review of four major distinct types of human phospholipase A2. *Adv. Biol. Regul.* **67** (3), 212–218. https://doi.org/10.1016/j.jbior.2017.10.009
3. Sofogianni A., Alkagiet S., Tziomalos K. 2018. Lipoprotein-associated phospholipase A2 and coronary heart disease. *Curr. Pharm. Des.* **24** (3), 291–296. https://doi.org/10.2174/1381612824666180111110550
4. Talmud P.J., Holmes M.V. 2015. Deciphering the causal role of sPLA2s and Lp-PLA2 in coronary heart disease. *Arterioscler. Thromb. Vasc. Biol.* **35** (11), 2281–2289. https://doi.org/10.1161/ATVBAHA.115.305234
5. Dore E., Boilard E. 2019. Roles of secreted phospholipase A 2 group IIA in inflammation and host defense. *Biochim. Biophys. Acta Mol. Cell. Biol. Lipids.* **1864** (6), 789–802. https://doi.org/10.1016/j.bbalip.2018.08.017
6. Nikolaou A., Kokotou M.G., Vasilakaki S., Kokotos G. 2019. Small-molecule inhibitors as potential therapeutics and as tools to understand the role of phospholipases A2. *Biochim. Biophys. Acta Mol. Cell. Biol. Lipids.* **1864** (6), 941–956. https://doi.org/10.1016/j.bbalip.2018.08.009
7. Zambelli V.O., Picolo G., Fernandes C.A.H., Fontes M.R.M., Cury Y. 2017. Secreted phospholipases A_2 from animal venoms in pain and analgesia. *Toxins.* **9** (12), 406. https://doi.org/10.3390/toxins9120406
8. Scott D., White S., Otwinowski Z., Yuan W., Gelb M., Sigler P. 1990. Interfacial catalysis: The mechanism of phospholipase A2. *Science.* **250** (4987), 1541–1546. https://doi.org/10.1126/science.2274785
9. Winget J.M., Pan Y.H., Bahnson B.J. 2006. The interfacial binding surface of phospholipase A2s. *Biochim. Biophys. Acta.* **1761** (11), 1260–1269. https://doi.org/10.1016/j.bbalip.2006.08.002
10. Berg O.G., Gelb M.H., Tsai M.-D., Jain M.K. 2001. Interfacial enzymology: The secreted phospholipase A2-paradigm. *Chem. Rev.* **101** (9), 2613–2654. https://doi.org/10.1021/cr990139w
11. Nevalainen T.J., Eerola L.I., Rintala E., Laine V.J., Lambeau G., Gelb M.H. 2005. Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases A2 in human serum. *Biochim. Biophys. Acta Mol. Cell. Biol. Lipids.* **1733** (2–3), 210–223. https://doi.org/10.1016/j.bbalip.2004.12.012

12. Macdonald D.J., Boyle R. M., Glen A.C.A., Leslie C.C., Glen A.I.M., Horrobin D.F. 2015. The development of an ELISA for group IVA phospholipase A2 in human red blood cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. **94**, 43–48. https://doi.org/10.1016/j.plefa.2014.11.003

13. Topbas C., Swick A., Razavi M., Anderson N.L., Pearson T.W., Bystrom C. 2018. Measurement of lipoprotein-associated phospholipase A2 by use of 3 different methods: Exploration of discordance between ELISA and activity assays. *Clin. Chem*. **64** (4), 697–704. https://doi.org/10.1373/clinchem.2017.279752

14. Zhuo S., Wolfert R.L., Yuan C. 2017. Biochemical differences in the mass and activity tests of lipoprotein-associated phospholipase A 2 explain the discordance in results between the two assay methods. *Clin. Biochem*. **50** (18), 1209–1215. https://doi.org/10.1016/j.clinbiochem.2017.08.019

15. Šribar J., Križaj I. 2011. Secreted phospholipases A2 – Not just enzymes. *Acta Chimica Slovenica*. **58** (4), 678–688.

16. Donato L.J., Meeusen J. W., Callanan H., Saenger A.K., Jaffe A. S. 2016. Advantages of the lipoprotein-associated phospholipase A2 activity assay. *Clin. Biochem*. **49** (1–2), 172–175. https://doi.org/10.1016/j.clinbiochem.2015.09.002

17. Ong W.Y., Farooqui T., Kokotos G., Farooqui A.A. 2015. Synthetic and natural inhibitors of phospholipases A2: Their importance for understanding and treatment of neurological disorders. *ACS Chem. Neurosci*. **6** (6), 814–831. https://doi.org/10.1021/acschemneuro.5b00073

18. Vaskovsky V.E., Kostetsky E.Y., Vasendin I.M. 1975. A universal reagent for phospholipid analysis. *J. Chromatography A*. **114** (1), 129–141. https://doi.org/10.1016/S0021-9673(00)85249-8

19. Alekseeva A.S., Tretiakova D.S., Chernikov V.P., Utkin Y.N., Molotkovsky J.G., Vodovozova E.L., Boldyrev I.A. 2017. Heterodimeric V. nikolskii phospholipases A2 induce aggregation of the lipid bilayer. *Toxicon*. **133**, 169–179. https://doi.org/10.1016/j.toxicon.2017.05.015

20. Schebb N.H., Falck D., Faber H., Hein E.-M., Karst U., Hayen H. 2009. Fast method for monitoring phospholipase A2 activity by liquid chromatography–electrospray ionization mass spectrometry. *J. Chromatography A*. **1216** (27), 5249–5255. https://doi.org/10.1016/j.chroma.2009.05.020

21. Stephenson D.J., MacKnight H.P., Hoeferlin L.A., Park M.A., Allegood J.C., Cardona C.L., Chalfant C.E. 2019. A rapid and adaptable lipidomics method for quantitative UPLC-mass spectrometric analysis of phosphatidylethanolamine and phosphatidylcholine in vitro, and in cells. *Anal. Methods*. **11** (13), 1765–1776. https://doi.org/10.1039/C9AY00052F

22. Tan G., Tian Y., Addy M., Cheng Y., Xie Q., Zhang B., Liu Y., Chen P., Ruan R. 2017. Structural analysis of phosphatidylcholine using a thin layer chromatography-based method. *Eur. J. Lipid Sci. Technol*. **119** (7), 1600282. https://doi.org/10.1002/ejlt.201600282

23. Leslie C.C., Gelb M.H. 2004. Assaying phospholipase A2 activity. *Methods Mol. Biol. (Clifton, N.J.)*. **284**, 229–242.

24. Petrovic N., Grove C., Langton P.E., Misso N.L.A., Thompson P.J. 2001. A simple assay for a human serum phospholipase A2 that is associated with high-density lipoproteins. *J. Lipid Research*. **42** (10), 1706–1713.

25. Eba C., Okano A., Nakano H., Iwasaki Y. 2014. A chromogenic substrate for solid-phase detection of phospholipase A2. *Analyt. Biochem*. **447** (1), 43–45. https://doi.org/10.1016/j.ab.2013.11.007

26. Cerelli M.J., Grimm K., Duan X., Mulberg E., Jalilie M., Sekella P., Payes M., Cox H., Blick K.E., Fang K.C., Zychlinsky E. 2016. Evaluation of recombinant enzyme calibration to harmonize lipoprotein-associated phospholipase A 2 activity results between instruments. *Clin. Biochem*. **49** (6), 480–485. https://doi.org/10.1016/j.clinbiochem.2015.11.018

27. Alekseeva A.S., Korotaeva A.A., Samoilova E.V., Volynsky P.E., Vodovozova E.L., Boldyrev I.A. 2014. Secretory phospholipase A2 activity in blood serum: The challenge to sense. *Biochem. Biophys. Res. Commun*. **454** (1), 178–182. https://doi.org/10.1016/j.bbrc.2014.10.069

28. Benson M.A., Schmalzer K.M., Frank D.W. 2010. A sensitive fluorescence-based assay for the detection of ExoU-mediated PLA2 activity. *Clinica Chimica Acta*. **411** (3–4), 190–197. https://doi.org/10.1016/j.cca.2009.10.025

29. Currò M., Ferlazzo N., Risitano R., Condello S., Vecchio M., Caccamo D., Ientile, R. 2014. Transglutaminase 2 and phospholipase A2 interactions in the inflammatory response in human Thp-1 monocytes. *Amino Acids*. **46** (3), 759–766. https://doi.org/10.1007/s00726-013-1569-y

30. Machado-Aranda D., Wang Z., Yu B., Suresh M.V., Notter R.H., Raghavendran K. 2013. Increased phospholipase A2 and lyso-phosphatidylcholine levels are associated with surfactant dysfunction in lung contusion injury in mice. *Surgery*. **153** (1), 25–35. https://doi.org/10.1016/j.surg.2012.05.043

31. Abe A., Rzepecki P.W., Shayman J.A. 2013. A fluorogenic phospholipid for the detection of lysosomal phospholipase A2 activity. *Analyt. Biochem*. **434** (1), 78–83. https://doi.org/10.1016/j.ab.2012.11.004

32. Wichmann O., Gelb M.H., Schultz C. 2007. Probing phospholipase A2 with fluorescent phospholipid substrates. *ChemBioChem*. **8** (13), 1555–1569. https://doi.org/10.1002/cbic.200600462

33. Popov A.V., Mawn T.M., Kim S., Zheng G., Delikatny E.J. 2010. Design and synthesis of phospholipase C and A 2 -activatable near-infrared fluorescent smart probes. *Bioconjug. Chem*. **21** (10), 1724–1727. https://doi.org/10.1021/bc100271v

34. Maity G., Bhattacharyya D. 2005. Assay of snake venom phospholipase A2 using scattering mode of a spectrofluorimeter. *Curr. Sci*. **89** (6), 1004–1008.

35. Horton K.L., Stewart K.M., Fonseca S.B., Guo Q., Kelley S.O. 2008. Mitochondria-penetrating peptides. *Chem. Biol*. **15** (4), 375–382. https://doi.org/10.1016/j.chembiol.2008.03.015

36. Liu Y., Cheng D., Lin I.-H., Abbott N.L., Jiang H. 2012. Microfluidic sensing devices employing in situ-formed liquid crystal thin film for detection of biochemical interactions. *Lab on a Chip.* **12** (19), 3746–3753.
https://doi.org/10.1039/c2lc40462a

37. Huang C., Zhou L., Liu Y., Lai L. 2006. A continuous fluorescence assay for phospholipase A2 with non-tagged lipid. *Analyt. Biochem.* **351** (1), 11–17.
https://doi.org/10.1016/j.ab.2006.01.021

38. Radvanyi F., Jordan L., Russo-Marie F., Bon C. 1989. A sensitive and continuous fluorometric assay for phospholipase A2 using pyrene-labeled phospholipids in the presence of serum albumin. *Analyt. Biochem.* **177** (1), 103–109.
https://doi.org/10.1016/0003-2697(89)90022-5

39. Mallat Z., Steg P.G., Benessiano J., Tanguy M.L., Fox K.A., Collet J.P., Dabbous O.H., Henry P., Carruthers K.F., Dauphin A., Arguelles C.S., Masliah J., Hugel B., Montalescot G., Freyssinet J., Asselain B., Tedgui A. 2005. Circulating secretory phospholipase A2 activity predicts recurrent events in patients with severe acute coronary syndromes. *J. Am. Coll. Cardiol.* **46** (7), 1249–1257.
https://doi.org/10.1016/j.jacc.2005.06.056

40. Mallat Z., Benessiano J., Simon T., Ederhy S., Sebella-Arguelles C., Cohen A., Huart V., Wareham N., Luben R., Khaw K., Tedgui A., Boekholdt M. 2007. Circulating secretory phospholipase A2 activity and risk of incident coronary events in healthy men and women: The EPIC-NORFOLK study. *Arteriosclerosis, Thrombosis and Vascular Biology*. **27** (5), 1177–1183.
https://doi.org/10.1161/ATVBAHA.107.139352

41. Tabaei S.R., Rabe M., Zetterberg H., Zhdanov V.P., Höök F. 2013. single lipid vesicle assay for characterizing single-enzyme kinetics of phospholipid hydrolysis in a complex biological fluid. *J. Amer. Chem. Soc.* **135** (38), 14151–14158.
https://doi.org/10.1021/ja4046313

42. Alekseeva A.S., Tretiakova D.S., Melnikova D.N., Molotkovsky U.G., Boldyrev I.A. 2016. Novel fluorescent membrane probe 2,3;5,6-bis(cyclohexyl)-BODIPY-labeled phosphatidylcholine. *Russ. J. Bioorgan. Chem.* **42** (3), 305–309.
https://doi.org/10.1134/S1068162016030031

43. Tretiakova D.S., Alekseeva A.S., Galimzyanov T.R., Boldyrev A.M., Chernyadyev A.Y., Ermakov Y.A., Batishchev O.V., Vodovozova E.L., Boldyrev I.A. 2018. Lateral stress profile and fluorescent lipid probes. FRET pair of probes that introduces minimal distortions into lipid packing. *Biochim. Biophys. Acta, Biomembr.* **1860** (11), 2337–2347.
https://doi.org/10.1016/j.bbamem.2018.05.020

44. Tretiakova D., Onishchenko N., Boldyrev I., Mikhalyov I., Tuzikov A., Bovin N., Evtushenko E., Vodovozova E. 2018. Influence of stabilizing components on the integrity of antitumor liposomes loaded with lipophilic prodrug in the bilayer. *Colloids Surf. B, Biointerfaces.* **166**, 45–53.
https://doi.org/10.1016/j.colsurfb.2018.02.061

45. Liu X.-Y., Nakamura C., Yang Q., Miyake J. 2001. Phospholipase A2-catalyzed membrane leakage studied by immobilized liposome chromatography with on-line fluorescent detection. *Analyt. Biochemistry.* **293** (2), 251–257.
https://doi.org/10.1006/abio.2001.5136

46. Liu X.-Y., Nakamura C., Hasegawa M., Miyake J. 2003. Effect of lipid composition on phospholipase A2-catalyzed membrane leakage in immobilized liposomes: Sensitization for polychlorinated biphenyls detection with antibody affinity column tandem with fluorescent liposome column. *Electrophoresis.* **24** (18), 3165–3171.
https://doi.org/10.1002/elps.200305530

47. Liu X.-Y., Nakamura C., Nakamura N., Hirano T., Shinbo T., Miyake J. 2005. Detection of polychlorinated biphenyls using an antibody column in tandem with a fluorescent liposome column. *J. Chromatography A.* **1087** (1–2), 229–235.
https://doi.org/10.1016/j.chroma.2005.03.013

48. Chen H., Lim S.K., Chen P., Huang J., Wang Y., Palaniappan A., Platt M., Leidberg B., Tok A. 2015. Reporter-encapsulated liposomes on graphene field effect transistors for signal enhanced detection of physiological enzymes. *Phys. Chem. Chem. Phys.* **17** (5), 3451–3456.
https://doi.org/10.1039/C4CP04644G

49. Li J., Zhang Y., Ai J., Gao Q., Qi H., Zhang C., Cheng Z. 2016. Quantum dot cluster (QDC)-loaded phospholipid micelles as a FRET probe for phospholipase A2 detection. *RSC Advances.* **6** (19), 15895–15899.
https://doi.org/10.1039/C5RA25292J

50. Chen S.-H., Hsu Y.-P., Lu H.-Y., Ho J.A. 2014. Gold/Phospholipid nanoconstructs as label-free optical probes for evaluating phospholipase A2 activity. *Biosensors and Bioelectronics.* **52**, 202–208.
https://doi.org/10.1016/j.bios.2013.08.017

51. Guo C., Zhang Y., Li Y., Xu S., Wang L. 2019. ^{19}F MRI nanoprobes for the turn-on detection of phospholipase A2 with a low background. *Analyt. Chem.* **91** (13), 8147–8153.
https://doi.org/10.1021/acs.analchem.9b00435

Translated by E. Makeeva